Exposure to Hydrocarbons and Renal Disease: An Experimental Animal Model

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ABSTRACT

The association between hydrocarbon exposure and chronic glomeru-
lonephritis is still a controversial scientific issue. Recent epidemiological
evidence suggests a role of exposure to hydrocarbons in the progression
of glomerulonephritis towards chronic renal failure. The present experi-
mental study on rats has been designed to assess the possible role of sty-
rene in the progression of adriamycin (ADR) nephrosis, a well known
model of renal fibrosis following nephrotic syndrome induced by ADR.
Female Sprague-Dawley rats were exposed to styrene, 300 ppm, 6 h/day,
5 days/week for 12 weeks (group 1); treated with ADR, 2 mg/Kg, i.v.,
twice on day 1 and day 15 of the study (group 2); Additional groups of
animals received both the styrene and ADR treatments (group 3) or serv-

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ed as controls (group 4). The urinary excretion of total and single pro-
teins (albumin, Retinol-Binding Protein (RBP), Clara Cell 16 Kd protein
(CC16), fibronectin) was measured monthly, whereas histopatology and
determinations requiring blood sampling were carried out at the end of
the experiment.

A progressive increase in total proteinuria, falling in the nephrotic
range already by the 6th week was observed in ADR-treated groups. Sty-
rene exposure caused up to a 3- to 5-fold increase as compared to con-
trols. Co-exposure to ADR and styrene also resulted in a proteinuria
much greater than that caused by ADR alone. The interactive effect of
styrene and ADR was statistically significant for albuminuria and uri-
nary fibronectin. A similar response was observed for glomerular filtra-
tion rate at the end of the experiment, styrene-exposed animals showing
hyperfiltration as compared to their respective control group. At the end
of the experiment, histopathological scoring for interstitial infiltration
and fibrosis was also significantly higher in styrene-treated animals as
compared to their respective control groups. In ADR-treated rats, low
molecular weight proteinuria (l.m.w.p.) was only slightly affected, sug-
gesting minimal tubular dysfunction associated with extensive tubular at-
rophy. However, styrene-exposed animals showed l.m.w.p. higher than
their respective controls. In summary, in this animal model we were able
to confirm both styrene-induced microproteinuria, mainly albuminuria
and minor increases in l.m.w.p., observed among occupationally exposed
workers and the role of hydrocarbon exposure as a factor accelerating
the progression of renal disease suggested by epidemiological investiga-
tions in patients suffering from chronic renal disease. Whereas in rats
exposed to styrene only, microproteinuria was stable over time and mi-
nor histopathological changes were noted at the end of the experiment,
evidence of a role of solvent exposure in the progression of ADR ne-
phropathy was obtained in terms of both renal dysfunction and intersti-
tial fibrosis. The mechanistic basis of styrene-ADR interaction is unclear.
However, experimental evidence is consistent with epidemiological find-
ings suggesting the need to avoid solvent exposure in patients suffering
from renal diseases.

Key Words: Biomarkers; Hydrocarbons; Kidney; Glomerulonephritis;
Adriamycin; Styrene

INTRODUCTION

The possible pathogenetic role of solvent exposure in the development of chronic glo-
merulonephritis is still a controversial scientific issue nearly 90 years after the first case
report (1) and more than 20 years after the first case-referent study (2) suggesting such an
association. Almost all published case-referent studies (11 out of 13) consistently show
an increased rate ratio or odds ratio (5.0 on average, range 2.8-8.9) (2–12), whereas the
two «negative» studies (13,14) are affected by major methodological shortcomings and
by a high prevalence of solvent-exposed controls rather than a low prevalence of solvent
exposure among cases.

A number of cross-sectional studies have been carried out in groups of workers exposed to relatively low concentrations of single solvents or mixtures (15–25). In shoemakers exposed to C5-C7 alkanes, Mutti et al. (15) found an increased excretion of proteins, lysozyme and β-glucuronidase suggesting mild, reversible tubular damage. Askergren and colleagues (16,17) found increased albuminuria and erhythrocyturia in workers exposed to aromatic solvents, mainly styrene. In oil refinery workers exposed to low concentrations of complex mixtures, urinary albumin and the brush-border antigen BB-50 were increased (20).

Neither individual solvents nor mixtures were identified as potential toxic agents in most studies. However, in a recent English study (25), the relative risk for petroleum products, greasing/degreasing agents and paints and gluing agents was estimated to be 15.5, 5.3 and 2.0, respectively.

