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LABORATORY STUDY

Modification of Immunoreactive EGF and EGF Receptor After Acute Tubular Necrosis Induced by Tobramycin or Cisplatin

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ABSTRACT

Acute tubular necrosis induced by aminoglycoside antibiotics and various other nephrotoxins is followed by a regenerative process which leads to the restoration of damaged tubules. Several lines of evidence indicate that tubular regeneration is mediated by polypeptide growth factors such as epidermal growth factor (EGF). Previous studies devoted to cisplatin nephrotoxicity have shown that this agent causes tubular cystic degeneration possibly related to an

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impairment of renal tissue repair. Thus, we examined on a comparative basis the time course of the regenerative response subsequent to tubular damage induced by tobramycin or cisplatin, particular attention being paid to renal EGF and its receptor. Female Sprague–Dawley rats (160–180 g body weight) were treated during 4 consecutive days with daily doses of 200 mg/kg tobramycin i.p. (BID) or 2 mg/kg cisplatin (once a day). Sham-treated rats were given 0.9% NaCl i.p. following the same protocol. Groups of experimental animals (n = 5–10) were terminated at increasing time intervals (1, 4, 7, 14, 21, 60 days) after cessation of treatment. One hour prior to sacrifice, each individual received i.p. 200 mg/kg 5-bromo-2'-deoxyuridine (BrdU) for the immunohistochemical demonstration of cell proliferation. Blood was collected at the time of sacrifice in order to assess glomerular filtration rate by measuring serum creatinine and BUN levels. Kidneys were analyzed with respect to total EGF determined by RIA in renal tissue homogenates, and soluble EGF was assayed in extracts prepared by centrifugation. Renal tissue was processed for the immunohistochemical detection of S-phase cells, of EGF, of EGF receptors, and of the intermediate filament vimentin, the latter being used as a marker of epithelium dedifferentiation. In absence of nephrotoxic alterations, EGF was immunolocalized in distal tubules, whereas EGF receptor immunostaining was seen in proximal tubules cells. Vimentin immunostaining was confined to glomeruli and blood vessels. Tobramycin and cisplatin caused acute tubular necrosis in proximal convoluted tubules and proximal straight tubules, respectively. Tissue damage was accompanied by renal dysfunction reflected by an elevation of serum creatinine and BUN levels. Tubular necrosis was followed by a proliferative response indicative of tubular regeneration. Regenerative hyperplasia was associated with a reduction of total immunoreactive EGF due to a decrease of tissue-bound proEGF. Tubules undergoing regenerative repair were characterized by a disappearance of EGF receptors and the presence of immunoreactive vimentin. In tobramycin-treated rats, renal dysfunction lasted for 4–7 days and was fully reversible, as indicated by the return of serum markers to normal values. Accordingly, tubular regeneration had led to the restoration of tubular epithelium in approximately 7–14 days. Moreover, the reduction of proEGF in renal tissue, and the loss of EGF receptors and vimentin expression in regenerating tubules occurred as a sequence of transient events so that kidneys had resumed a normal appearance by 14 days. In contrast with what was observed after exposure to tobramycin, cisplatin administration induced a protracted renal impairment probably related to a defective tissue repair. Kidneys of treated animals displayed a persistent depletion of immunoreactive proEGF. In addition, cystic tubules developing in the long term after exposure to cisplatin remained devoid of EGF receptors and exhibited continuous vimentin expression. The current study suggests that renal tubules damaged by cisplatin cannot undergo normal regeneration and probably remain in a dedifferentiated state.

INTRODUCTION

Acute renal failure characterized by an abrupt decline of glomerular filtration rate is generally associated with morphological evidences of tubular necrosis, particularly in proximal segments of the nephron (1, 2). Tissue damage may result from renal ischemia or from exposure to various nephrotoxic compounds, some of them used for therapeutic or diagnostic purposes. Nephrotoxicity is encountered as an adverse effect in several groups of drugs widely differing in structure and mechanism of action (3, 4), and sometimes is regarded as a major dose-limiting toxicity.

In clinics, both aminoglycoside antibiotics such as gentamicin and tobramycin, and platinum-based anticancer agents, in particular cisplatin, are known to be nephrotoxic (5–7). Moreover, these drugs have been repeatedly shown to induce acute tubular necrosis and renal failure in experimental animals. Beside the fact that tobramycin and cisplatin do not share any common feature with respect to their structure and toxicity mechanism, they also offer an interesting contrast regarding the reversibility of drug-induced renal dysfunction and the evolution of tubular damage after renal intoxication.

Functional impairment induced by aminoglycosides and many other nephrotoxins appears mostly reversible, and the return to a normal function occurs concomitantly with a repair of necrotic lesions by regenerative hyperplasia (2, 6, 8–10). In contrast, cisplatin has been reported to cause irreversible renal dysfunction in patients (5, 11–16). Moreover, animal studies have revealed that protracted functional impairment induced by cisplatin is accompanied by persisting morphological abnormalities in renal tissue (17, 18). In previous studies, we hypothesized that cisplatin nephrotoxicity might not only lead to tubular necrosis, but also result in a deficiency in the mechanisms underlying renal tissue repair (19, 20).

Tubular regeneration entails important changes in cell proliferation and differentiation, and is undoubtedly submitted to a variety of control mechanisms. Most significant progress made in the understanding of this complex process concerns the possible involvement of polypeptide growth factors as regulators of renal cell proliferation/differentiation (21, 22). Although current evidence indicates that tubular regeneration might require the participation of several growth factors (22), the bulk of experimental data reported to date points to the particular importance of epidermal growth factor (EGF). In particular, we and others demonstrated previously that EGF administration stimulates kidney regenerative response and accelerates the recovery of renal function in animals undergoing renal failure of ischemic or nephrotoxic origin (23–28). Previous work of our group also suggests that tubular regeneration involves an enzymatic processing of renal proEGF into diffusible EGF, thus allowing the latter to interact with EGF receptors on proximal tubular cells (10, 29–31). Therefore, the present study was initiated in order to compare the regenerative response elicited by the administration of tobramycin and cisplatin, with particular attention paid to EGF and its receptor in kidney tissue.

METHODS

Animals and Treatment

Experimental studies were conducted on female Sprague–Dawley rats (160–180 g body weight) supplied by a commercial breeding farm (Iffa Credo, L'Arbresle, France). Upon their arrival, the animals were distributed in groups of 5 individuals per cage. They were

allowed a minimum of 1 week of acclimatazation before starting any treatment. During this period and until termination, the rats were housed in a central facility submitted to a regular 12-h light/dark cycle and were provided with food and tap water *ad libitum*.

The animals were treated i.p. with the drug formulation available for parenteral administration in clinical practice. Tobramycin (Obracin®) was given twice a day at a daily dosage of 200 mg/kg and cisplatin (*cis*-diamminedichloroplatinum II) once a day at a daily dosage of 2 mg/kg. Both treatments were administered over 4 days. Before each drug injection the animals were weighed in order to adjust the volume of drug solution (approx. 0.5 mL and 0.8 mL for tobramycin and cisplatin, respectively) according to body weight. Sham-treated rats received i.p. 0.9% NaCl on the same schedule of administration.

Groups of experimental animals and controls were terminated by decapitation 1, 4, 7, 14, 21, and 60 days after the end of treatment. One hour before sacrifice each individual received i.p. 200 mg/kg 5-bromo-2′-deoxyuridine (BrdU) for pulse labeling of DNA and immunocytological demonstration of S-phase cells. At the time of sacrifice, blood was collected from the stump for the measurement of serum creatinine and BUN. After laparotomy, right and left kidneys were quickly excised and bisected. Tissue corresponding to renal cortex/OSOM was dissected from right kidneys and snap-frozen in dry ice whereas left kidneys were immersed in Duboscq–Brazil fixative for light microscopy. Necropsies of right kidneys were stored frozen until biochemical analysis.

Immunochemical Measurement of EGF in Renal Tissue

The levels of total and soluble immunoreactive EGF in renal tissue were determined by a double-antibody nonequilibrium radioimmunoassay using rabbit polyclonal anti-rat EGF antiserum. Frozen tissue samples were transferred to ice-cold distilled water in a volume: tissue weight ratio of 8 mL/g and homogenized in a Potter–Elvehjem device equipped with a teflon pestle. All subsequent steps were carried out at 0–4°C. After a 30-min incubation, each sample was split and mixed with concentrated phosphate-buffered saline (0.5 M phosphate, 1 M NaCl, pH 7.4) with or without 10% Triton X-100, added in a volume ratio of 1:10. Total EGF was estimated in detergent-containing homogenates after a further incubation of 30 min. For the determination of soluble EGF, samples without detergent were spun at 100.000 × g for 30 min, and supernatants were recovered and used as soluble fractions.

Radioimmunoassay of renal EGF was performed on triplicate samples, in comparison with purified rat EGF used as a reference. Anti-EGF antiserum (100 μL, 1:20,000 dilution) was added to test tubes containing 100 μL of either tissue extract or standard (0.005–10 ng/mL rat EGF) and the mixtures were allowed to stand for 16 h. Fifty microliters of murine ^{125}I-EGF (200,000 cpm/mL) were then added to each tube. After a further incubation of 3 h, 500 μL of goat anti-rabbit IgG conjugated to cellulose beads (3 mg protein/g cellulose) were mixed thoroughly with each sample. Immunocomplexes were allowed to form during a period of 2 h at 4°C and the mixtures were centrifuged at 1000 × g for 10 min. Supernatants were discarded and pellets were suspended in 3 mL phosphate buffered saline (0.05 M phosphate, 0.1 M NaCl, 0.25% bovine serum albumin, pH 7.4). Samples were spun for 5 min as above, supernatants were discarded, and the radioactivity present in pellets was measured in a LKB minigamma counter.

Biochemical Assays

Protein concentrations were evaluated by the method of Lowry et al. (32), using bovine serum albumin as standard. Serum was decanted after completion of blood clotting at room

temperature. Serum creatinine and BUN were measured by Jaffe's method (with deproteinization) and the urease method, respectively. Both assays were performed using commercial kits and according to the instructions of the suppliers.

