Distribution of epidermal growth factor in the kidneys of rats exposed to amikacin

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**Distribution of epidermal growth factor in the kidneys of rats exposed
to amikacin.** The distribution of epidermal growth factor (EGF) was
examined by immunocytochemistry in the kidneys of rats exposed to
amikacin, an aminoglycoside antibiotic causing tubular necrosis at high
dose. Five-animal groups were treated for 4 or 10 days with amikacin at
daily doses of 15, 40, 80 or 200 mg/kg. The drug was delivered i.p. twice
a day. One hour before termination, each rat received an i.p. injection
of [³H] thymidine to evaluate DNA synthesis in renal tissue. After
sacrifice, the kidneys were processed for morphological (semithin and
paraffin sections) and biochemical analysis (measurement of DNA
synthesis by [³H] thymidine incorporation in vivo). Amikacin induced
in proximal tubules a dose-related lysosomal phospholipidosis, which
was assessed by the morphometric evaluation of altered lysosomes
("myeloid bodies") on semithin section. However, frank evidence of
acute tubular necrosis was only observed in rats receiving amikacin at
a daily dose of 200 mg/kg. Concomitantly with the development of
tubular necrosis, there was a rise in the rate of cell turnover, reflected
by an increase of DNA synthesis in renal tissue. This sign of tubular
regeneration was accompanied by a redistribution of EGF immunoreac-
tivity, as revealed by immunocytochemical staining. Within renal
cortex of control rats, EGF immunoreactivity predominantly appeared
in distal tubules and collecting ducts (97% of examined tubular sec-
tions). In contrast, in treated animals where the renal cortex displayed
evidence of tubular necrosis/regeneration, EGF immunoreactivity was
frequently associated with proximal tubules (more than 30% of exam-
ined tubular sections, as compared to 3% in controls). This change in
the topography of EGF immunoreactivity suggests that the growth
factor might be involved in the process of tissue repair consecutive to
drug-induced tubular necrosis.

Although the kidney is prone to hypoxic or nephrotoxic insult
eventually resulting in tubular necrosis [1], injury is usually
followed by a tissue repair reaction which tends to compensate
for the loss of tubular epithelium. Renal tubular regeneration is
a well known phenomenon in pathology [2], and has been
characterized both qualitatively and quantitatively in experi-
mental studies on animals exposed to various nephrotoxins [3,
4]. It involves an increase of cell turnover, occurring primarily,
but not exclusively, in the area of tubular epithelium which has
suffered injury [5–7]. Frequently, tubular regeneration is also
associated with a transient dedifferentiation of epithelial cells,
and is particularly conspicuous in the proximal sections of the
nephron [8–10]. In addition, the stimulation of epithelial cells
proliferation is often accompanied by a mild fibroblastic hyper-
plasia in renal interstitium [10, 11].

In contrast with the renal compensatory growth observed
after unilateral nephrectomy [12, 14], tubular regeneration has
remained mostly unexplored with regard to its mechanism.
Several experimental facts suggest, however, that the process is
submitted to a biological control, probably involving endoge-
nous regulators. First, the increase of cell proliferation must
involve a transition from quiescence (G0 phase) to G1 phase of
the cell cycle, since in the normal state the rate of cell turnover
is very low in renal tissue [15]. Second, the extent of the
proliferative response remains commensurate with the degree
of tubular injury and does not entail detectable increase in renal
mass [16, 17]. Third, the wave of cell divisions and the apparent
loss of differentiation which follow tubular necrosis are gener-
ally transient, the kidney returning to its basal cell kinetics and
the tubular cells regaining their differentiation characters after
repair of the lesions [8, 9]. Finally, the proliferative activity
associated with tubular regeneration seems to occur indepen-
dently of circulating factors [18].

During the last decade the concept has emerged that the
division and differentiation of eucaryotic cells are controlled by
polypeptide growth factors [19, 20] acting in paracrine or
autocrine fashion. For the kidney in particular, several growth
factors have been proposed to regulate cell proliferation [21].
Recently, two studies have shown that the administration of
epidermal growth factor (EGF) enhances tubular regeneration
and accelerates the recovery of normal renal function following
postischemic acute renal failure [22, 23]. Thus, the present
study examines the distribution of EGF immunoreactivity in
renal tissue after treatment with amikacin, a nephrotoxic ami-
noglycoside antibiotic.