A more recent example is the European collaborative study on laundry workers exposed to organic solvents, mostly perchloroethylene (PCE) (26). A large battery of markers was used, including single high and low molecular weight proteins, kidney-derived antigens and enzymes, and prostanoids were measured in urine. Creatinine, β_2-microglobulin, laminin fragments, and anti-glomerular basement membrane were also measured in serum. PCE-exposed workers excreted more high molecular weight proteins (albumin, transferrin), more brush-border antigens, urinary fibronectin and tissue nonspecific alkaline phosphatase. The higher excretion level of glycosaminoglycans and Tamm-Horsfall glycoprotein also approached statistical significance. Serum anti-glomerular basement membrane antibodies, expressed as a percentage of a positive serum from a patient with Goodpasture's syndrome, as well as laminin fragments, were significantly higher in PCE-exposed workers. Serum creatinine and β_2-microglobulin overlapped in the examined groups, thus excluding any major impairment of renal function. This, as other cross-sectional investigations and case referent studies also failed to show any dose-effect or dose-response relationship. One exception is the study of Ng and coworkers on toluene-exposed workers (24).

In laboratory animals, exposures to hydrocarbons have sporadically produced glomerular lesions, but this has generally occurred in the presence of concomitant tubulointerstitial damage (27,28). Although the role of tubulointerstitial injury is now acknowledged as a key factor in the progression of renal diseases, the relevance of the male rat model to the human situation is questionable, due to differences in the delivery of hydrocarbon metabolites to the target. Some such metabolites bind β_2-microglobulin and accumulate in the segment S2 of proximal tubules. At variance with spontaneous nephropathy in the old-rat, the protein-chemical complex precipitates in S2 epithelial tubular cells as angular crystals rather than as spherical lysosomes (29). Such a selective accumulation of insoluble and indigestible chemical-protein complex eventually leads to cell necrosis. However, the male rat β_2-microglobulin is a species- and sex-specific protein, found in much lower concentrations in female rats, and apparently lacking a counterpart in other species, including humans. In a rat model of PCE-induced nephropathy, tubular accumulation of β_2-microglobulin precipitating in the form of insoluble crystals gave rise to a selective damage of the segment S2 of proximal tubules and was correlated with albuminuria, a widely accepted marker of glomerular dysfunction. Smaller but significant increases in albuminuria associated with low molecular weight proteinuria were found in female rats. Thus, in this rat model, exposure to PCE caused glomerular proteinuria of tubular origin (30).

Despite a large base of data, only in part summarized here, whether organic solvents induce or are associated with chronic renal disease or cancer still remains a question

without satisfactory answers. Two recent nested prospective studies (based on a follow-up of cases and controls) suggest a role of solvent exposure in the progression of glomerulonephritis towards chronic renal failure (31,32). The cases still exposed after the diagnosis of either chronic renal failure or chronic glomerulonephritis showed a poorer prognosis in terms of deterioration rate and stability of proteinuria, respectively.

The present study was designed in the rat to test the hypothesis that hydrocarbon exposure may interact with factors underlying progressive renal disease. Adriamycin (ADR)-induced nephrosis was chosen as a suitable animal model, characterized by heavy proteinuria, followed by progressive glomerular sclerosis and tubulo-interstitial fibrosis.

MATERIALS AND METHODS

Styrene (99% pure) was purchased from Aldrich Chemicals (Milan, Italy). Adriamycin (ADR) was from Farmitalia Carlo Erba (Milan, Italy). Adult female Sprague-Dawley rats weighing 150–180 g were obtained from Charles River (Calco, Italy). The animals were randomised to four treatment groups each composed of 10 rats: (i) sham; (ii) styrene inhalation; (iii) ADR treatment; (iv) ADR treatment followed by styrene exposure.

The animals were housed two per cage under conditions of constant temperature and humidity and automatic photocycle. All animals were maintained on a 21% casein diet (Atromin-Rieper, Vandoies, Italy) according to a standardised recipe and were allowed free access to water. During inhalation exposure, animals were individually housed and deprived of food, but drinking water was available *at libitum*. All rats were sacrificed under CO_2 narcosis by decapitation 15 weeks after the first ADR dose or 13 weeks of styrene inhalation. One hour before sacrifice, all animals received i.p. 200 mg/Kg BrdU (5-Bromo-2'-deoxyuridine, Sigma, St. Louis, Mo.) for the immunocytochemical demonstration of S-phase cells. All experiments were performed in compliance with the European Community Guide for the Care and Use of Laboratory Animals.

