Uptake of Nanoparticles by Rat Glomerular Mesangial Cells *in Vivo* and *in Vitro*

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Glomerular mesangial cells play a major role in the structure of capillary loops, generation of mediators of inflammation, and uptake of macromolecules. We demonstrate here that isobutylcyanoacrylate nanoparticles loaded with actinomycin D (ADNP) concentrate in rat mesangial cells *in vitro* and *in vivo*, as compared to the free drug (AD). In normal rats injected with 20 μg of ^{3}H-ADNP or ^{3}H-AD, the uptake ratios ^{3}H-ADNP/^{3}H-AD measured in whole kidneys at 30 and 120 min were 2.2 ± 1.0 and 2.3 ± 0.9, respectively. The same ratios calculated for isolated rat glomeruli and tubules, were 4.1 ± 0.5 and 0.8 ± 0.2 at 30 min, and 2.6 ± 0.5 and 0.6 ± 0.3 at 120 min, respectively. In the glomeruli, the absolute uptake of ^{3}H-ADNP corresponded to 7.5 (30 min) and 1.8 (120 min) % I.D./100 mg of protein. In rats with experimental glomerulonephritis, the uptakes of ^{3}H-ADNP and ^{3}H-AD by the glomeruli were 6.9 and 4.0 times higher than in normal rats, respectively. *In vitro* experiments demonstrated up to 5 times higher uptake by glomerular mesangial cells than by epithelial cells. Uptake was maximum after 60 min, higher at 37°C than at 4°C, dependent on the presence of fresh serum and inhibited by cytochalasin-B. Drug targeting by nanoparticles is thus possible to renal cells involved in inflammatory processes, especially mesangial cells and macrophages. Nanoparticles could also be useful for lowering drug concentration in tubular cells, to reduce any tubular toxicity. Targetting of the mesangial cells is of particular interest for drugs such as corticosteroids capable of reducing glomerular inflammation in various pathological conditions.

KEY WORDS: nanoparticles; polyalkylcyanoacrylate; endocytosis; mesangial; targetting; kidney.

INTRODUCTION

The classical function of glomerular mesangial cells is to provide a structural support for capillary loops. Further, these cells play a role in response to vasoactive agents, synthesis and degradation of basement membrane components, generation of autacoids, growth factors and other mediators of inflammation, and uptake of macromolecules (1).

The endocytosis of macromolecules and immune complexes by mesangial cells in culture is associated with generation of PGE2 and platelet activating factors (1), and release of reactive oxygen (2). Similarly, the phagocytosis of opsonized zymosan by cultured mesangial cells is associated with the generation of prostaglandins, lipoxygenase products and reactive oxygen species (3). Therefore, the mesangial cells could contribute to local injury, in conjunction with resident macrophages (1,4). Accordingly, drug targetting into mesangial cells (or resident macrophages) may represent a new approach to reduce glomerular inflammation in various pathological conditions. In the present study, we tested if the elevated endocytic activity of mesangial cells could trigger preferential glomerular accumulation of a drug carried by nanoparticles over that of the free form. We selected polyisobutylcyanoacrylate nanoparticles (NP), because of the main following properties (5–9): 1. easy and reproducible preparation; 2. homogeneity and stability of the obtained suspension, with particles of about 150 nm in diameter, even after freeze-drying; 3. preferential uptake by cells exhibiting elevated endocytic properties, such as cancer cells and cells of the mononuclear phagocyte system; 4. degradation by an enzymatic, mainly intracellular process involving esterases, the hydrolysis rate depending on the length of the alkyl chain; 5. possibility of entrapping various kinds of drugs in the cyanoacrylic network, allowing a controlled delayed release inside the target cell; 6. possibility of coating proteins such as monoclonal antibodies onto the surface of formed nanoparticles.

Actinomycin D (AD) was chosen as a representative drug since it is easily incorporated into NP (10,11) and since it inhibits DNA, RNA and protein synthesis *in vitro* and *in vivo*.