Methods

Animals and treatment

Female Sprague-Dawley rats were used throughout the
study. The animals were purchased from a commercial breeding
farm (Iffa-Credo, l'Arbresle, France) and allowed one week of

acclimatation before starting the treatment. During the acclimatation and treatment periods, the rats were maintained in an animal facility with a regular 12-hour light/dark cycle, and had free access to tap water and rodent chow (type AO4, U.A.R., Villemoisson-sur-Orge, France). The weight of the animals, measured prior to the first amikacin dosing, was found roughly similar for all individuals (mean weight: 204 g, with less than 5% deviation from that value).

Amikacin solutions were prepared in 0.9% NaCl from the ready-to-use preparation available for clinical practice (Amukin®, Laboratoires Bristol Benelux, Brussels). Five-animal groups were treated for 4 or 10 days with amikacin at daily doses of 15, 40, 80 or 200 mg/kg. The drug was delivered i.p. in two daily injections (9:00 a.m. and 6:00 p.m.), the volume of injected solution (approx. 0.5 ml) being adjusted each time according to body weight. Control animals received an equivalent amount of 0.9% NaCl following the same protocol.

One hour prior to sacrifice, 200 μCi of [methyl-^{3}H] thymidine (40 Ci/mmol, Amersham International, Amersham, UK) was injected i.p. to each rat, for the measurement of DNA synthesis in renal tissue.

Sacrifice and sampling of renal tissue

The animals were terminated by decapitation 15 hours after the last drug administration. Following laparotomy, the kidneys were quickly exposed. After bisection, one half of the right kidney was immersed in Duboscq-Brazil fixative for light microscopy. Renal cortex was separated from the other half by sharp dissection and diced in 1 mm^3 blocks after immersion in glutaraldehyde fixative for electron microscopy (2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4). The left kidney was also bisected and the renal cortex separated by dissection. Kidney cortex from the left kidney was snap-frozen in dry ice and stored at $-20°C$ until biochemical analysis.

Preparation of specimens for morphological analysis

Paraffin sections. Kidney samples fixed in Duboscq-Brazil fluid were rinsed twice in 70% ethanol and once in butanol. After proper dehydration following routine procedure, the samples were embedded in Paraplast plus® paraffin. Sections of 4 to 5 μm thickness were cut on a Reichert Autocut 2040 microtome equipped with a glass knife, and mounted on glass slides. For standard morphological examination, renal tissue was stained with periodic acid-Schiff, hemalun and luxol fast blue.

Morphological demonstration of EGF immunoreactivity. EGF immunoreactivity was revealed on paraffin tissue section by an immunogold-silver staining procedure, as described previously [24]. Dewaxed paraffin sections were rehydrated with phosphate-buffered saline, pH 7.4 (PBS) and pretreated for 20 minutes with 5% normal goat serum in the same buffer, to prevent nonspecific adsorption. Thereafter, the sections were incubated overnight at 4°C in presence of rabbit anti-mouse EGF (BioMakor, Rehovot, Israel) (1:150 dilution in PBS containing 5% normal goat serum). Treatment with the primary antibody was followed by a one hour exposure to goat anti-rabbit IgG antiserum adsorbed on 5-nm colloidal gold particles (Auro Probe LM, Janssen Biotech, Olen, Belgium) (1:50 dilution in the same buffer). After several rinses in PBS and distilled water to eliminate the excess of labeled antibody, immunogold

labeling was enhanced by short-term treatment (6 to 10 min) with silver staining reagent (Inten SE M, Janssen Biotech, Olen, Belgium). Finally, silver enhancement was arrested by rinsing in distilled water.

According to the supplier's specification, polyclonal anti-EGF antibody was raised against highly purified EGF from mouse submaxillary glands, thus minimizing the risk of cross reactions. To check the specificity of the immunocytochemical labeling, the primary antibody was replaced by non-immune rabbit serum or by anti-EGF antiserum preincubated with an excess of antigen (70 μg/ml purified EGF). These controls were negative. In addition, we verified that anti-EGF antiserum gave positive staining in sections of mouse submaxillary gland. Finally, the same anti-EGF antiserum was used to reveal EGF immunoreactivity in mouse renal tissue. The staining pattern was similar to that reported in this study for the kidney of control rats.

Semithin sections. Renal cortex specimens were kept in buffered glutaraldehyde solution for four hours at 4°C. The blocks were thereafter rinsed overnight in 0.1 M cacodylate buffer, pH 7.4 containing 4% sucrose. After three rinses in buffered sucrose solution, the blocks were postfixed in 2% osmium tetroxide (2% OsO$_4$ in 0.04 M veronal-acetate buffer, 5.6% sucrose, pH 7.4), dehydrated in graded acetone solutions and rinsed in propylene oxide. The samples were embedded in Epon 812 and semithin sections (1 to 2 μm thick) were cut with a glass knife on a LKB type 4801A ultramicrotome. Sections were mounted on glass slides and stained with toluidine blue before examination.