TREATMENTS

ADR

Rats belonging to groups (iii) and (iv) were given 2 subsequent doses of ADR (2 mg/Kg b.w.): on days 1 and 15, respectively. ADR was dissolved in 0.5 mL sterile physiologic solution and infused slowly into the tail vein (33). Control rats were injected i.v. with 0.5 mL saline.

Styrene Exposure

Groups (ii) and (iv) were exposed to 300 ppm of styrene for 13 weeks, 6 h/day, 5 days/week, using a dynamic exposure chamber of 1 m³, as previously described (33–34).

LABORATORY INVESTIGATIONS

Kidney Function Tests

Urine samples were collected in cooling vials over 18 h over NaN_3 as preservative. Urine volume was measured. Aliquots were frozen (-80°C) until analysis. Albumin was

measured by competitive ELISA (35) using monospecific antisera and rat proteins. Rat albumin was purchased from Calbiochem (La Jolla, Calif., USA) and rabbit antirat albumin - IgG fraction - was from Bio Science Products (Emmenbrücke, Switzerland). Rat retinol-binding protein (RBP) was measured by a sensitive 'sandwich' ELISA (36) using specific antiserum produced in our laboratory. Briefly, RBP was isolated from the urine of rats poisoned with chromium (VI) salts to induce tubular proteinuria; RBP was purified from urine by affinity chromatography on human transtiretin linked to Sepharose. Antirat RBP antibodies, obtained from the serum of rabbits repeatedly injected and purified with RBP, were used to set up an immunoassay using microtitration plates, biotinylated antibodies being used to reveal RBP in biological samples (working range from 0.8 to 50 ng/mL). Total proteinuria was evaluated by the Coomassie G250 dye binding assay (37) using bovine serum albumin as a standard. Urinary and serum CC16 as well as cystatin C were determined by latex immunoassay (LIA) (38). Fibronectin was measured by solid phase EIA based on its binding to collagen (39).

Histological examination

One μm sections were stained with PAS-hemalun-luxol fast blue or with hematoxylin-fuchsin-Heidenhain blue(Orange G + anilin blue). The dye anilin blue seemed well suited for the study of fibrosis since it stains deep blue the collagen (I) fibres and pale blue other connective tissues (except elastin). The slides were examined independently by two observers (G .T. and D.N.) who were kept unaware of the treatment until final pooling of the results. To standardise the evaluation procedure, an additional lens engraved with a 5 × 5 grid was inserted into a 10X eyepiece. For each histological structure, tissue alterations in the kidney were graded along arbitrary scales according to the following criteria:

- Glomerular Sclerosis
  0 (no departure from natural morphology); 1 (1 to 25% of the glomerular tuft showing thickening of basement membrane and/or hyalinisation); 2 (26 to 50% of the glomerular tuft); 3 (>50 % of the glomerular tuft). At least 20 glomeruli per section at 400X magnification have been evaluated.

- Interstitial Fibrosis
  The scoring of interstitial fibrosis was obtained by the observations of ten microscopic fields per section at a magnification of 100X (total scanned surface of 13.5mm^2 for each section): 0 (no departure from normal morphology); 1 (1 to 25% of the field occupied by hyaline connective tissue and/or by structures (glomeruli or tubules sections) surrounded by thickened basement membranes; 2 (26 to 50% of the field); 3 (>50 % of the field).

The following scoring was performed by the observation of the entire tissue section,corresponding to approximately 5–10 microscopic fields per section at 100X magnification:

- Interstitial Cellular Infiltrates
  0 (no clusters of interstitial cells); 1 (1 to 5 clusters of interstitial cells); 2 (6 to 10); 3 (more than 10).

- Cystic Tubules

(dilated tubules lined by a flattened epithelium): 0 (no cystic tubules); 1 (1 to 5 cystic tubules); 2 (6 to 10); 3 (more than 10)

- Hyalinosis
  0 (no departure from normal morphology); 1 (1 to 5 tubule sections filled with hyaline material); 2 (6 to 10); 3 (more than 10).

- Distribution and Density of Proliferating Cells
  For each animal, one paraffin section was processed for the immunocytochemical detection of BrdU in the nuclei of S phase cells as described previously (40).

A total of 20 microscopic fields (10 in the cortex and 10 in the medulla) were scanned in each section at a magnification of 400X (corresponding to a scanned area of 1.68 mm^2 and approx. 300E10^3 nuclei). The number of proliferating cells was observed for the different structures of the cortex (glomeruli, proximal and distal tubules, collecting ducts and interstitium) or of the medulla (proximal and distal tubules, collecting ducts and interstitium). For each section, the global labeling index was the total number of proliferating cells per mm^2 of kidney tissue.