Morphological Methods

Tissue specimens fixed in Duboscq–Brazil were dehydrated in graded ethanol solutions, finished in butanol, and embedded in paraffin wax (Paraplast Plus®) following routine procedure. Sections of 4- to 5-μm thickness were produced on a Reichert Autocut 2040 microtome. For histopathological examination, tissue was stained with periodic acid–Schiff, hemalun and luxol fast blue.

Immunohistochemical methods were applied to dewaxed paraffin sections rehydrated in distilled water. Cells in S phase were revealed by the immunolabeling of BrdU incorporated into DNA. To unmask hidden epitopes, sections were pretreated with trypsin and HCl, as described previously (10). Tissue sections were thereafter incubated in presence of a mouse anti-BrdU monoclonal antibody in phosphate-buffered saline (0.04 M $Na_2H\ PO_4$, 0.01 M KH_2PO_4, 0.12 M NaCl, pH 7.2–7.4). The same buffer composition was used along all subsequent steps of this and following histochemical procedures. Exposure to anti-BrdU monoclonal antibody was followed by an incubation with biotinylated rabbit anti-mouse IgG antibodies. Immunocomplexes were revealed as described below.

EGF was immunostained in tissue section by application of the same rabbit anti-rat EGF antiserum as that used in radioimmunoassay. This was followed by exposure to biotinylated swine anti-rabbit IgG antibodies.

EGF receptors were immunolocalized in renal tissue by using a rabbit antiserum raised against a 12-amino acid sequence (residues 985–996) located within the intracellular domain of human EGF receptor (33). Rabbit immunoglobulins were detected as described above by applying a second layer of biotinylated antibodies.

Vimentin immunostaining was performed after pretreatment of tissue sections with trypsin (0.1 mg/mL; 20 min). We used mouse monoclonal anti-pig vimentin as a primary antibody. The second layer consisted of biotinylated rabbit anti-mouse IgG antibodies.

In all cases, biotinylated antibodies were demonstrated by exposure to biotinylated peroxidase–avidin (ABC) complexes (34). Bound peroxidase activity was visualized by incubation in a solution of 0.02% 3,3′-diaminobenzidine, 0.01% H_2O_2. Sections were finally counterstained with periodic acid–Schiff, hemalun, and luxol fast blue for histological examination. The specificity of immunostaining was checked by performing positive and negative controls, as described previously (31).

Morphometric Analysis

Paraffin sections processed for the immunohistochemical demonstration of BrdU-labeled DNA or of EGF immunoreactivity were submitted to an interactive computer-assisted morphometric analysis in order to obtain a quantitative assessment of S-phase cells and of distal tubules containing immunoreactive EGF. As described previously (10, 30), sections (one slide per experimental animal) were scanned by a high-resolution color video camera mounted on a Zeiss Axioplan microscope and connected to an IBM-compatible microcomputer. Microscopic fields were analyzed using softwares designed for grey level discrimination, object counting, and surface calculation (Systèmes d'Analyses Microscopiques à Balayage Automatique—SAMBA; Alcatel TITN Answare, Grenoble, France). The relative areas occupied by tubular profiles staining for EGF were estimated on 10

microscopic fields at 50× magnification (approx. total surface: 8.5 mm^2). The numbers of labeled nuclei per unit surface were evaluated on 10 microscopic fields at 100× magnification (approx. total surface: 2.1 mm^2).

Statistical Analysis

Analysis of experimental data was first run on a program allowing fully factorial analysis of variance (ANOVA). In cases of p values lower than 5%, treated groups were compared to controls by the Tukey post hoc test. For selected groups, effects of tobramycin and cisplatin were compared using Student's or Cochran's t tests. Level of statistical significance was arbitrarily set at $p \leq .05$.

Drugs and Reagents

Tobramycin (Obracin®) and cisplatin (Platinol®) were courteously supplied by E. Lilly-Benelux (Brussels, Belgium) and Laboratoire Bristol Benelux (Brussels, Belgium), respectively. Rabbit polyclonal antiserum raised against rat EGF was obtained and checked for its specificity as described previously (10, 35). Anti-EGF receptor antiserum was a kind gift from R. M. Kris (Department of Pharmacology, New York University School of Medicine, New York) and was elicited by using as an immunogen a 12-residue peptide corresponding to positions 985–996 near the carboxyterminus of human EGF receptor (33). Anti-BrdU and anti-vimentin monoclonal antibodies, biotinylated rabbit anti-mouse IgG antibody, biotinylated swine anti-rabbit IgG antibody, and ABC complexes came from Dakopatts (Glostrup, Denmark). Commercial kits for serum creatinine and BUN assays were purchased from Boehringer (Mannheim, Germany) and Merck (Darmstadt, Germany), respectively. ^{125}I-labeled EGF came from Amersham Belgium (Gent, Belgium). Goat anti-rabbit IgG antibody was conjugated to cellulose beads according to the protocol described by Axen et al. (36).

RESULTS

During the 4-day period of drug administration, the body weight gain of tobramycin-treated rats appeared lower than that observed in animals receiving saline (3.0 ± 2.7% vs. 7.4 ± 0.9%), and animals exposed to cisplatin actually lost 8.3 ± 2.6% of their initial weight. Two weeks posttreatment, the mean body weight of rats dosed with cisplatin was still approx. 20% lower than that of controls or tobramycin-treated animals. This effect of cisplatin on the growth rate was attributed to the well-known gastrointestinal toxicity of the anticancer agent. Other than this, we did not encounter gross manifestation of toxicity or animal loss related to drug injection.

Examination of kidney paraffin sections revealed morphological signs of acute tubular necrosis resulting from drug-induced renal toxicity. The appearance of tissue damage was similar to that reported in previous studies on aminoglycosides (see Ref. 10 and references therein) or cisplatin (19, 20) nephrotoxicity. In tobramycin-treated rats, tubular necrosis preferentially affected proximal convoluted tubules of renal cortex and culminated at days 4 and 7, although some animals sacrificed at day 1 also exhibited morphological evidence of tubular injury. However, at days 14, 21, and 60, renal tissue had become almost unremarkable, except for the occasional presence of moderate tubular dilatation

infiltration. Contrasting with what was seen after tobramycin administration, tubular necrosis due to cisplatin exposure was more prominent in proximal straight tubules of OSOM. Necrotic injury was particularly severe at day 4 and was followed by progressive cystic tubular degeneration (17–19). Two months after treatment with cisplatin, renal OSOM of treated animals was still mostly occupied by tubular cysts between which one could note the frequent occurrence of peritubular infiltrates.

As shown by the monitoring of BUN and serum creatinine (Fig. 1), nephrotoxic insult was accompanied by a reduction of glomerular filtration rate. In rats receiving tobramycin, serum creatinine rose sharply to reach at day 1 a concentration approx. twice that measured in sham-treated animals. During the following days, creatinine level returned to the normal range [Fig. 1(a)]. BUN concentration followed a parallel course, with peaks at days 1 and 4 and a subsequent return toward baseline level [Fig. 1(b)]. Serum creatinine and BUN measurements in cisplatin-treated animals disclosed a different pattern. Serum creatinine increased slowly but steadily and attained by day 14 a plateau twice above control level [Fig. 1(c)]. In a quite similar fashion, BUN rose to a maximum level (approx. 6 times the mean control value) at day 14 and only showed thereafter a limited tendency to decrease (Fig. 1(d)]. Thus, in rats sacrificed at day 60, both serum creatinine and BUN were still significantly higher than control values. Although the progression of kidney dysfunction obviously differed depending on the treatment, comparison of peak levels of serum creatinine and BUN (tobramycin-treated rats, day 1 vs. cisplatin-treated rats, day 14) did not disclose a significant difference (Student's t test).

Drug-induced tubular necrosis elicited a process of tubular regeneration which was assessed by the immunohistochemical demonstration of BrdU incorporation into DNA and the morphometric estimation of S-phase cells in renal tissue. Exposure to tobramycin was followed by a burst of proliferative activity in both renal cortex and OSOM, the mitogenic response showing a peak at day 4. The rate of cell proliferation declined subsequently and became virtually equivalent to control values by day 14 [Fig. 2(a)]. As compared to animals which received tobramycin, the proliferative response appeared slightly delayed in cisplatin-treated rats [Fig. 2(b)] but also peaked at day 4. Rate of cell proliferation progressively abated past that time point. Although at day 60 no statistically significant difference was observed between cisplatin-treated and control rats, mitotic activity still appeared slightly higher in the kidneys of the former animals. Thus, at that time point, renal cell proliferation following cisplatin exposure remained higher ($p < .05$, Cochran's t test) compared with that measured in tobramycin-treated rats.

Since several lines of evidence indicate that EGF acts as a positive mediator in the process of renal tubular regeneration, we applied immunochemical and immunohistochemical approaches in order to uncover possible modifications of this growth factor during the course of tubular necrosis and repair. Distinct forms of immunoreactive EGF have been identified in rat renal tissue (29). Soluble immunoreactive EGF that is detected in the kidney consists of native EGF (undistinguishable from mature EGF secreted by submaxillary glands) and of EGF lacking the carboxyterminal arginine, both of which appear biologically active. Tissue-bound immunoreactive EGF most likely corresponds to the high molecular weight precursor (proEGF) synthesized within distal tubules of the kidney (37). It has been shown that treatment of this fraction with trypsin generates a single form of immunoreactive EGF (29). In the present study, we used a simplified procedure to estimate total EGF since preliminary experiments showed that 30-min Triton extraction combined with a modification of the separation system in RIA (double-antibody solid-phase