Morphological analysis

Semiquantitative assessment of drug-induced injury. Sublethal alterations and tubular necrosis associated with amikacin nephrotoxicity were submitted to single-blind evaluation following an approach similar to that developed previously for the study of cisplatin nephrotoxicity [17], except that only proximal tubules were considered. Briefly, one paraffin section per experimental animal was examined by an observer who was kept unaware of the treatment. Twenty 0.084 mm^2 fields were scanned at 400-fold magnification and morphological abnormalities in proximal tubules located in each field were graded along an arbitrary scale: 0, no departure from normal morphology as observed in control animals; 1, abnormal vacuolization, PAS-positive inclusions or focal cell necrosis; 2, at least one necrotic tubular section (more than half of the necrotic tubular section(s) remaining identifiable); 3, same as 2, but less than half of the necrotic section(s) remaining identifiable; 4, totally necrotic tubule(s) of unidentifiable origin. For each animal, an average score was calculated to express the level of tubular injury.

Morphometric evaluation of lysosomes in proximal tubules. The relative size of lysosomes in proximal tubules was evaluated by a computer-aided morphometric approach, using a software specifically designed for color analysis and morphometry (Système d'Analyses Microscopiques à Balayage Automatique, SAMBA, Alcatel TITN Answare, Grenoble, France), and relied on the fact that, in semithin sections, lysosomes exhibit a blue coloration after staining with toluidine blue. One tissue section per animal was examined at 400-fold magnification on a Zeiss Axioplan light microscope equipped with a high-resolution JVC KY.15 color video camera. Approximately
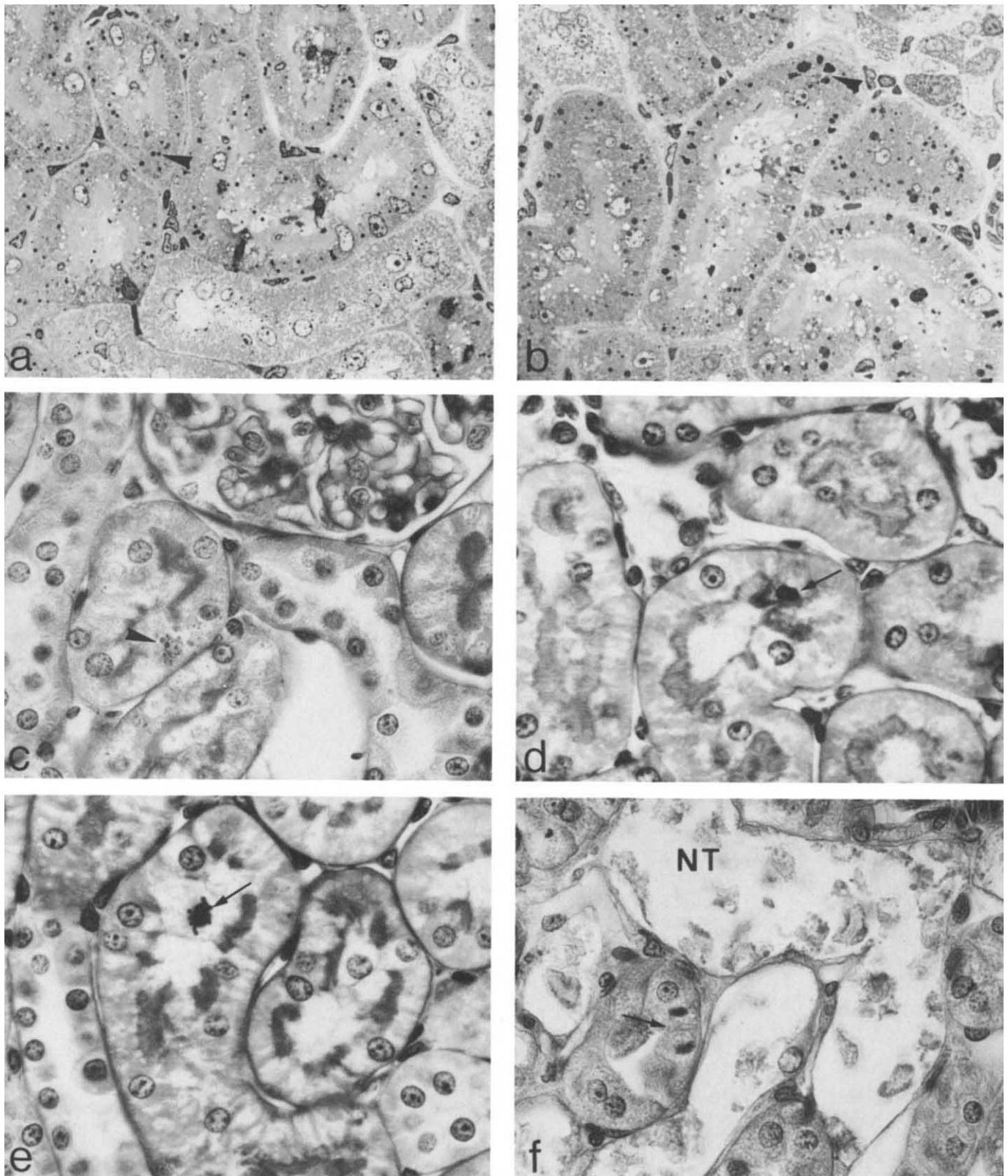
Fig. 1. *Morphological alterations and tubular necrosis induced by amikacin in rat kidney.* **A** and **B** illustrate the light microscopic appearance of renal cortex on semithin sections obtained from control and treated (40 mg/kg · day, 4 days) animals, respectively. After amikacin administration, lysosomes (arrowheads) increased in size and exhibited a heterogenous content (× 540). **C** through **F** are paraffin sections of kidneys from treated rats. **C.