STATISTICAL ANALYSIS

Parametric statistical analysis was carried out on biochemical and functional variables. Log-transformed values were used when necessary to obtain a normal distribution, which was verified using the Kolmogorov-Smirnov. Differences among groups were primarily assessed using a two-way ANOVA and Student's t test for paired data. Linear regression analysis was carried out using the least-square method (Pearson's correlation). Non parametric tests were applied to morphometric evaluations (Mann-Whithney U test).

RESULTS

ADR caused severe toxicity and high mortality rates (50%) in both treated groups. Surviving rats showed poor general conditions and a decrease in body weight by about 25% as compared to both controls and styrene exposed animals.

Renal markers are summarized in Table 1. In ADR-treated rats, total proteinuria was in the nephrotic range. Very high albumin excretion rates were associated with a dramatic increase in the urinary excretion of fibronectin. Co-exposure to styrene resulted in even higher excretion rates of all such proteins, whereas animals only exposed to styrene showed a two or three-fold increase in total proteins, albumin and fibronectin as compared to controls. In ADR-treated rats, geometric mean of albuminuria was 35 times higher than in controls, and a 3.5-fold increase was apparent in styrene-treated as compared with control animals. Co-exposure to ADR and styrene resulted in a further increase (by 30%) in the albumin values recorded in rats given ADR only. Statistical analysis indicated significant effects of ADR and styrene, and a significant ADR*styrene interaction ($p = 0.036$). The effects of ADR on urinary fibronectin were even greater, the geometric mean of ADR-treated rats being two orders of magnitude higher than control values. A further increase (by 50%) in urinary fibronectin has been observed in rats co-exposed to styrene. Two-way ANOVA indicated a significant effect of ADR, borderline

Table 1

*Urinary Excretion of Plasma-and Tissue-Derived Proteins According to the Applied Factor. Values are Geometric Means of Urinary Excretion Rates. A Two-Way ANOVA Model Indicate That Both Styrene and ADR, Though at Different Extent, and That a Styrene * ADR Interaction Occurs For High Molecular Weight Proteins.*

Urinary Excretion of Plasma and Tissue-Derived Proteins

Independent Variables (Factors)		Total proteins (ng/min)	F	p	Albumin (ng/min)	F	p	Fibronectin (ng/min)	F	p	RBP[1] (pg/min)	F	p	CC16[2] (opg/min)	F	p
STYRENE	ADR															
STYRENE	NO	1,829			274.1			0.77			125.6			61.5		
NO	4 mg/Kg	15,924			9,845.8			83.63			1,124.0			912.0		
	NO	3,019			981.8			1.67			118.5			57.2		
300 ppm	4mg/Kg	21,512			12,664.0			121.95			1,769.8			1201.7		
ANOVA		F	p		F	p		F	p		F	p		F	p	
Effect	Styrene	3.8	.055		11.8	.001		3.34	.070		.68	.411		.1	.781	
	ADR	108.3	.000		153.5	.000		180.37	.000		157.69	.000		131.7	.000	
	Interaction	.6	.454		4.5	.036		4.38	.039		1.60	.207		.5	.498	
	Model	37.7	.000		53.6	.000		50.95	.000		51.63	.000		44.1	.000	