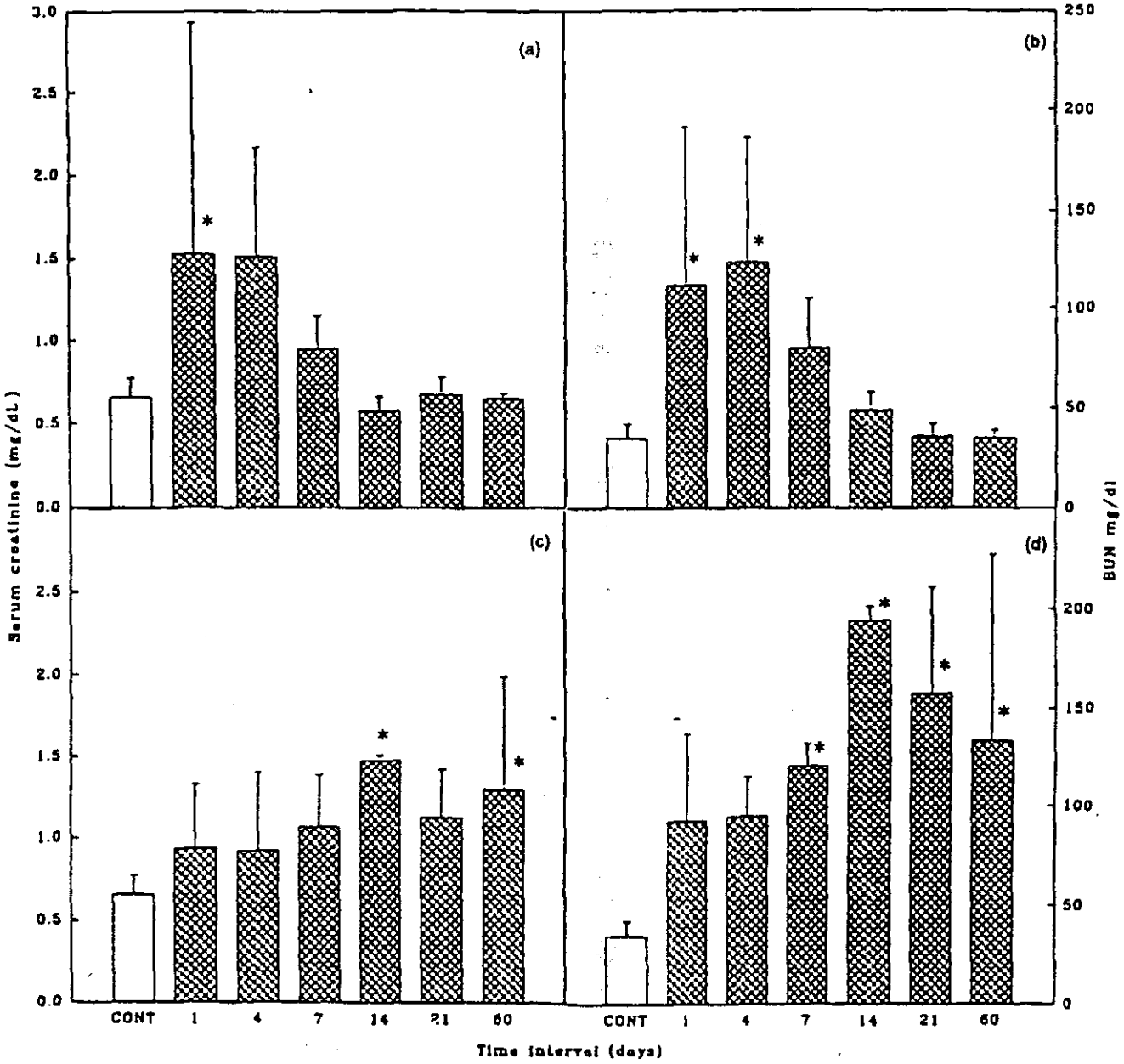


Figure 1. Assays of serum creatinine (a, c) and of BUN (b, d) in animals terminated at different time intervals after 4-day exposure to tobramycin (a, b) or cisplatin (c, d) (cross-hatched columns: treated rats; open columns: sham-treated rats). Columns refer to the mean values found in different experimental groups, with the standard deviations indicated by the vertical bars. Statistical significance (*$p < .05$ as compared to controls) was evaluated by ANOVA and Tukey post hoc test.

trypsin treatment. Figure 3 illustrates displacement curves generated by the inclusion of either samples at increasing concentrations in the RIA incubation mixture. Soluble renal EGF produces a displacement parallel to the standard curve obtained with EGF purified from rat submaxillary glands. In the case of renal homogenates, most likely containing a mixture of soluble and proEGF, the displacement curve departs slightly from the standard curve. Levels of soluble and total immunoreactive EGF measured in renal tissue of control and drug-treated animals appear in Figure 4. Even though comparison of RIA data must be made with caution because of different immunoreactivities, examination of the figure reveals that soluble EGF accounts for only a minor (approx. 4%) fraction of total EGF, at

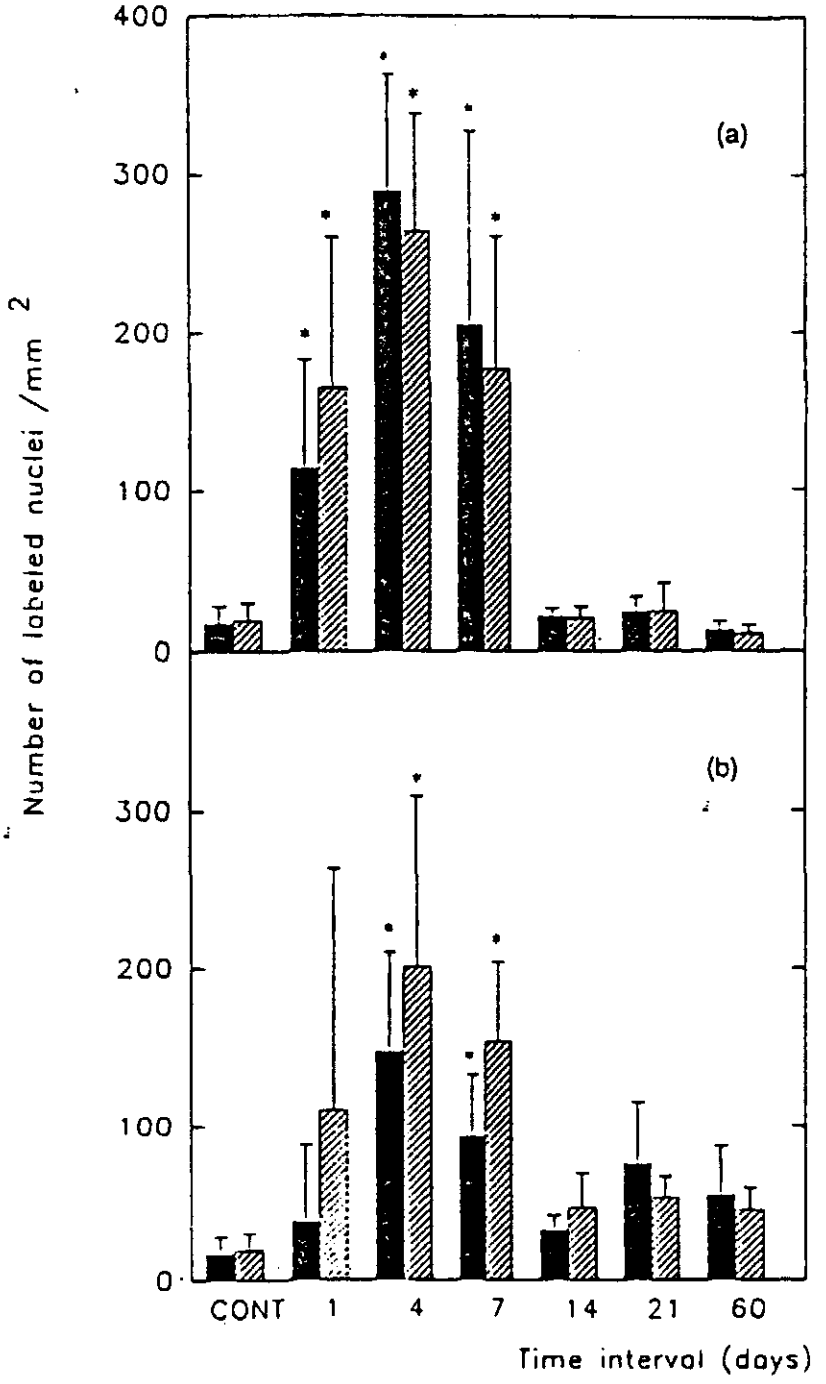


Figure 2. Morphometric evaluation of cell proliferation in kidneys of rats sacrificed at different time intervals after treatment with tobramycin (a) or cisplatin (b) (CONT: sham-treated animals). Each animal received i.p. 200 mg/kg BrdU 1 h prior to sacrifice for the immunohistochemical demonstration of DNA synthesis. S-phase cells [see, e.g., Fig. 6(c)] were revealed by using an anti-BrdU monoclonal antibody, as described in Methods. Frequencies of labeled nuclei per unit surface in renal cortex (filled columns) and OSOM (striped columns) were determined by computer-assisted morphometry. Columns give the mean values of different experimental groups, with the standard deviations indicated by the vertical bars. Statistical analysis as in Figure 1 (*$p < .05$ as compared to controls).

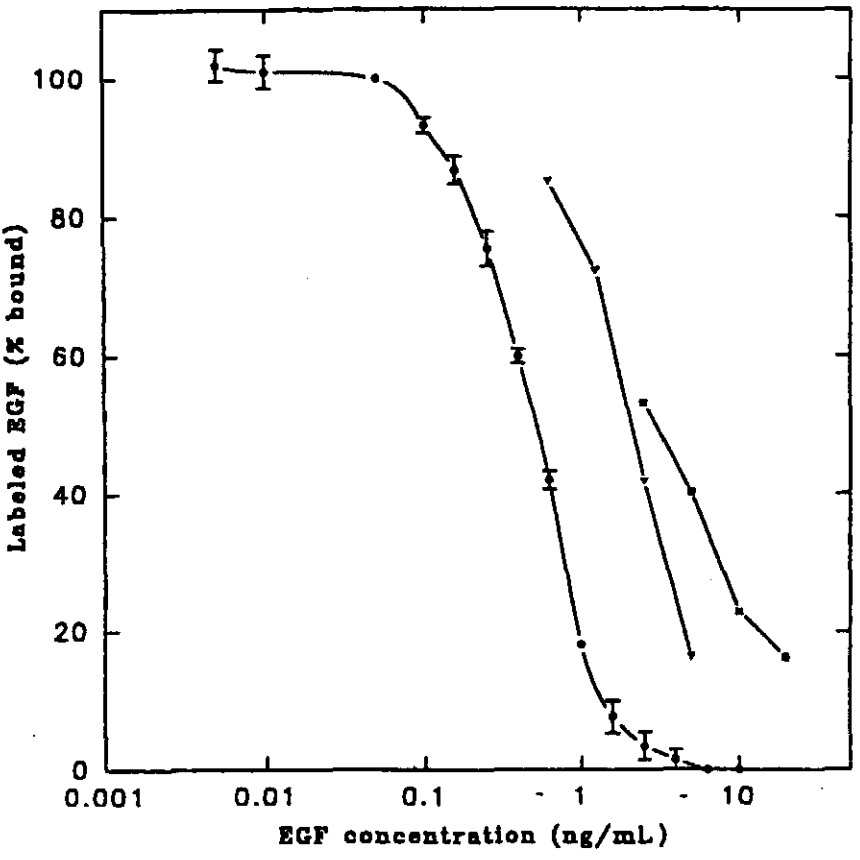


Figure 3. Displacement curves generated by the addition of different EGF-containing samples to the EGF radioimmunoassay mixture (see Methods). ●: EGF purified from rat salivary glands (reference curve); ▼: soluble fractions of kidney homogenates (dilutions 1:16 to 1:2); ■: total kidney homogenates (dilutions 1:128 to 1:16). Each symbol is the mean of triplicate measurements, vertical bars in the reference curve indicating standard deviations. Curves obtained with renal tissue samples were arbitrarily set on the graph and do not necessarily correspond to EGF concentrations on the abscissa.