** PAS (+) vacuoles (arrowhead) in a proximal tubular cell (80 mg/kg · day amikacin, 10 days). **D.** Focal cell necrosis (pycnotic nucleus, arrow) in a proximal tubule (80 mg/kg · day amikacin, 4 days). **E.** Mitotic figure (arrow) in a proximal tubule (80 mg/kg · day amikacin, 4 days). **F.** Tubular necrosis (NT). The arrow indicates a mitotic figure in a neighboring tubular section (200 mg/kg · day amikacin, 10 days) (× 645).
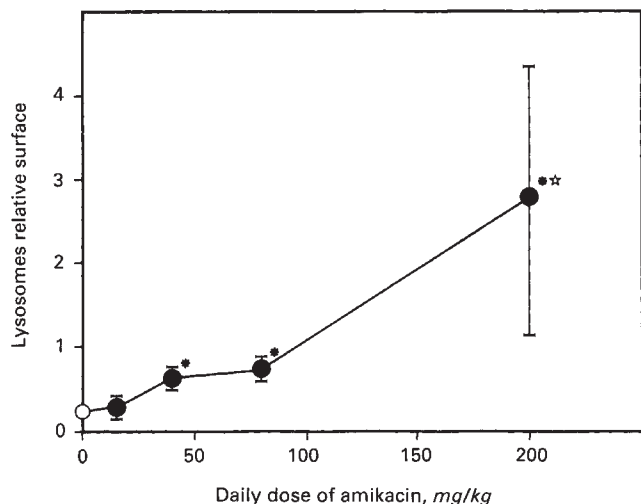
Fig. 2. *Increase of size of lysosomes in kidney proximal tubules of rats treated with amikacin (4 days, closed circles).* The total area occupied by lysosomes in proximal tubular cells was evaluated by morphometric approach and expressed relative to tubular sections (see **Methods** sections). Each symbol refers to the mean value calculated for the group (N = 5), the vertical bar indicating the standard deviation. The open circle corresponds to control animals. Statistical difference between data ($P < 0.05$, Student's t-test) is shown by the asterisks (values significantly higher than mean control value) and the star (value significantly higher than all other values).

10 fields were successively scanned by the camera and processed by a MVP-AT/NP imaging system implemented in an IBM-compatible microcomputer (Compaq type AT 386/25). Proximal tubular sections picked at random for morphometric analysis were delineated in interactive mode and their area was computed. In each tubular section, lysosomes were detected by setting a lower threshold in gray levels (green channel) and the aggregated area of these organelles was calculated relative to the area of the corresponding tubular section. Approximately 25,000 μm^2 of tubular sections were thus analyzed per experimental animal.

Measurement of DNA synthesis in renal tissue

The rate of DNA synthesis was evaluated by measuring the extent of [^{3}H] thymidine incorporation into renal tissue DNA, as described previously [25]. Briefly, renal tissue samples were homogenized in distilled water and DNA was isolated by acid precipitation [26]. The concentration and radioactivity of the nucleic acid were measured by colorimetry [27] and liquid scintillation counting, respectively. The amount of incorporated [^{3}H] thymidine was inferred from DNA specific radioactivity.