MATERIALS AND METHODS

Nanoparticles

Isobutyl cyanoacrylic "nude" NP were prepared as previously described (5). Briefly, the monomer (Ethnor, Paris, France) was added at room temperature to an aqueous solution (100 μl/10 ml) of 10^{-3} M H_3PO_4 containing 1% dextran 70 and 5% glucose, under continuous mechanical stirring. After 30 min of polymerization, the resulting NP formed a stable and homogeneous milky suspension (mean diameter of the particles: 150 nm; polydisperity index: 0.12). Actinomycin D-NP (ADNP) were also prepared according to published methods (10,11). Eight mg of AD (Merck-Sharp and Dohme, Brussels, Belgium) were added under continuous stirring to an aqueous solution (100 ml) of 0.01 M HCl containing 0.5% Tween 20 and 4 ml of monomer (isobutylcyanoacrylate, Sopar, Belgium). After polymerization (30 min), the suspension was buffered to pH 7 with Tris-base and passed through a 0.8 μm filter. Under those conditions, 1 ml of ADNP suspension contained 60 μg of AD and an average of 1.2×10^{12} NP (10,11). Tritiated ADNP (^{3}H-ADNP) were similarly obtained by mixing 40 μl of labeled AD (Amersham, Buchs, UK; specific activity 5–15 Ci/mmol or 4–12 μCi/μg) with 20 μl of monomer (11). Under those experimental conditions, the entrapment of ^{3}H-AD by NP reached about 60% of the present dose (11), and the specific activity of the radiolabelled tracer reached about 60 nCi/μg NP. Solutions of free AD or ^{3}H-AD, containing all the same re-

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Table I. ^{3}H-ADNP and Free ^{3}H-AD Uptake by Different Organs at 30 min.

	Spleen	Liver	Lungs	Whole	Kidneys glomeruli	Tubules
^{3}H-AD	2.0 ± 0.7	1.2 ± 0.5	1.5 ± 0.8	2.5 ± 0.7	1.8 ± 0.6	2.5 ± 0.5
^{3}H-ADNP	22.4 ± 3.5	31.4 ± 4.0	11.2 ± 2.8	5.5 ± 2.4	7.5 ± 1.6	2.0 ± 0.6
^{3}H-ADNP/^{3}H-AD	11.2 ± 3.1	26.2 ± 3.1	7.5 ± 1.6	2.2 ± 1.0	4.1 ± 0.5	0.8 ± 0.2

Whole organs: % I.D./g of wet tissues
Glomeruli and tubules: % I.D./100 mg of proteins.

agents except the cyanoacrylic monomer, and mixtures of free drug and unloaded NP were also used as controls.

In vivo experiments

Ten normal Sprague-Dawley rats (150–200 g) were injected i.v. with preparations containing 20 μg of either ^{3}H-ADNP or ^{3}H-AD of identical specific activities. Thirty or 120 min later, 5 rats were killed and different organs were excised. Tissue samples (50 mg) were then solubilized using 1 ml of 0.5 N quaternary ammonium hydroxide in toluene and counted in a liquid scintillation counter. Glomeruli and tubules were isolated from kidneys by differential sieving (12) and were similarly processed for ^{3}H counting. Results were expressed as percentage of injected dose per g of the whole organ or per 100 mg of glomerular or tubular proteins and as ratios between the uptakes of ^{3}H-ADNP and of ^{3}H-AD in those organs or tissues. The protein content was determined according to the method of Lowry et al. (13), using bovine serum albumin as a standard. In another series of experiments, 2 groups of 3 rats each received 20 μg of unlabeled ADNP or AD. Proteinuria was then controlled every day for 7 days by the method of Kingsbury and Clarck (14). Rats were then sacrificed and their kidneys were examined by both light and transmission electron microscopy (15).