[1] RBP = Retinol-Binding Protein
[2] CC16 = 16 Kd Clara Cell protein
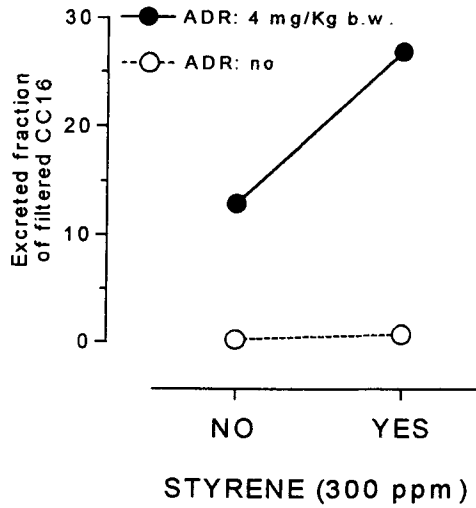
Figure 1. At the end of the experiment, the excreted fraction of filtered CC16 in ARD-treated animals is increased by several orders of magnitude as compared to controls. Although styrene exposure causes only minor changes, its interaction with ADR is quite apparent, resulting in much higher values as compared to values recorded in animals treated with ADR alone.
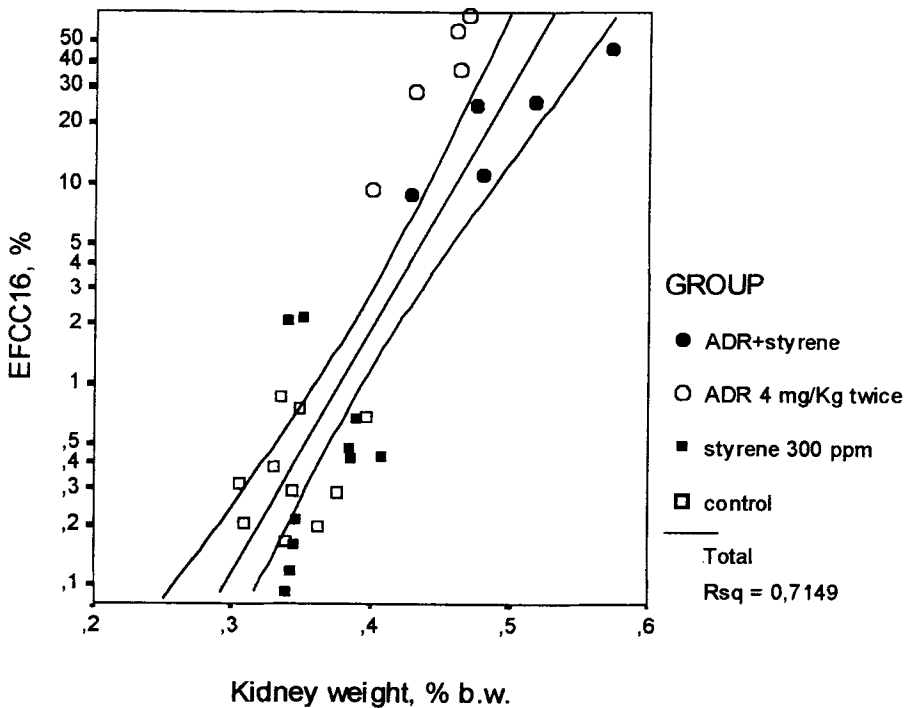
Figure 2. Relationship between urinary CC16 and kidney weight at the end of the experiment (13th week).
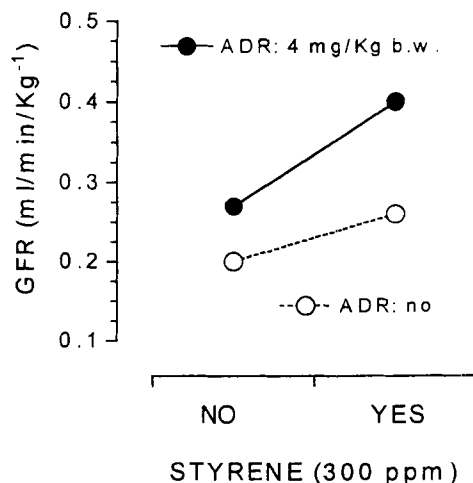
Figure 3. At the end of the experiment, GFR is increased by 50% both in styrene- and in ADR-exposed rats, and it is increased by about 100% in rats co-exposed to both ADR and styrene.

significance for styrene-induced changes ($p = 0.07$), and a significant ADR*styrene interaction ($p = 0.039$). Geometric means of serum-derived proteins with low Mr also exhibited 10- to 20- fold increases as compared to control values. Although styrene exposure alone did not modify protein excretion rates, its combination with ADR further enhanced (by 50–100%) ADR-induced low molecular weight proteinuria, but the ADR*styrene interaction was not statistically significant.

The excreted fraction of filtered low M_r proteins (EF) was calculated at the end of the experiment. While the EF_{RBP} was very low in all experimental groups ($4 \times 10^{-5} - 4 \times 10^{-3}$) apparently due to its combination with transtiretin in serum, EF_{CC16} showed marked differences among groups. Styrene exposure caused a 3-fold increase in EF_{CC16} as compared to controls. In animals treated with both ADR and styrene, EF_{CC16} was also three times higher than in ADR-treated rats, the latter being two orders of magnitude greater than control values (Figure 1). At the end of the experiment, EFCC16 was closely related to the kidney weight relative to body weight (Figure 2). Such an increase in EF_{CC16} was at least in part attributable to an increased filtered load. Accordingly, while glomerular filtration rate was increased by 50% both in rats exposed to styrene and in ADR-treated rats, co-exposure to styrene and ADR resulted in a further 50% increase as compared with animals treated with each factor separately (Figure 3).