Statistical analysis

The data were compared statistically on the basis of Student's t-test.

Results

Effect of treatment on animal growth and viability

Even at high dose, amikacin administration was well tolerated by the experimental animal. No rat was lost during the treatment period or even showed overt evidence of systemic toxic reaction. During the 10 day period of aminoglycoside

dosing, a mean weight gain of 8.7% was observed in the animals receiving a daily dose of 200 mg/kg amikacin (8.4% in controls).

Lysosomal phospholipidosis and histopathological alterations

The kidneys of treated animals were examined by light microscopy to disclose signs of sublethal alterations and of drug-induced tubular necrosis. After four days of amikacin administration proximal tubular cells already exhibited the morphological abnormality typical of aminoglycoside-induced phospholipidosis [28–30], as revealed by examination of semi-thin sections of renal cortex. Lysosomes appeared enlarged, irregular in shape and showed a heterogenous content (Fig. 1 A and B). The global size of the lysosomes, evaluated by morphometric analysis, increased in parallel with the daily dosage of amikacin (Fig. 2).

Although signs of aminoglycoside-induced phospholipidosis (particularly the heterogeneity of lysosomal content) were already apparent in animals treated with the lowest dose (15 mg/kg) of amikacin, frank tubular necrosis was only observed in paraffin sections of kidneys from animals treated for 10 days with 200 mg/kg amikacin (Fig. 1F). At lower doses and in animals treated for four days, proximal tubules displayed mild alterations, such as an accumulation of PAS(+) vacuoles in epithelial cells (Fig. 1C), focal cell necrosis (Fig. 1D) and evidence of increased cell turnover (frequent occurrence of mitotic figures; Fig. 1E).

### Proliferative response and distribution of EGF immunoreactivity

Figure 3 shows the rate of DNA synthesis measured in the renal cortex tissue of rats treated with various doses of amikacin. Consistently with the fact that mitotic figures appeared repeatedly in proximal tubules of treated rats, amikacin administration led to a stimulation of cell proliferation in the kidney with a concomitant increase of DNA synthesis. At low doses and/or after the four-day administration, amikacin only exerted a minimal effect, but the aminoglycoside produced a more substantial increase of DNA synthesis (more than three-fold the mean control value) when given at high doses and/or for 10 days. Although the histological nature of proliferating cells was not determined in the present experiments, previous studies [16] on the proliferative response occurring in rat kidney after amikacin administration indicate that this reaction primarily involves proximal tubular cells, with only a minor contribution of interstitial cells.

When the rate of DNA synthesis was plotted against the degree of drug-induced morphological alterations and tubular necrosis, the regression analysis gave a high correlation coefficient ($r = 0.93$), indicating a parallelism between tissue injury and the proliferative response (Fig. 4).