In order to test the renal uptake in inflammatory conditions and to investigate further the renal toxicity of NP, ADNP, and AD in the rat, a proliferative glomerulonephritis was induced in 12 other rats (150–200 g), according to a previously described protocol (16): briefly,

- on day −7, female Sprague-Dawley rats received i.p. 2 mg of rabbit IgG mixed with Complete Freund's adjuvant.
- on day 0, they were injected i.v. with 1 mg of rabbit anti-rat glomerular basement membrane IgG antibodies.
- between days +2 and +5, many mononuclear phagocytes infiltrated the glomeruli and, simultaneously, a pathological proteinuria occurred.

Unlabelled or ^{3}H-labelled NP, AD (20 μg), ADNP (20 μg AD) or LP (0.9% NaCl, as a control) were respectively injected i.v. into 3 rats, 72 h after induction of the glomerulonephritis (day +3). Countings and proteinuria measurements were performed as described previously.

In vivo Experiments

Rats glomerular mesangial and epithelial cells were cultured and characterized as previously described (15). Different concentrations (from 1.3 to 13 pmol per ml) of ^{3}H-ADNP or of unlabeled ADNP containing 1.2×10^{10} NP per ml were either untreated or mixed for one hour at 37°C with 1 ml of autologous fresh rat serum. These solutions were then added to culture flasks of either epithelial or mesangial cells and incubated for 15, 30, 45, 60 or 120 min at 4°, 10°, 20°, 30° or 40°C. At the end of the incubations, the cells were extensively washed with RPMI medium and thereafter processed either for transmission electron microscopy (ADNP) (15) or for liquid scintillation counting (^{3}H-ADNP). For this latter purpose, the cells were detached from culture flasks by 2.5 mM EDTA, .5 N quaternary ammonium hydroxyde-toluene solution (.75 ml) and were thereafter counted in a liquid scintillation counter. The protein content was also measured by the method of Lowry et al. (13). Control cells were incubated with comparable concentrations of ^{3}H-AD. Similar experiments were also conducted using mesangial cells pretreated for 15 min. at 37°C with cytochalasin B at a final concentration of 10 μM, this drug being dissolved in 1% dimethylsulfoxide. In this case, control cells were incubated with dimethylsulfoxide only. Results were expressed as ratios between the percentage of ^{3}H-ADNP and of ^{3}H-AD accumulated per mg of cellular protein. Three culture flasks were used for each experimental condition.

RESULTS

In vivo experiments

Thirty min after i.v. injection of 20 μg of ^{3}H-ADNP or ^{3}H-AD into normal rats, concentration of ^{3}H-ADNP in the

Table II. ^{3}H-ADNP and Free ^{3}H-AD Uptake by Normal and Glomerulonephritic Kidneys at 120 min.

	Whole kidneys (% I.D./g of wet tissues)		
A	Nl	Gn	Gn/Nl
^{3}H-AD	0.8 ± 0.2	2.0 ± 0.3	2.7
^{3}H-ADNP	1.8 ± 0.8	8.0 ± 1.2	4.6
^{3}H-ADNP/^{3}H-AD	2.3 ± 0.9	4.0 ± 1.0	1.7

	Glomeruli (% I.D./100 mg of proteins)			Tubules (% I.D./100 mg of proteins)
B	Nl	Gn	Gn/Nl	Nl
^{3}H-AD	0.7 ± 0.3	2.8 ± 0.8	4.0	0.9 ± 0.3
^{3}H-ADNP	1.8 ± 0.5	12.8 ± 2.6	6.9	0.5 ± 0.3
^{3}H-ADNP/^{3}H-AD	2.6 ± 0.5	4.6 ± 1.3	1.7	0.6 ± 0.3

Nl: normal rats.
Gn: rats with experimental glomerulonephritis.