least in the kidneys of untreated rats. This is consistent with the fact that, in normal conditions, renal EGF exists mostly in the precursor form. As shown in Figures 4(a) and 4(c), treatments with tobramycin or cisplatin induced a substantial reduction in the amount of total EGF, the trough values being statistically similar (Student's t test) in both cases. During the recovery period following exposure to tobramycin, concentrations of total EGF eventually returned to values equivalent to those found in controls [Fig. 4(a)]. In contrast, after cisplatin administration total EGF remained significantly lower throughout the whole period of observation (i.e., up to 2 months) [Fig. 4(c)]. Thus, at day 60 total EGF was significantly lower ($p < .05$, Cochran's t test) in the case of cisplatin-treated rats compared with animals exposed to tobramycin. No statistically significant departure from control values appeared when soluble EGF was assayed in renal tissue of treated rats, although a moderate decrease was noted 7 days after tobramycin injection and throughout the whole period of observation following cisplatin dosing.

In accordance with previous studies (10, 30, 37) EGF was immunolocalized in distal convoluted tubules and thick ascending loops of Henle. When estimated by morphometric analysis, immunostained tubular profiles were found to occupy approximately 3.5% of tissue section area in renal cortex of controls. Treatment with either tobramycin or cisplatin

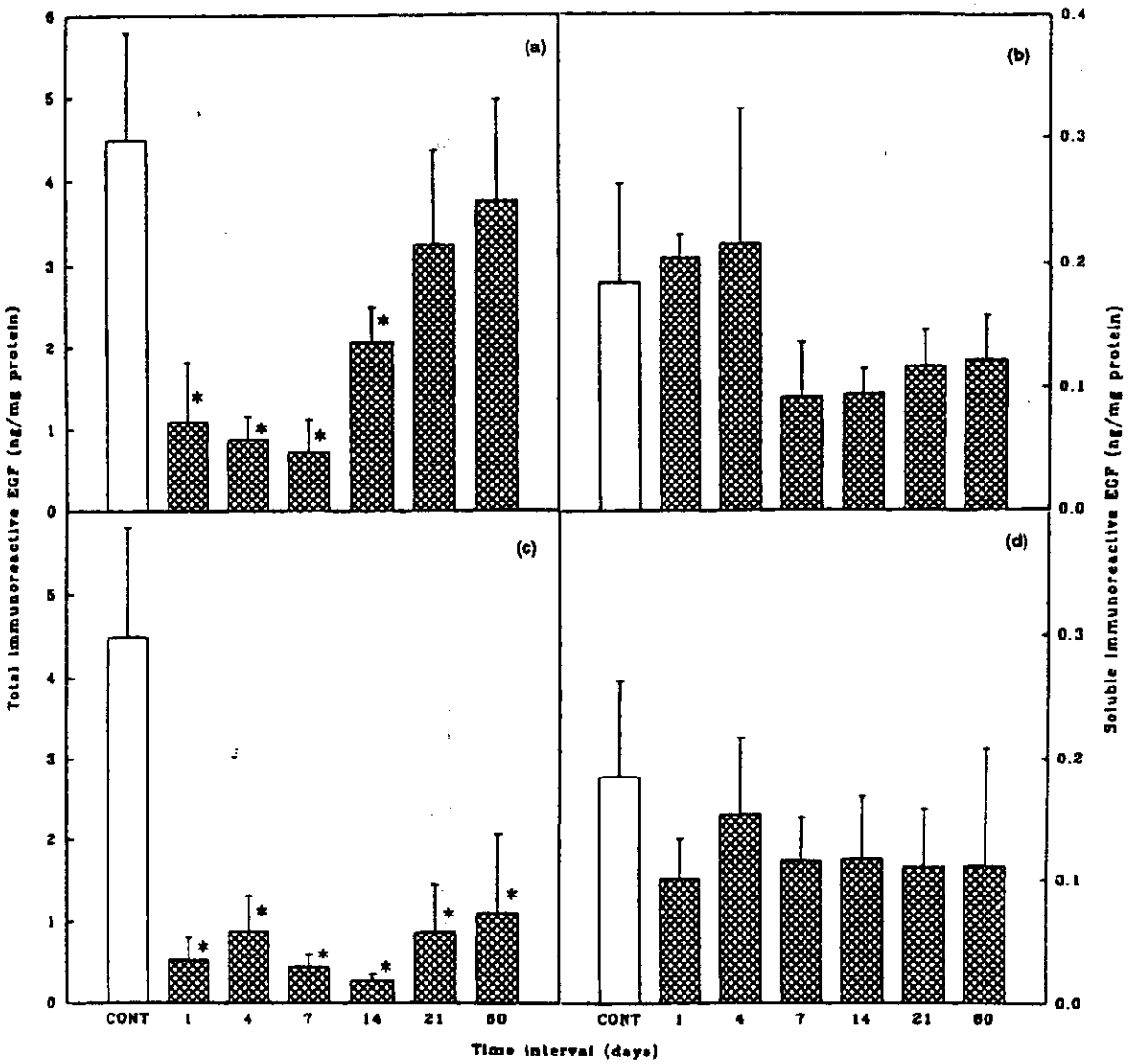


Figure 4. Values of immunoreactive EGF in the kidneys of rats sacrificed at different time intervals after tobramycin (a, b) or cisplatin (c, d) administration. Renal EGF was determined by radioimmunoassay using purified salivary gland EGF as a standard (see Fig. 3), in Triton-treated renal tissue homogenates [(a, c): total EGF] or in supernatants recovered after centrifugation [(b, d): soluble EGF]. Cross-hatched and open columns refer to treated and sham-treated animals, respectively. Columns give the mean values of different experimental groups, with the standard deviations indicated by the vertical bars. Statistical analysis as in Figure 1 (*$p < .05$ as compared to controls).

led to a sizable reduction of EGF immunostaining, particularly evident in cortical tissue (Fig. 5). Thus the relative area covered by immunolabeled tubular profiles remained significantly lower than mean control value during a period of 2 weeks following tobramycin exposure. Thereafter, EGF immunostaining gradually resumed a normal appearance [Fig. 5(a)]. In contrast, in animals treated with cisplatin, the quenching of EGF immunostaining persisted during the whole period of observation [Fig. 5(b)].

Consistent with a recent report from our group (31), immunohistochemical staining with an antiserum raised against the EGF receptor demonstrated the presence of the latter on the

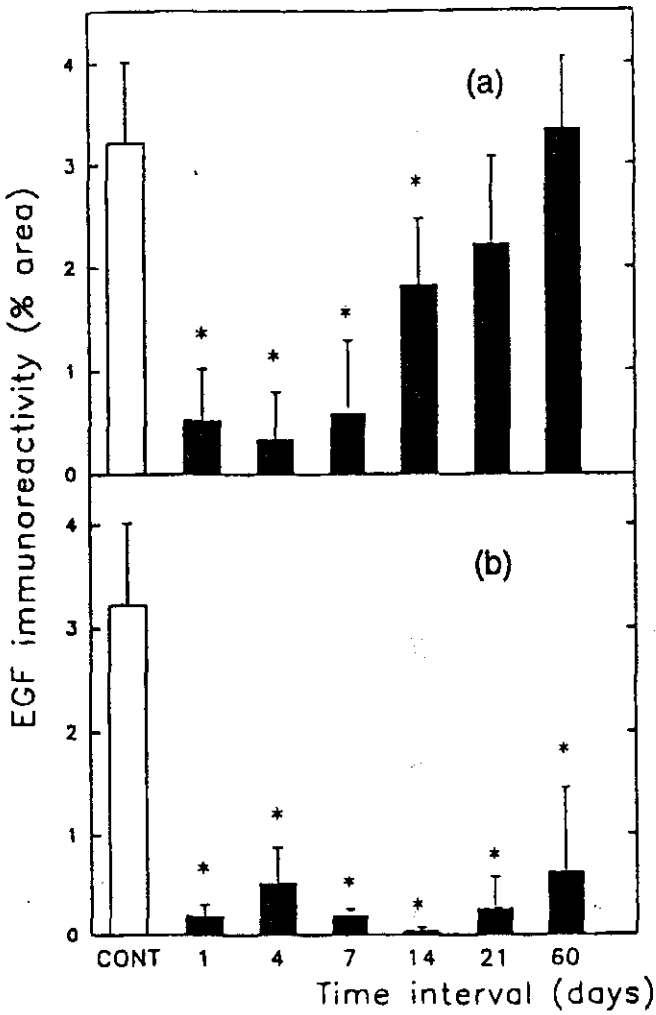


Figure 5. Morphometric evaluation of distal tubules displaying EGF immunoreactivity in renal cortex of rats sacrificed at different time intervals after exposure to tobramycin [(a), filled columns] or cisplatin [(b), filled columns]. Open columns: sham-treated rats. Tissue sections were processed for the immunohistochemical demonstration of EGF as described in Methods, and relative areas occupied by immunostained tubular profiles were determined by computer-assisted morphometry. Columns give the mean values of different experimental groups, with the standard deviations indicated by the vertical bars. Statistical analysis as in Figure 1 (*$p < .05$ as compared to controls).