As illustrated in Figure 5, the immunocytochemical procedure clearly demonstrated the presence of anti-EGF reactive material within epithelial cells of renal tubules. In control animals, EGF immunoreactivity was mostly confined to distal tubules and collecting ducts located in the inner stripe of outer medulla, but could also be found occasionally within renal cortex, associated with distal tubules (Fig. 5 A and B). Among tubular sections reacting positively with anti-EGF, few (3% or less of stained sections) belonged to the proximal section of the
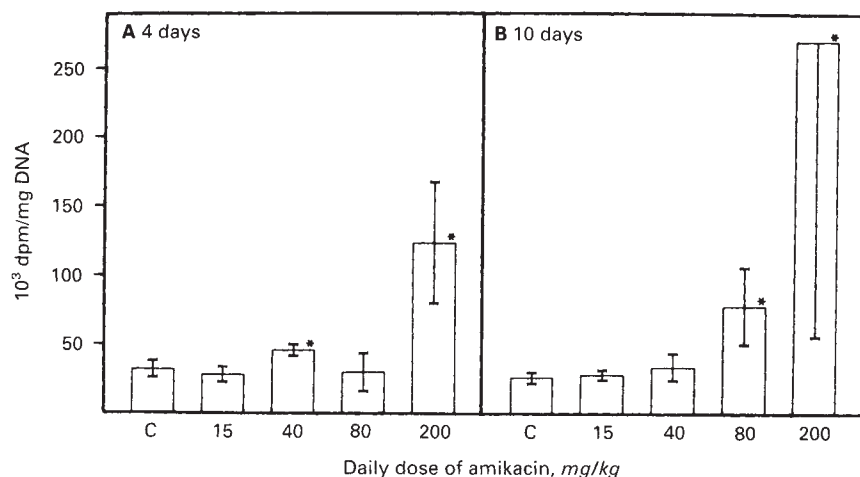
Fig. 3. *Rate of DNA synthesis in renal cortex tissue of rats treated with amikacin.* DNA synthesis was estimated by the extent of [^{3}H] thymidine incorporation into DNA (**Methods**). Each column refers to the mean value of each group ($N = 5$), with the standard deviation shown by the vertical bar. Statistical difference ($P < 0.05$, Student's test) between treated and control (c) rats is indicated by asterisks.
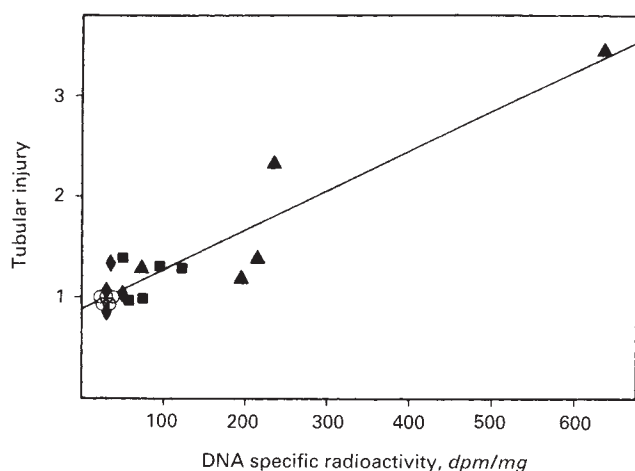
Fig. 4. *Relationship between the rate of DNA synthesis (abscissa) and the degree of tubular injury (sublethal alterations and tubular necrosis evaluated on paraffin sections,* **Methods***) (ordinate).* The analysis was performed on animals treated for 10 days with daily doses of 15 mg/kg amikacin (○), 40 mg/kg amikacin (◆), 80 mg/kg amikacin (■) and 200 mg/kg amikacin (▲). The correlation coefficient r was calculated by regression analysis; $r = 0.93$.
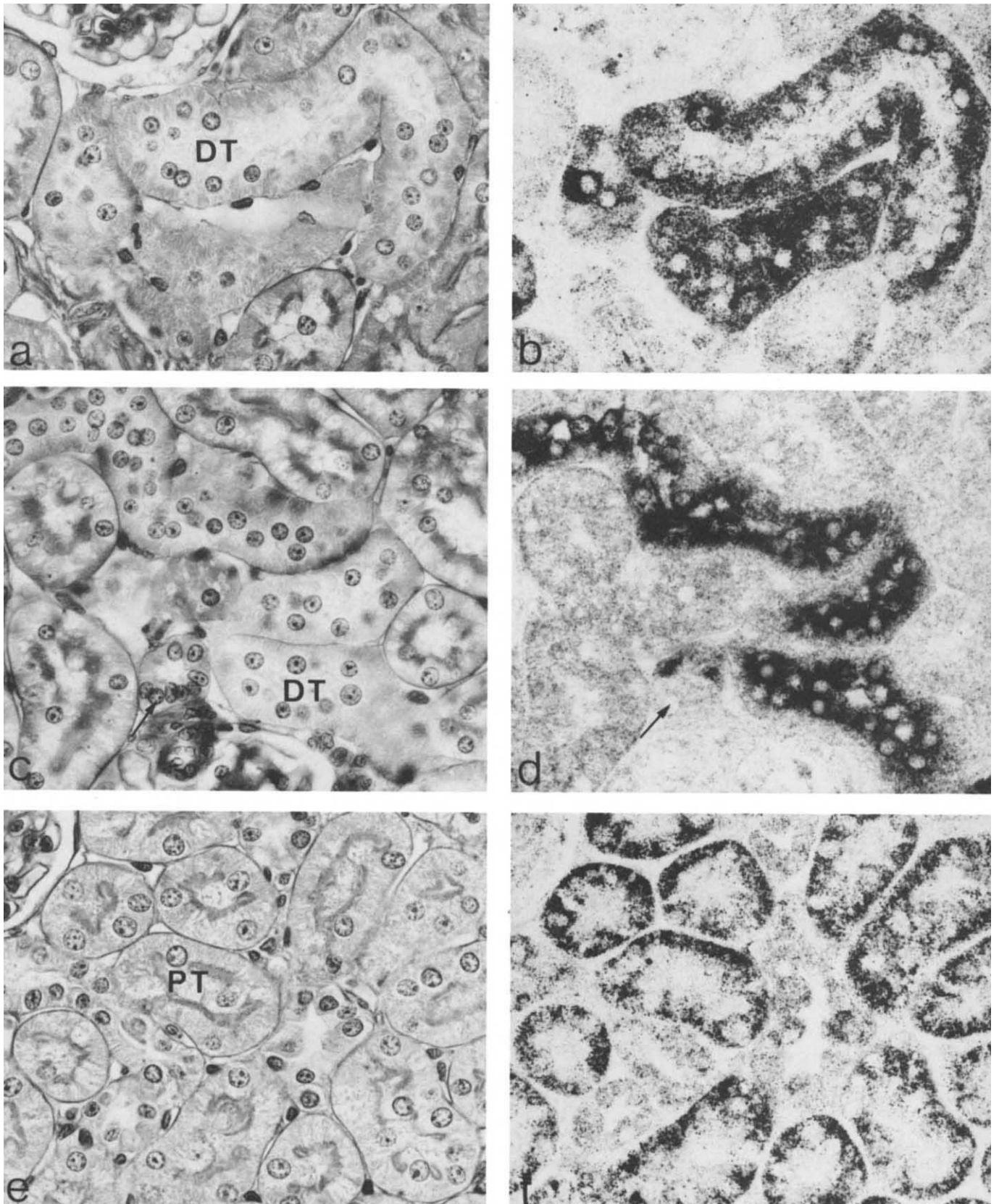
Discussion