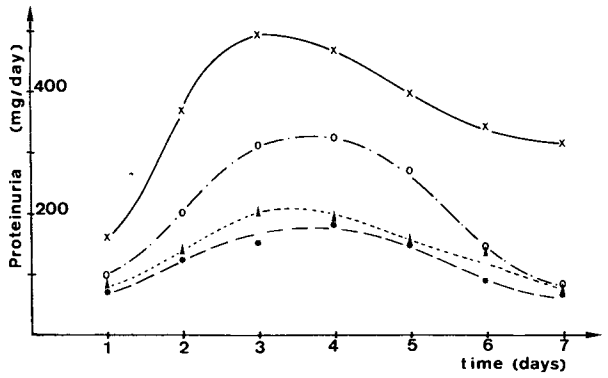

Fig. 1. *In vivo* renal toxicity of free and NP-associated actinomycin D in rats with acute glomerulonephritis. Proteinuria induced by AD (-*-), NP (-o-), ADNP (-x-) and saline (-▲-) was measured daily for 7 days. Unloaded NP induced a short but 2 times more intense proteinuria than saline (control) and free AD. ADNP provoked a large and long lasting proteinuria, that remained very significant on the 7th day.

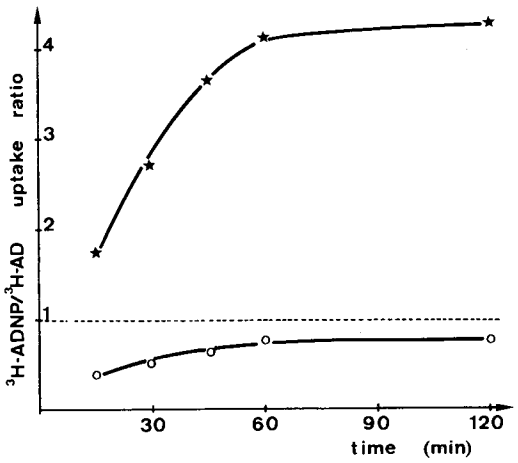

Fig. 3. *In vitro* ^{3}H-ADNP/^{3}H-AD uptake ratios for glomerular epithelial (-o-) or mesangial cells (-*-) as a function of the incubation time. The incubation temperature was 37°C and the AD concentration 6.5 pmol/ml.

kidneys (5.5% I.D./g) was far lower than in the liver, the spleen and the lungs (Table 1). The mean ^{3}H-ADNP/^{3}H-AD organ uptake ratios reached 26, 11 and 7 in these organs, respectively. In contrast, this ratio was only 2.2 ± 1.0 in the kidneys; owing to the standard deviation, this appears as of only borderline significance in terms of drug targetting to the whole organ. A preferential uptake of ^{3}H-ADNP was noted in the glomerular fraction (ratio 4.1 ± 0.5), in contrast to the tubular fraction (ratio 0.8 ± 0.2; table 1). At 120 min, this ratio was similar in whole kidneys (2.3 ± 0.9) and somewhat reduced for the glomerular (3.1 ± 0.5) and the tubular (0.6 ± 0.3) subfractions (Table 2A). The glomerular uptake had little effect on drug concentration in the whole organ, which was also compensated by the low tubular uptake of ^{3}H-ADNP. These data therefore demonstrate the existence of both targetting into rat glomeruli and exclusion from the tubules.

In order to determine whether the preferential glomerular accumulation of ADNP *in vivo* could trigger a pathological proteinuria, 20 μg of ADNP or of free AD were separately injected i.v. into 2 series of 6 normal rats each. No significant proteinuria (<10 mg/day) appeared in each instance during the 7 day observation period. Similarly, free NP (1.2×10^{10} NP/ml) injected i.v. into 3 other rats did not induce any pathological proteinuria within 7 days. At sacrifice on day 7, transmission electron microscopy did not display any evidence of ultrastructural alterations in these 3 groups of rats (data not shown).