epithelium of proximal tubules [Fig. 6(b)]. Immunolabeling was particularly conspicuous at the contraluminal pole of epithelial cells. In addition, juxtaglomerular cells of afferent arterioles exhibited a strong reaction with anti-EGF receptor antiserum. Nephrotoxic insult did not immediately result in the disappearance of immunoreactive EGF receptor in proximal tubules, since some immunostaining could still be observed in tubular sections showing evidence of necrosis or sublethal injury. However, in tobramycin-treated rats [Fig. 6(d)] as well as in animals exposed to cisplatin [Fig. 7(b)], tubular sections endowed with proliferative activity and/or lined by undifferentiated epithelium appeared virtually devoid of immunoreactive EGF receptors. After exposure to tobramycin, the disappearance of EGF receptor immunostaining was particularly evident at days 4 and 7. Subsequently, immunoreactive EGF receptors reappeared along with the redifferentiation of tubular epithelium [Fig. 6(f)], i.e., after completion of regenerative hyperplasia. In contrast, tubular profiles lacking immunoreactive EGF receptors were observed throughout the

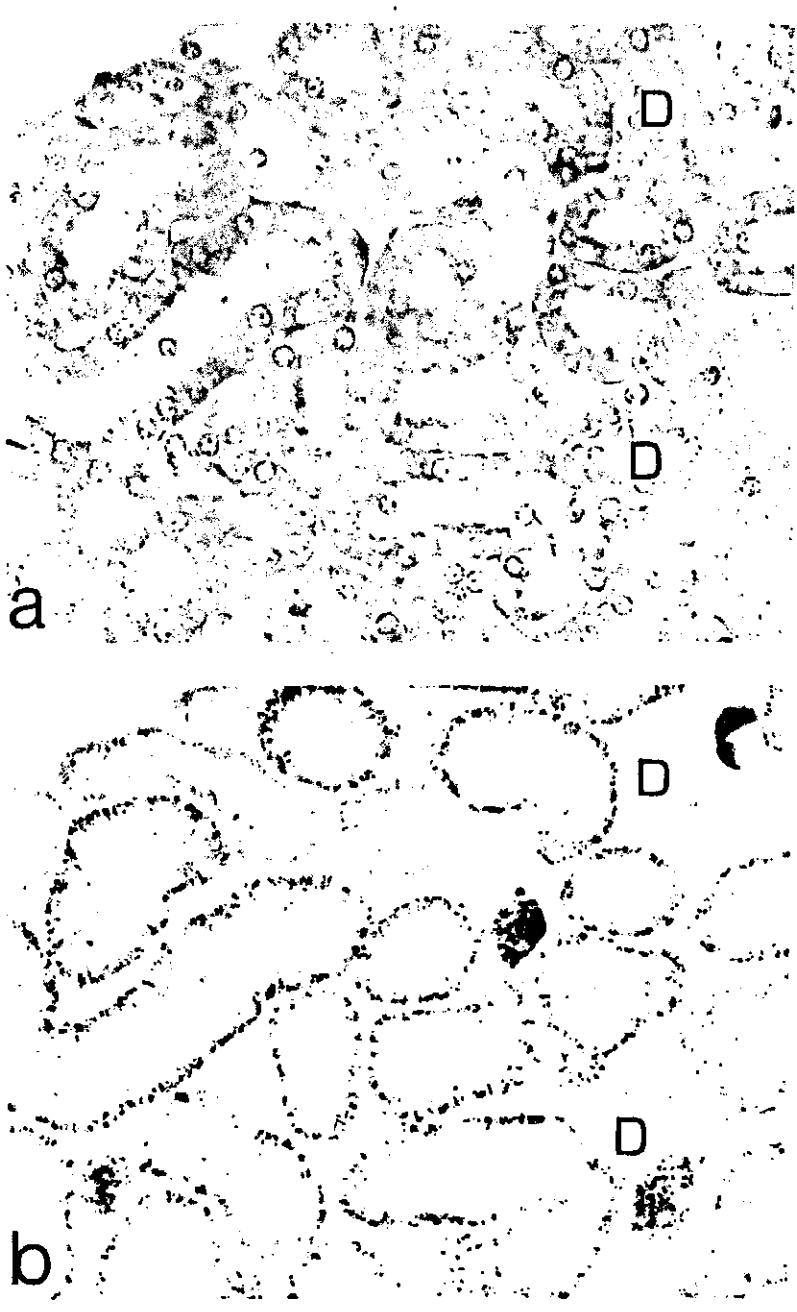


Figure 6. Immunohistochemical demonstration of EGF receptors in renal tissue. Each set of micrographs—(a-b); (c-d); (e-f)—shows corresponding fields on consecutive tissue sections. In panels (b), (d), and (f), EGF receptors were immunostained as described in Methods. In panels (a), (c), and (e), tissue sections were processed to reveal S-phase cells by the immunohistochemical detection of BrdU incorporated into DNA. Micrographs (a-b), (c-d), and (e-f) correspond to kidney necropsies collected from a control rat or from tobramycin-treated animals sacrificed 7 or 21 days after drug exposure, respectively. All proximal tubules profiles appearing in micrograph (b) exhibit immunoreactive EGF receptors, whereas no immunostaining is seen in distal tubules (D). Seven days after tobramycin exposure, i.e., during the regenerative response, some tubular profiles show numerous labeled nuclei [(c), arrowheads] and are also devoid of differentiation characters. Disappearance of EGF receptor immunostaining is observed in these immature, regenerating tubules [(d), R]. Twenty-one days after treatment with tobramycin—thus well beyond the episode of tubular regeneration—the incidence of S-phase cells has fallen to baseline level [compare (e) and (a)], and proximal tubules have recovered immunoreactive EGF receptors (f) (×356).

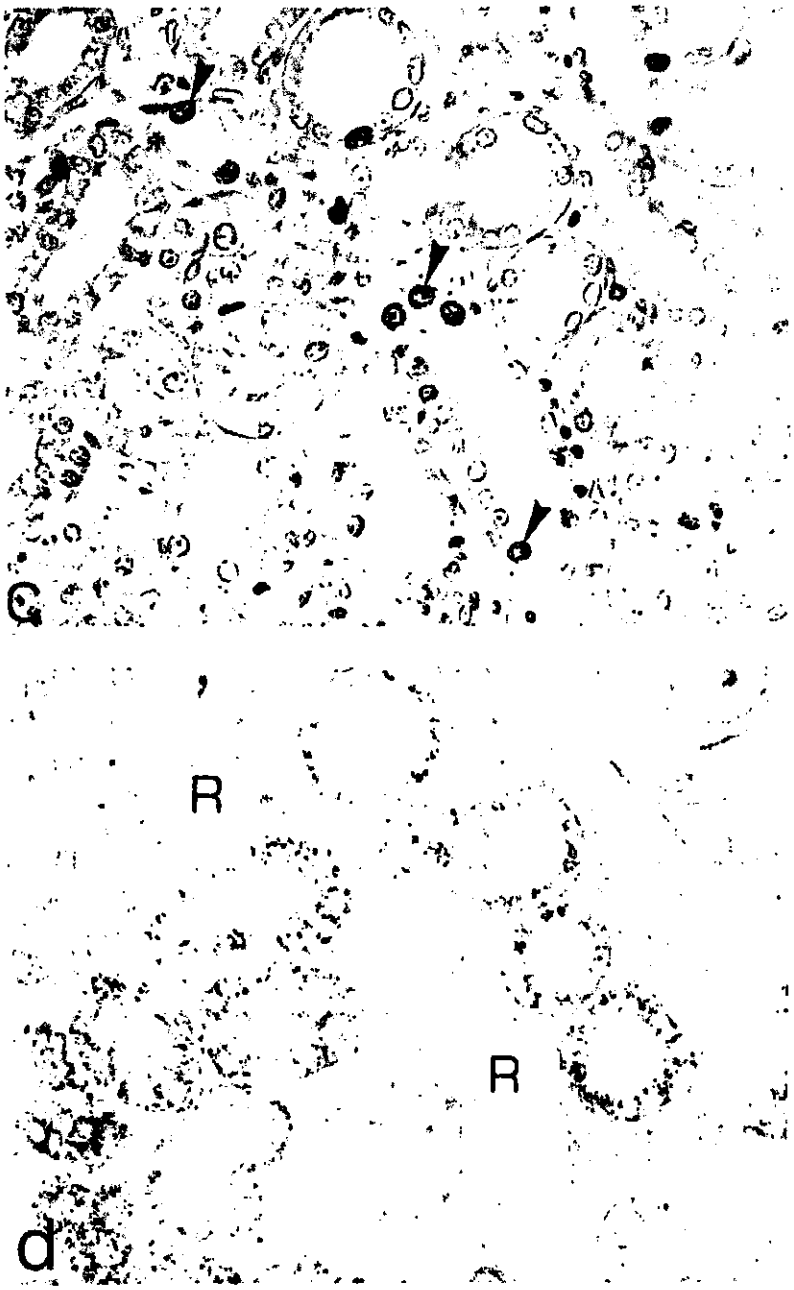


Figure 6. Continued.

period of observation following treatment with cisplatin [Figs. 7(d) and 7(f)]. In particular, cystic tubules, atrophic tubules, and tubules which had not regained differentiation characters remained negative after EGF receptor immunostaining.