A variety of nephrotoxic compounds are known to cause acute tubular necrosis [31–34]. In this study, amikacin was selected as a model because it belongs to the group of aminoglycoside antibiotics, drugs for which the mechanism of nephrotoxicity is well documented [reviewed in 35–37].

Aminoglycosides accumulate within proximal tubular cells [38] where they induce a lysosomal phospholipidosis [9, 28–30, 39], this metabolic disturbance resulting from an impairment of phospholipid catabolism [40–42]. The causal relationship between phospholipid accumulation in proximal tubules and the ensuing tubular necrosis is particularly well substantiated by the effect of protective agents [43–47], namely anionic polypeptides which both prevent the development of aminoglycoside-induced phospholipidosis and alleviate tubular injury. From comparative in vitro and in vivo studies, it has become clear that different aminoglycoside derivatives, varying in their propensity to interfere with phospholipid enzymatic hydrolysis, are also endowed with a different nephrotoxic potential [29, 48, 49]. In this respect, amikacin has been demonstrated to behave as a mild nephrotoxin.

Therefore, the use of amikacin as a model to study tubular regeneration after nephrotoxic tissue injury presents further advantage. The administration of ascending doses enables one to create the whole spectrum of renal insult (from mere cytological abnormality to frank tubular necrosis). However, it becomes feasible to differentiate early metabolic alterations from cell death. Indeed our results show that, at low doses of amikacin, the proximal tubule already shows evidence of drug-induced phospholipidosis, whereas the administration of higher doses is necessary to obtain tubular necrosis.

This point appears particularly crucial since, in amikacin-treated rats, a sizeable change in the localization of EGF immunoreactivity was not observed concomitantly with the formation of myeloid bodies (the morphological manifestation of the lysosomal phospholipidosis), but only occurred in the kidneys showing evidence of tubular necrosis and regeneration.

The staining pattern that we obtained after EGF immunolocalization in the kidneys of control rats did not show major differences with that appearing in the previous studies of Salido et al on the mouse kidney [50, 51], except for the presence of

nephron. No drastic change in the pattern of EGF immunoreactivity was found in treated animals, up to 80 mg/kg · day. After the four-day treatment with 200 mg/kg · day amikacin, distal tubules located in renal cortex exhibited strong positive reaction after immunocytochemical staining (Fig. 5 C and D). Strikingly, in animals dosed for 10 days with amikacin at 200 mg/kg · day, the topography of EGF immunoreactivity appeared drastically modified within kidney cortex, since a sizeable proportion (>30%) of stained section were identified as proximal tubules (Fig. 5 E and F). In addition, the intracellular distribution of anti-EGF reactive material was different in cells of proximal tubules, as compared to those of distal tubules and collecting ducts. Whereas in the latter, the immunocomplex seemed to occupy the whole cytoplasm (Fig. 5D), in the former, EGF immunoreactivity was predominantly located in the basal part of epithelial cells (Fig. 5F).