In rats with **experimental glomerulonephritis** (Gn), the preferential accumulation of ^{3}H-ADNP as compared to free ^{3}H-AD was more marked than in normal rats (NI) in both the whole kidneys and the glomerular fraction (ratio ^{3}H-ADNP/^{3}H-AD$_{Gn}$/^{3}H-ADNP/^{3}H-AD$_{NI}$ = 1.7) (Table 2). In toxicity experiments, proteinuria appeared in all rats and was maximum on the third day (Fig. 1). Animals having received saline (0.9% NaCl), free AD or unloaded NP behaved similarly

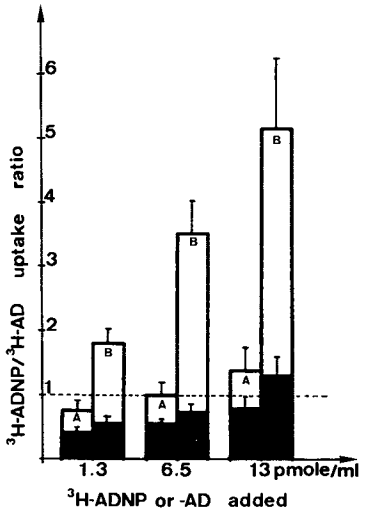

Fig. 2. *In vitro* ^{3}H-ADNP/^{3}H-AD uptake ratios obtained using glomerular epithelial (black histograms) or mesangial (white histograms) cells incubated for 60 min at 37°C with three AD concentrations in the absence (A) or the presence (B) of fresh rat serum.

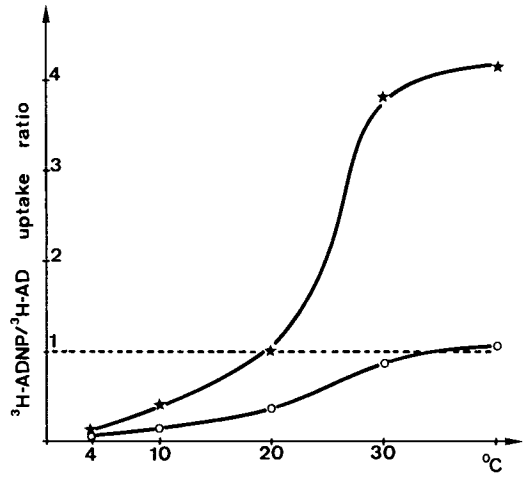

Fig. 4. *In vitro* ^{3}H-ADNP/^{3}H-AD uptake ratios for glomerular epithelial (-o-) or mesangial cells (-*-) as a function of the temperature. The incubation time was 60 min and the AD concentration 6.5 pmol/ml.