In kidneys of sham-treated rats, only glomeruli, cortical blood vessels (endothelial and smooth muscle cells), and vasa rectae of renal medulla displayed positive reaction upon vimentin immunostaining. Thus, no morphological evidence of vimentin expression was found in epithelium of renal tubules. Vimentin immunostaining remained present in these histological entities of the kidney after nephrotoxic injury. However, postnecrotic tubular regeneration was also associated with the appearance of immunoreactive vimentin in tubular epithelium, concomitant with the loss of differentiation characters. In tobramycin-treated rats, tubules showing vimentin immunostaining were particularly prominent at day 7 [Fig. 8(a)], and virtually disappeared thereafter. In a similar fashion, immunoreactive

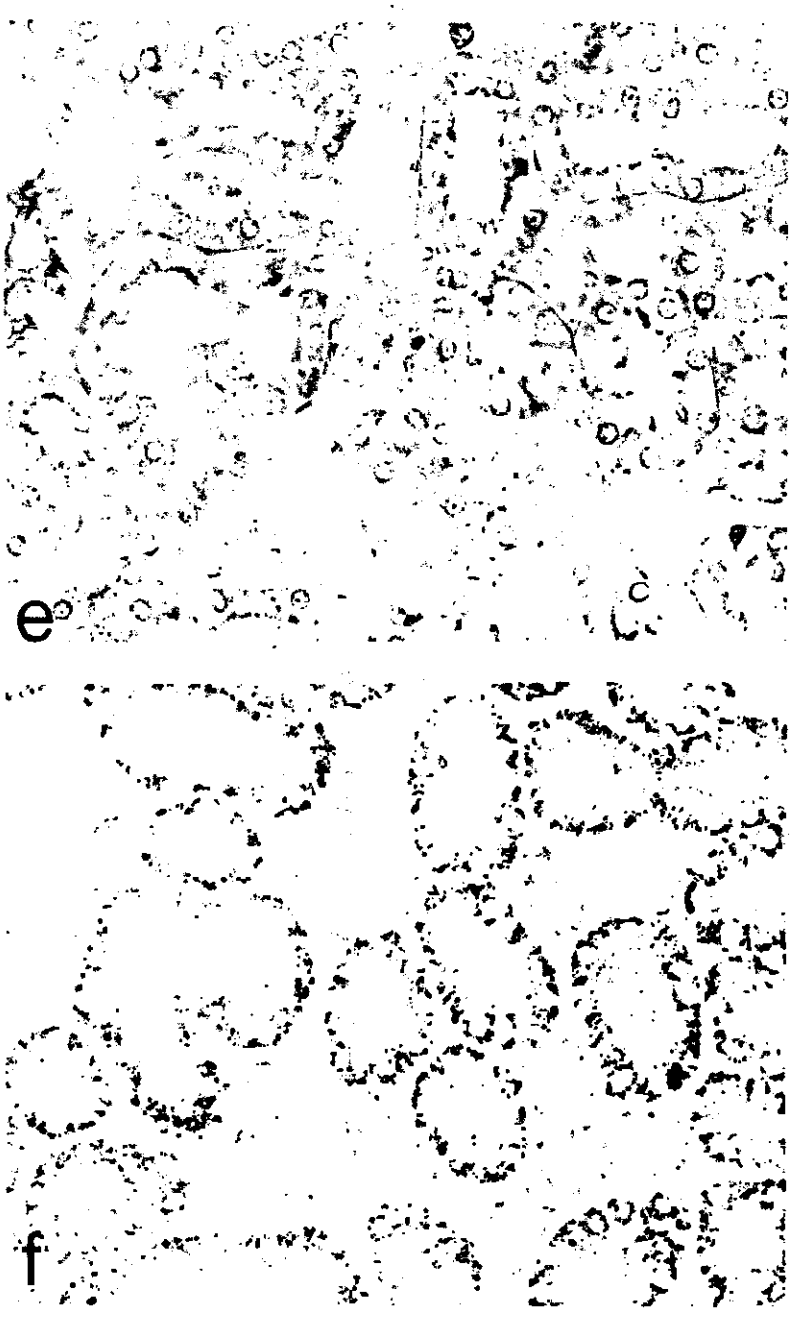


Figure 6. Continued.

vimentin only became conspicuous in tubular epithelium 7 days after cisplatin exposure [Fig. 8(b)]. However, in the latter case vimentin expression persisted in tubular epithelium for a protracted period of time. In rats sacrificed 2 months posttreatment, cystic tubules [Fig. 8(d)] and tubules with evidence of incomplete repair and/or redifferentiation [Fig. 8(c)] frequently stained for vimentin.

DISCUSSION

In most cases, tubular necrosis due to renal ischemia or exposure to nephrotoxic agents elicits a regenerative process leading to the complete restoration of tubular epithelium in segments which have undergone damage (38, 39). Accordingly, acute renal failure related

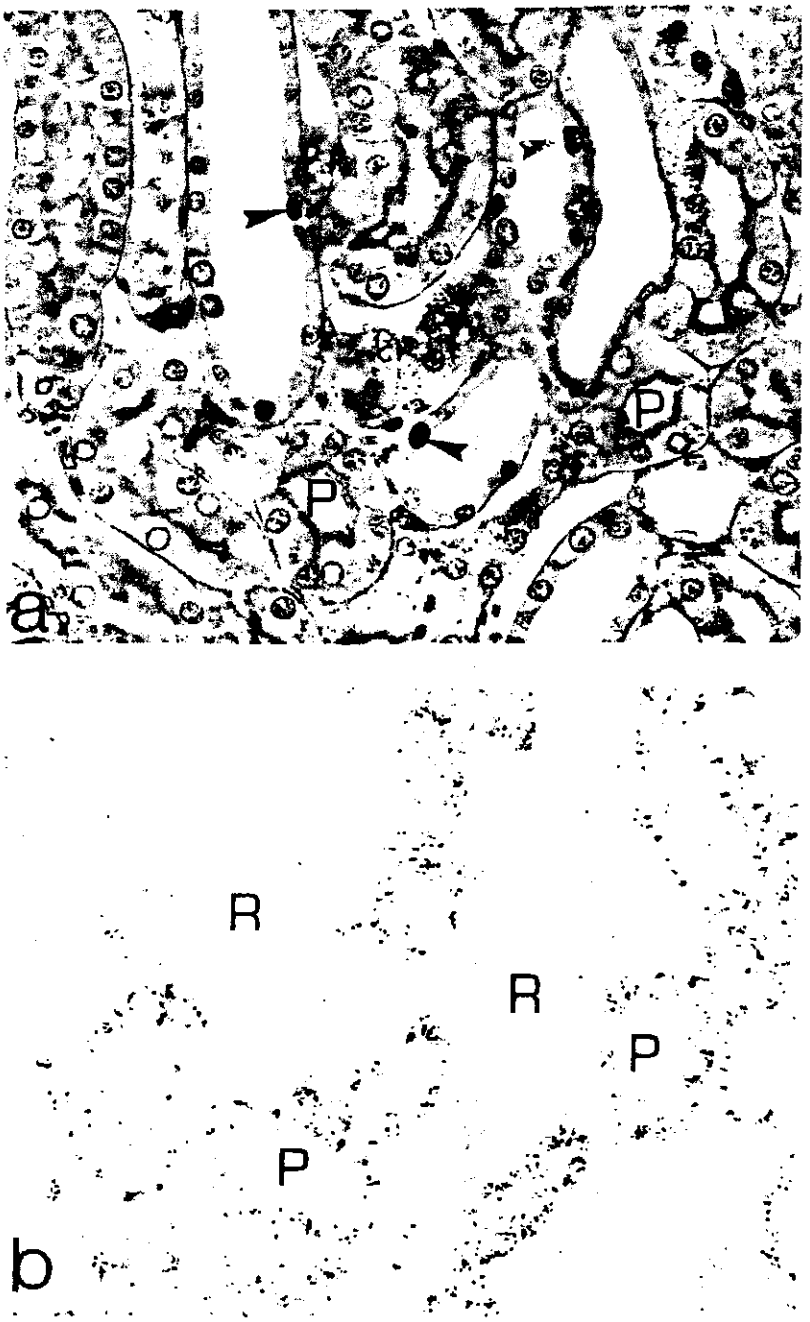


Figure 7. Immunohistochemical demonstration of EGF receptors in kidneys of animals treated with cisplatin and sacrificed 1 (a, b), 21 (c, d), or 60 (e, f) days after drug exposure. Presentation of micrographs as in Figure 6. At day 1, EGF receptor immunostaining (b) can still be observed in many profiles of proximal tubules (P). Tubular sections with an immature appearance and signs of high proliferative activity [(a), arrowheads] appear negative for EGF receptor immunostaining (R). At day 21 (c-d), cystic tubules (CT) or tubules with evidence of incomplete repair (I) are devoid of immunoreactive EGF receptors (CT, I), whereas morphologically normal proximal tubules (P) exhibit immunostaining. Sixty days after cisplatin administration (e-f), cystic tubules (CT) are still characterized by a quasi-absence of EGF receptor immunostaining. EGF receptors are also lacking in tubular profiles with evidence of incomplete repair [(e-f), arrows] (×356).