Fig. 5. *Immunocytochemical demonstration of EGF immunoreactivity in the kidneys of control (a and b) and treated (c–f) rats.* Consecutive paraffin sections of renal tissue were either stained by conventional histological method (periodic acid-Schiff, hemalun and luxol fast blue, a, c, e) or processed to reveal the presence of anti-EGF reactive material (b, d, f). a and b. Presence of EGF immunoreactivity in distal tubules. c and d. Strong positive reaction of distal tubular cells with anti-EGF antibody (rat treated with 200 mg/kg amikacin, 4 days). The arrows point to the macula densa, which does not show any immunolabeling. e and f. EGF immunoreactivity associated with proximal tubules (rat treated with 200 mg/kg amikacin, 10 days).

EGF-like immunoreactive material in collecting ducts, which was not reported by these authors. Although an explanation for that apparent discrepancy still remains to be found, we think that it is more likely to originate from a species difference rather than from artifacts in our immunocytochemical procedure. Indeed, the occurrence of EGF in collecting ducts has been recently confirmed at the ultrastructural level, by using electron microscopy immunocytochemistry (Toubeau et al, manuscript in preparation).

The redistribution of EGF immunoreactivity reported here appears consistent with other studies and provides additional evidence for a role of EGF as a positive mediator during the tissue repair of tubular necrosis. EGF receptors have been identified on the basolateral membrane of proximal tubular cells [52, 53]. Moreover, established epithelial cell lines of renal origin and tubular cells in primary cultures respond to the mitogenic effect of EGF in vitro [52, 54]. Finally, the administration of EGF has been reported recently to promote the repair of renal tubules after hypoxic or nephrotoxic injury [22, 23, 55].

At this stage of the study, the interpretation of our observations still remains difficult, since the immunocytochemical approach that we used does not give any information on the molecular structure and the origin of the immunoreactive material. Although substantial EGF synthesis has been demonstrated in the kidney by in situ hybridization [56], the growth factor is produced within this organ as a high molecular weight precursor, which under normal circumstances does not appear to be processed to give the low molecular weight form of EGF [57]. Furthermore, in the normal kidney, EGF exists as a membrane bound protein [51, 58] predominantly located in the thick ascending limb of Henle and in distal convoluted tubules. Thus, the origin of the EGF immunoreactivity which is found at the level of proximal tubules during tubular regeneration remains to be uncovered. It is worth pointing out, however, that urinary EGF exclusively originates from the kidney [59–61] and that serum level of EGF is very low, two facts which argue against an extrarenal (for example, the salivary glands) origin for the EGF present in renal parenchyma.

Additional questions remain to be answered concerning the high incidence of proximal tubules reacting positively with anti-EGF antibody during tubular regeneration. Recently, Norman et al [23] have demonstrated an increased binding of exogenous ^{125}I-EGF in renal tissue during the episode of tissue repair which follows ischemic acute tubular necrosis. This observation leads us to postulate that the EGF immunoreactivity exhibited by proximal tubules might be due to the interaction of endogenous EGF with the receptors present at the surface of proximal tubular cells and might be related to a phenomenon of receptor upregulation [62].

Whether or not there is an increase of EGF concentration within renal tissue during tubular regeneration remains an open issue. Recently, Safirstein et al [63, 64] have examined by hybridization techniques the level of preproEGF mRNA in the kidneys of rats after cisplatin administration or induction of renal ischemia, and found a decline in the amount of mRNA, concomitant with the acute renal failure resulting from nephrotoxic or hypoxic injury. This finding suggests that the regenerative response of the kidney is not accompanied by an increased de novo synthesis of EGF. Moreover, these authors demonstrated in the experimental animals experiencing renal dysfunc-

tion a decline of EGF urinary excretion and increased EGF binding to surface receptors of renal tubular cells. Although renal tubular regeneration appears associated with a decreased expression of the gene coding for preproEGF (or an increased degradation of the corresponding mRNA), that does not exclude the possibility that EGF might be involved as a positive mediator in this process. For instance, during the proliferative response which follows tubular necrosis, mature EGF could be released in a diffusible form from enzymatic processing of the membrane-bound high molecular weight precursor. Indeed, in vitro proteolysis of EGF precursor purified from renal tissue has been shown to result in the appearance of a smaller polypeptide of a size similar to that of mature EGF and reacting with anti-EGF antiserum [65].

Taken altogether, the experimental facts available to date suggest that, during tubular regeneration, EGF precursor might be converted at distal tubular level into a low molecular weight, diffusible form and attain proximal tubules through the interstitial space. Although this concept is still highly speculative, it deserves further experimental probing.

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