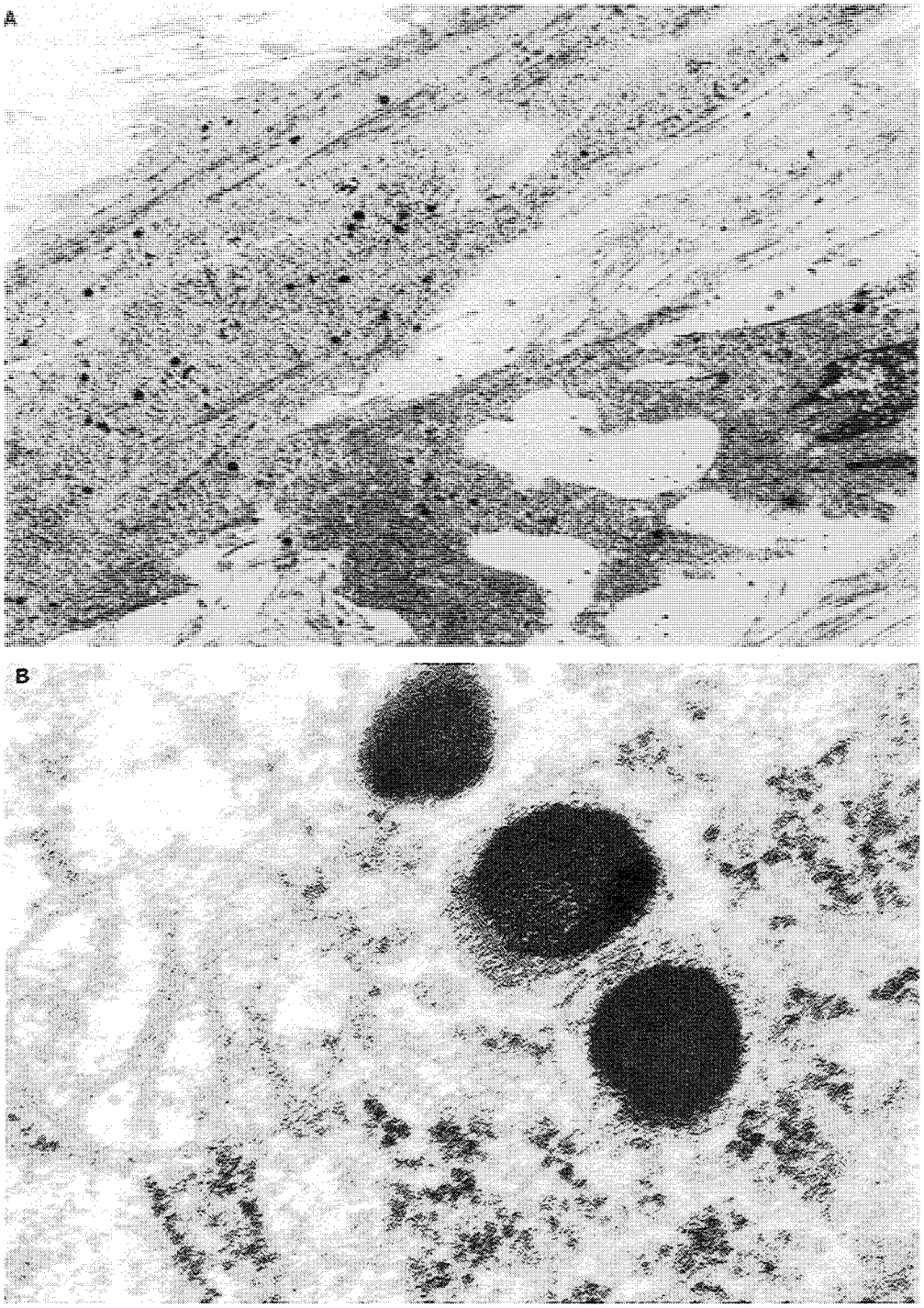

Fig. 5. A) Transmission electron microscopy of mesangial cells incubated with nanoparticles loaded with unlabelled actinomycin D in the presence of rat serum. The rounded and dense structures are considered as nanoparticles since they exhibit a 200 nm diameter and since they are never observed in the absence of nanoparticles (original magnification ×5,000); B) Transmission electron microscopy of a mesangial cell incubated with nanoparticles loaded with unlabelled actinomycin D in the presence of rat serum. Note the presence of nanoparticles into lysosomal vacuoles (original magnification ×120,000).

and proteinuria came back to the initial level within 7 days. However, unloaded NP induced a transient but 2 times more intense proteinuria than the 2 other series. ADNP provoked large and long lasting proteinuria over 7 days. Therefore, AD incorporated into NP provoked more renal injury in the acute experimental conditions tested than free AD, and unloaded NP displayed intermediate toxicity.