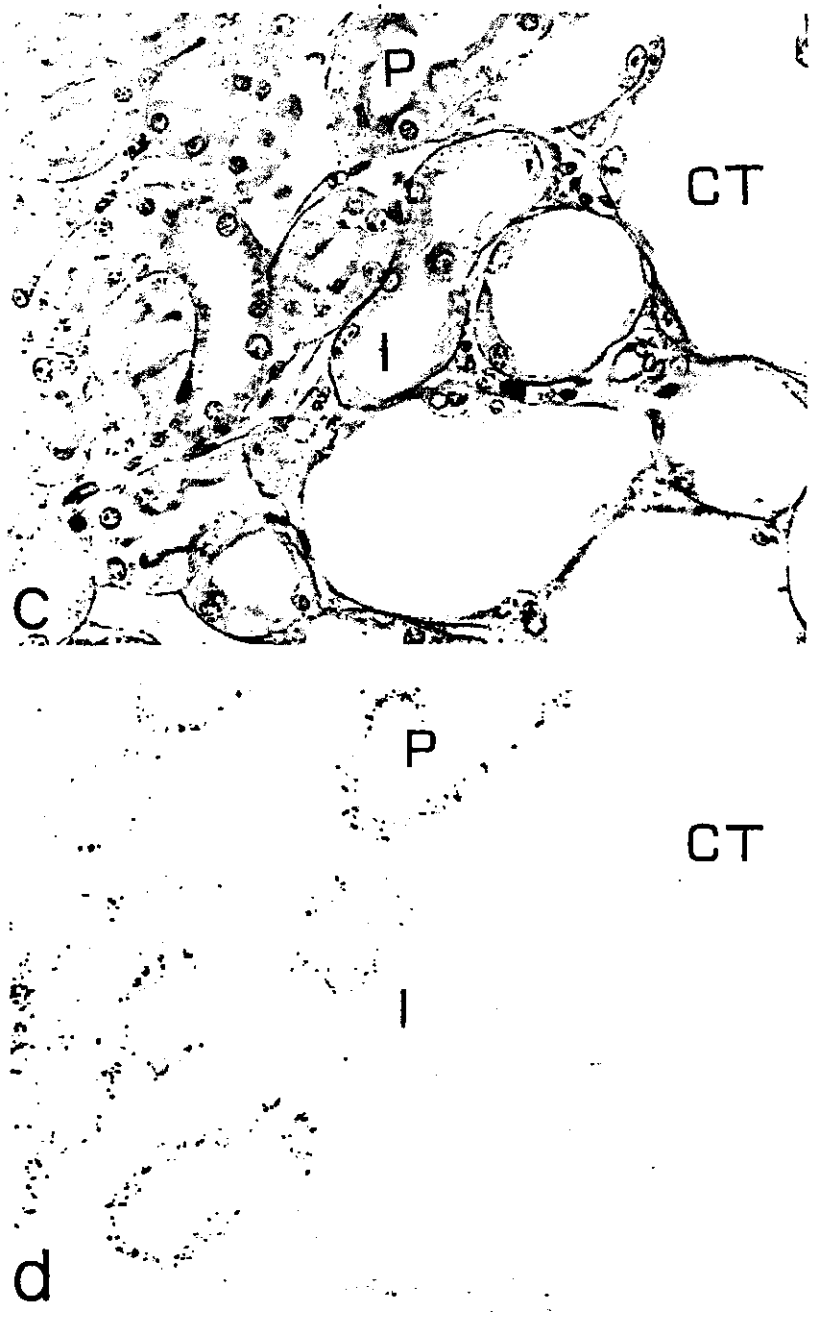
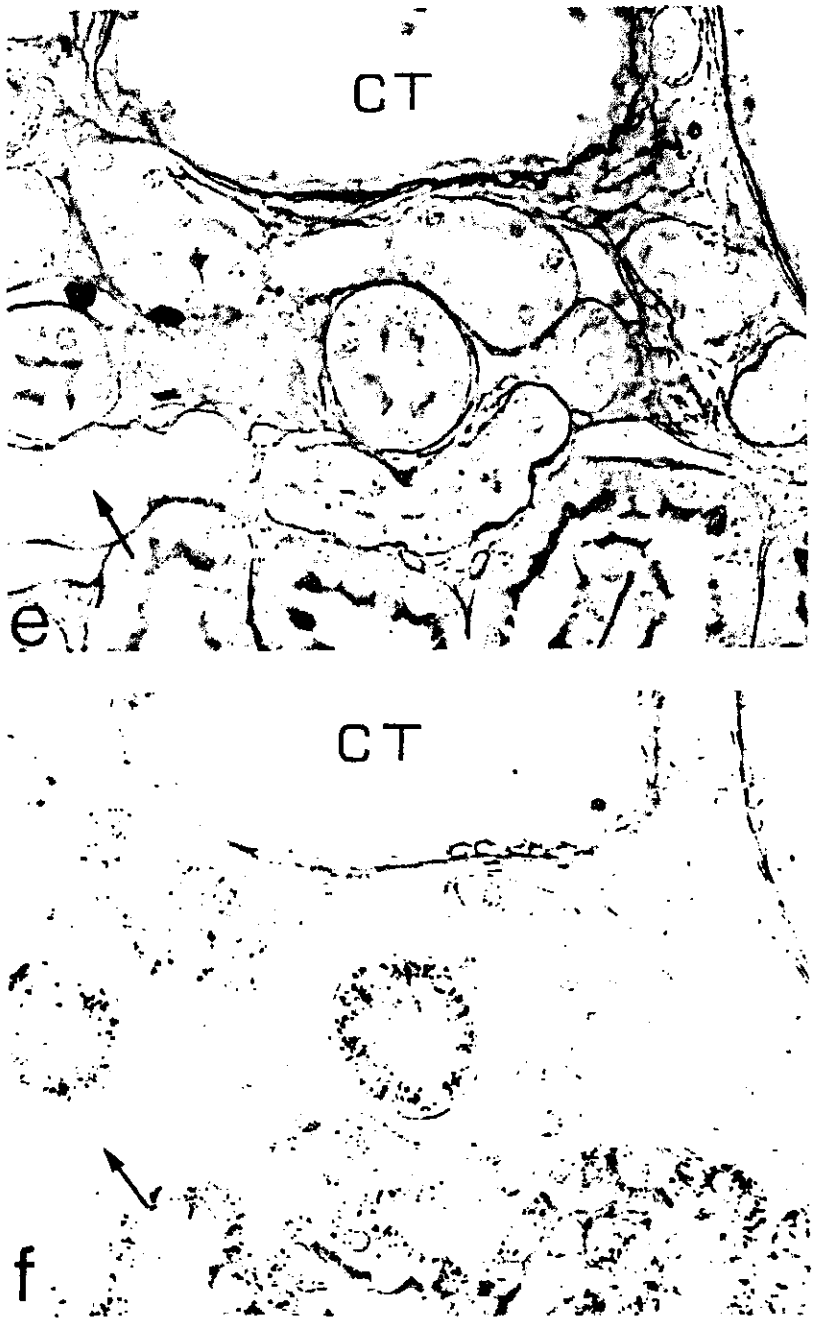


Figure 7. Continued.

to tubular insult and consisting of a fall in glomerular filtration rate is typically reversible, as reflected by the rapid return of functional markers to baseline values. In this regard, cisplatin stands as an exception since in both clinical studies and animal experiments this drug has been found to induce persistent renal dysfunction (5, 40). This was confirmed by our own measurements of BUN and serum creatinine, which showed only a transient increase in tobramycin-treated rats whereas animals exposed to cisplatin exhibited a marked tendency to develop chronic functional impairment, indicated by a sustained elevation of these functional markers.

As revealed by the observations of Dobyan et al. (17, 18) and of our group (19, 20), persisting renal dysfunction caused by cisplatin administration to experimental animals is



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associated with a progressive deterioration of tubular epithelium particularly affecting proximal straight tubules and culminating in the development of tubular cysts. In a previous study (19), the extent and duration of renal tissue repair in the kidneys of rats exposed to cisplatin was evaluated by measuring the rate of DNA synthesis in renal tissue. We found that the initial episode of tubular necrosis was followed by a protracted increase of DNA synthesis, in contrast with what has been reported for other nephrotoxic agents such as mercuric chloride (41) or gentamicin (10). Here also, the immunohistochemical analysis of cell proliferation suggests that kidneys damaged by cisplatin do not completely return to a normal rate of cell turnover even though interindividual variations (and maybe other factors such as tissue edema or the presence of cystic tubules) cause a lack of statistical

significance. Along with the long-lasting morphological abnormalities and renal dysfunction, these data suggest that cisplatin nephrotoxicity involves somehow a defect in the control mechanisms underlying postnecrotic tubular regeneration.

The repair of tubular necrosis occurs through regenerative hyperplasia and is also accompanied by a temporary dedifferentiation of tubular epithelium. From a mechanistic point of view, this process appears particularly complex since it implies a controlled modification of cell behavior. It undoubtedly depends on cell–cell interactions and interactions between cells and the extracellular matrix, and also requires the participation of extracellular mediators acting as physiological regulators. In this respect, recent advance has led to the unraveling of changes in gene expression related to regenerative hyperplasia (42, 43). Other reports also point to extracellular signals, namely growth factors, which might play a role in the control of cell proliferation and/or differentiation in the kidney (22). In this study, we have chosen to concentrate on EGF since various experimental data support the view that it could contribute as a renotrophic factor to tubular regeneration. Among these data are the facts that EGF binding sites have been demonstrated on proximal tubular epithelium, and that cultured kidney-derived epithelial cells respond to the mitogenic effect of EGF *in vitro* (44–46). EGF has also been shown to enhance tubular regeneration and accelerate the recovery of renal function after acute renal failure of ischemic (23, 24) or nephrotoxic (25–28) origin.

In our experiments, EGF was demonstrated in renal tissue by immunohistochemistry, and also measured by radioimmunoassay in tissue homogenates and soluble fractions prepared by high-speed centrifugation. As discussed in a previous publication (30), EGF immunostaining in the kidney most probably reflects the membrane-bound precursor synthesized in distal tubules, with little if any contribution of soluble EGF. In a similar fashion, our radioimmunoassay data indicate that in control kidneys soluble EGF represents only a minor fraction of total EGF assayed in tissue homogenates. Therefore, we believe that the reduction of EGF immunostaining and the decrease of total immunoreactive EGF measured in kidneys of drug-treated rats is best accounted for by a diminution of proEGF. Two possible reasons can be advanced to explain this observation. On the one hand, hybridization studies of Safirstein et al. (47, 48) and of Verstrepen et al. (49) have revealed that acute tubular necrosis due to renal ischemia or resulting from treatment with cisplatin or gentamicin leads to a marked depletion of mRNA coding for preproEGF. Such a finding, which suggests a temporary switching off of preproEGF gene expression, has been taken as evidence against a role of EGF as a renotrophic factor during tubular regeneration (49). On the other hand, we have recently reported that, prior to the regenerative response subsequent to tubular necrosis induced by ischemia, the fall of proEGF is accompanied by a concomitant and commensurate elevation of soluble immunoreactive EGF (30). Increase of soluble renal EGF has also been demonstrated after treatment with mercuric chloride (50). These observations suggest a proteolytic conversion of proEGF into soluble, mature EGF (29) and have been put forth as an argument in favor of a paracrine action of EGF as a positive regulator of regenerative hyperplasia (30). Actually, data generated by hybridization techniques or obtained by radioimmunoassay are not necessarily conflicting since EGF might be required as a mitogenic signal only for a short period of time at the onset of tubular regeneration.