In vitro experiments

In order to further investigate the role of mesangial cells

in NP uptake, *in vitro* experiments were then conducted using 2 available rat glomerular cells in culture, i.e., epithelial and mesangial cells. The uptake ratio at 37°C of ^{3}H-ADNP and of free ^{3}H-AD by these cells are illustrated in figures 2 to 4. For epithelial cells in serum-free medium, the mean ADNP/AD uptake ratios ranged between 0.4 and 0.8, for AD concentrations ranging from 1.3 to 13 pmol per ml; the addition of serum to the incubation medium induced only a moderate increase in this ratio (from 0.5 to 1.3) under otherwise identical experimental conditions (Fig. 2). For mesangial cells, the mean ADNP/AD uptake ratios ranged between 0.8 and 1.4 in serum-free medium, whereas the addition of rat serum to the incubation buffer enhanced this ratio from 2 for low AD concentrations (1.3 pmol/ml) to 5 for high concentrations (13 pmol/ml) (Fig. 2). The kinetics of drug incorporation in the presence of fresh serum is shown in Fig. 3. For both types of glomerular cells, the maximal ADNP/AD ratio was reached after 60 min, whereas the half-maximum value was observed after 15–20 min. Fig. 4 illustrates the effects of various incubation temperatures on the ADNP/AD uptake ratios in the presence of fresh rat serum. For both epithelial and mesangial cells, the ADNP/AD uptake ratios measured after 60 min were higher at 37°C than at 4°C. However, the increase of this ratio at 37°C was much more obvious for the mesangial cells than for the epithelial cells (fig. 4). Finally, we noted that a prior cyto-B treatment of mesangial cells decreased by 75% at least the mean ^{3}H-ADNP/^{3}H-AD uptake ratio measured after 60 min at 37°C in the presence of fresh rat serum (data not shown).

Mesangial cells were incubated for 60 min at 37°C with 1.2×10^{10} ADNP, either in the presence or absence of autologous fresh rat serum. In the presence of serum, all cell sections examined by transmission electron microscopy showed many nanoparticles concentrated mainly into lysosomal vacuoles (Fig. 5a and 5b). In contrast, mesangial cells incubated with NP in serum-free medium or mesangial cells pretreated with cyto-B (10 μM) and thereafter incubated with NP in suspension in rat serum, only contained sparse NP (data not shown).

DISCUSSION

Targetting of ^{3}H-ADNP into glomeruli can result either from an active, -endocytic-, process mediated by glomerular cells or from a passive accumulation of NP into the capillary loops and the mesangial matrix during the filtration process (17), or from both. The preferential glomerular accumulation of ADNP in vivo was higher after 30 min than after 120 min, as has been noted in the organs exhibiting an elevated phagocytic activity, namely the spleen, the liver and the lungs (Fig. 1). This similar time course suggests that phagocytic activity of some glomerular cells may also be involved, at least in part, in the preferential glomerular uptake of ADNP.

Shingal et al. (18) have demonstrated that small colloidal gold particles (10 nm in diameter) are actively incorporated into mesangial cells in culture via a coated pit phagocytic mechanism with subsequent delivery of the particles to endosomes. Our *in vitro* data show that the phagocytic activity of rat glomerular mesangial cells is higher than that of rat glomerular epithelial cells and also concerns particles of more than 150 nm in diameter. As for small gold particles (18), the uptake of polyalkylcyanoacrylate NP was rapid (less than 60 min) and clearly dependent on the presence of fresh rat serum. In addition, it did not occur at 4°C and was suppressed by cytochalasin-B. These results confirm (18) that the endocytosis by mesangial cells of NP in suspension in rat serum is not mainly due to non-specific adherence but is rather a consequence of an active process ("mediated uptake") during which the cellular cytoskeleton is "mobilized", possibly through unknown receptors, e.g. Fc receptors (1,2) or C3 receptors (3).

Drug targetting by NP is well documented for cancer cells and for cells of mononuclear phagocyte system (19–21). The present study demonstrates that the incorporation of AD into NP also enhances its uptake by rat glomerular mesangial cells, as compared to free AD. The elevated endocytic activity of mesangial cells may therefore open the way for targetting these cells with anti-inflammatory drugs (such as corticosteroids). However, NP renal toxicity, not measurable in normal conditions, but significant in rats with acute glomerulonephritis, needs to be considered. Further, avoidance of tubular cells by ADNP is of interest if the drug has a high tubular toxicity (e.g., gentamycin).

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