At variance with what we found previously in postischemic kidneys (30), our measurements did not disclose a significant increase of soluble EGF in kidneys exposed to tobramycin or cisplatin. This raises the possibility that EGF might not be the most important mediator involved in the process of regenerative hyperplasia subsequent to

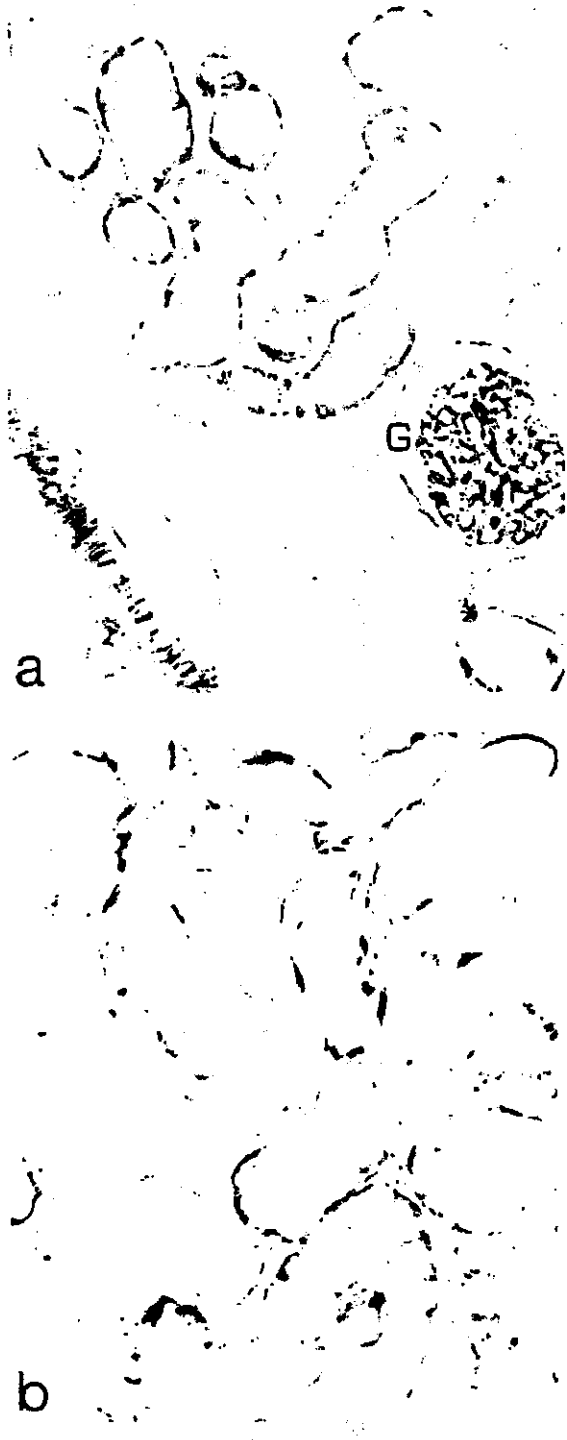

Figure 8. Immunostaining of vimentin in kidneys of animals treated with tobramycin (a) or cisplatin (b, c, d). Micrographs illustrate kidney necropsies collected 7 days (a-b) or 60 days (c-d) after drug exposure. Immunohistochemical staining of vimentin was performed as indicated in Methods. Under normal circumstances, vimentin is not present in tubular epithelium (not shown). Seven days after treatment with tobramycin (a) or cisplatin (b), numerous undifferentiated tubular profiles exhibit vimentin. Vimentin expression can also be noted in glomeruli [(a), G] and blood vessels [(a), V], but this is also observed in control kidneys. In kidneys exposed to tobramycin, tubular epithelium lose rapidly vimentin expression (not shown). In contrast, 60 days after exposure to cisplatin (c-d), vimentin immunostaining is still apparent in cystic tubules [(c-d), CT] or tubules devoid of differentiation characters [(c), I] (a, b, c: ×336; d: ×725).

Figure 8. Continued.

nephrotoxic insult. Indeed, other growth factors such as IGF-I (28, 51), HGF (52), or even FGF-1 (53) have been proposed as plausible candidates for the positive regulation of nephrogenic repair. However, one must also realize that while soluble EGF is produced by an enzymatic processing of the precursor, it is also likely to be taken up and degraded by target cells, such as proximal tubules cells which possess the receptor (see below). This latter phenomenon could prevent a significant accumulation of soluble EGF. Another possibility would be an elimination of soluble EGF in tubular fluid. Even though we did not assay urinary EGF, we consider that a massive loss of renal soluble EGF through that

pathway is hardly conceivable since it has been shown previously that the concentration of urinary EGF sharply decreases after tubular necrosis (48). Anyway, we surmise that, during the period which immediately follows tubular injury, the concentration of renal soluble EGF could obey a complex kinetics, making it hard to observe a significant accumulation of soluble EGF in every experimental circumstance. As compared to the ischemia model, increase of soluble EGF might also be more progressive, and thus more readily counterbalanced by EGF consumption in the tobramycin and cisplatin models where several days of treatment are required to induce substantial tubular necrosis. However, it must be kept in mind that soluble EGF does not undergo a decrease parallel and commensurate with that of total EGF, in contrast with what would have been expected if the variation of renal EGF had merely resulted from depressed transcription and protein synthesis.

If it is true that EGF exerts a mitogenic effect contributing to tubular regeneration, this implies that the growth factor must interact during this process with receptors displayed by target cells. In our study, we used a polyclonal antiserum raised against a 12-residue sequence within the cytoplasmic domain of the EGF receptor (33) in order to immunolocalize the latter in kidneys of experimental animals. Upon immunostaining of tissue sections in control kidneys, immunoreactive EGF receptors exhibited a quite specific and restricted distribution since they were confined to proximal tubules and juxtaglomerular cells. In proximal tubules cells, immunocomplexes were particularly apparent at the contraluminal side. This result confirms data that we obtained in a previous study (31) and appears in good agreement with EGF binding studies performed by other investigators (45, 54–56). The presence of immunoreactive EGF receptors in juxtaglomerular cells, which are the site of prorenin synthesis, emerges as an original finding and its possible physiological implications are still to be uncovered. It has been reported that angiotensin II, whose production is mediated by renin, potentiates EGF mitogenic effect on kidney proximal tubules cells (38). Maybe also of significance is the fact reported recently that EGF behaves as a potent inhibitor of renin secretion (57).

After renal tissue insult caused by tobramycin or cisplatin, tubular sections became negative for EGF receptor immunostaining. Strikingly, the disappearance of immunoreactive EGF receptors did not occur during the early phase of tubular necrosis but was more characteristic of regenerating tubular segments lacking differentiation characters and displaying signs of extensive proliferation. In kidneys exposed to tobramycin, alteration of EGF receptor expression was reversible and a normal pattern of immunostaining was recovered after completion of tubular regeneration, whereas after exposure to cisplatin, tubular sections—particularly cystic tubules and tubules lined by an undifferentiated epithelium—remained devoid of detectable EGF receptors. A transient disappearance of EGF receptors in tubular epithelium has already been found during tubular regeneration following ischemic tubular necrosis (31). As discussed previously, the apparent loss of EGF receptors could be due to a process of downregulation. Binding of EGF to its receptor is indeed followed by the endocytosis of the complex which is transferred to the lysosomal compartment. Although the kidney has not been examined in this respect, in liver parenchyma EGF–receptor complexes have been clearly shown to undergo degradation in lysosomes (58, 59). In agreement with a putative phenomenon of receptor downregulation is the fact that the disappearance of EGF receptor immunostaining occurs precisely in tubules displaying evidence of a regenerative response.

Other investigators have reported that acute tubular necrosis due to ischemia (48) or folic acid intoxication (55) is followed by an increase of EGF binding in renal tissue, i.e., a process of receptor upregulation. Our own data neither support nor invalidate these observations. Although an increase of immunoreactive EGF receptors might theoreti-

cally be reflected by stronger immunostaining, the intensity of immunohistochemical staining may also depend on other factors unrelated to the actual density of antigenic site and difficult to assess in an objective manner. On the other hand, since we clearly show that the disappearance of EGF receptors during the regenerative response only involves a fraction of renal tubules, this modification might be difficult to demonstrate in binding studies which are performed on membranes isolated from whole-kidney tissue.

As an alternative, the apparent loss of EGF receptors might be attributed to the dedifferentiation of tubular epithelium occurring in the course of tubular regeneration. To explore this possibility, renal tissue sections were processed for the immunohistochemical demonstration of vimentin. Earlier studies indeed indicate that this intermediate filament might be a useful marker of the epithelium dedifferentiation associated with tubular regeneration (60). In unaltered kidney, distribution of immunoreactive vimentin is restricted to glomeruli, cortical blood vessels, and medullary vasa rectae (31, 60). However, during the repair of nephrotoxic (60) or ischemic (31) tubular injury, a transient expression of vimentin can be evidenced in the epithelium of regenerating tubules. Our observations on tobramycin-treated rats confirm the fact that vimentin immunostaining appears in tubular epithelium during a short period of time during tubular regeneration and is most prominent after the peak of regenerative hyperplasia. A similar sequence of events has been found by Wallin et al. (60), who expressed the opinion that vimentin expression is more associated with cell motility than with cell proliferation. Moreover, vimentin expression clearly occurs well after the loss of EGF receptors. Thus, EGF receptors do not seem to disappear as a mere consequence of epithelium dedifferentiation. However, one must point out that, in the long term after cisplatin exposure, altered tubules remain positive for vimentin immunostaining and devoid of EGF receptors. This suggests that, in tubular epithelium damaged by cisplatin, a permanent loss of EGF receptor can be associated with an inability to redifferentiate.

The data reported here confirm and extend previous findings (10, 30, 31) on postnecrotic regenerative hyperplasia in the kidney. As illustrated by the example of tobramycin-induced insult, renal tissue repair is characterized by transient modifications of EGF and EGF receptors accompanying an increase of mitotic activity. Moreover, the cell dedifferentiation associated with the proliferative response also appears mostly reversible. One can reasonably surmise that a similar reversibility would be observed in other cases of nephrotoxin-induced injury. In sharp contrast, kidney tissue changes associated with cisplatin nephrotoxicity show a marked tendency to become permanent. In particular, some nephrons do not recover the capacity of producing EGF and EGF receptors, whereas tubular segments show sustained expression of an intermediate filament normally absent in differentiated epithelium. It is interesting to note that a defective expression of EGF gene has also been reported in some forms of inheritable murine polycystic kidney disease (61, 62). This unexpected evolution of nephrotoxic lesions after cisplatin remains intriguing but is consistent with the well-known interactions of this type of agent with cellular DNA (Ref. 63; see also discussion in Ref. 20). Thus, DNA damage caused by cisplatin might lead to irreversible changes in genomic expression, possibly resulting in a severe imbalance in the control of cell proliferation and differentiation in the kidney.

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