The time course of the urinary excretion of plasma proteins with high Mr is summarized in Figure 4. Styrene exposure was associated with an immediate increase in urinary albumin excretion rate, then stable over time (Figure 4a). Co-exposure to styrene and ADR did not increase albumin excretion rate as compared with ADR alone, which was associated with a sharp increase in albuminuria from the first month from ADR administration onwards. A similar trend was apparent for urinary fibronectin (Figure 4b), also showing a 3-fold increase in styrene-exposed animals as compared to the relevant control group (controls or ADR, respectively). Whereas the group treated with ADR showed a progressive increase in urinary fibronectin parallel to that of albuminuria, rats co-exposed
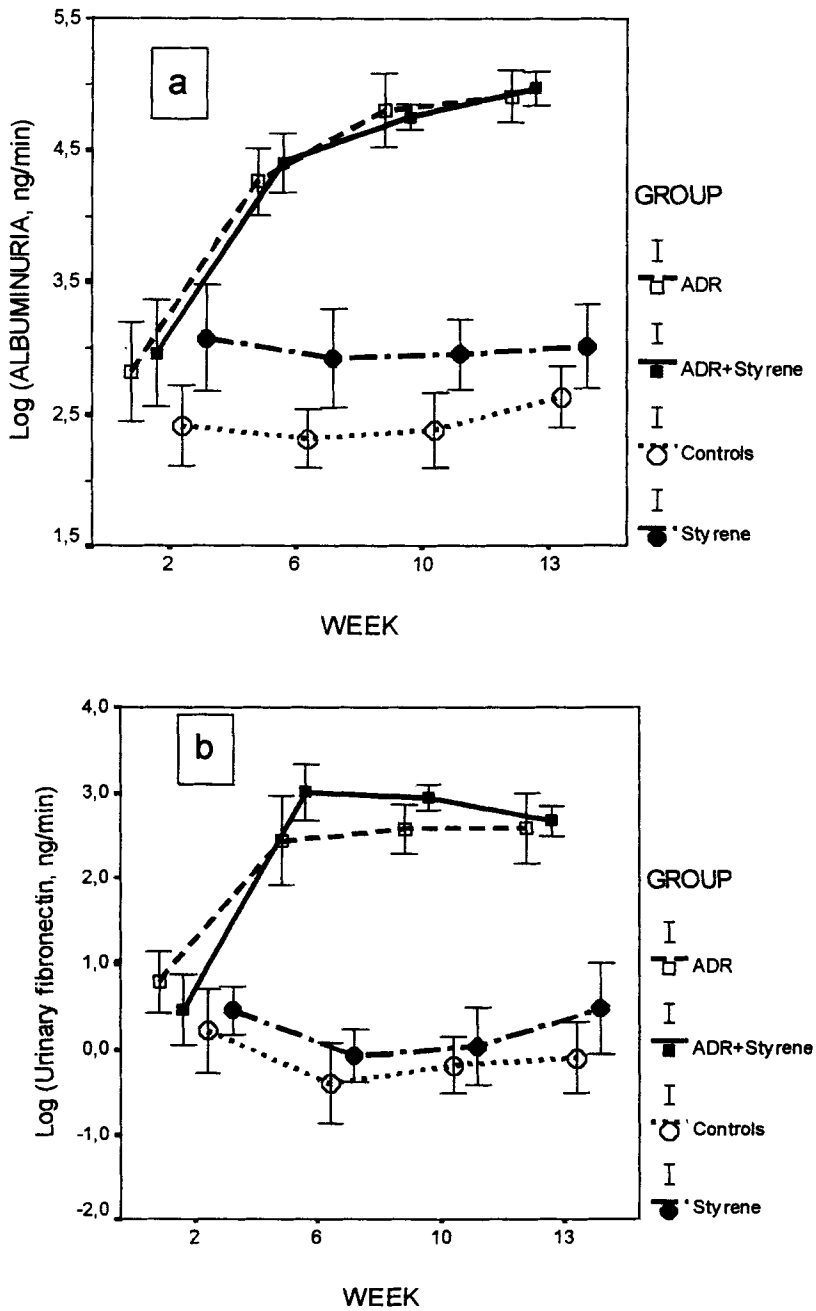
Figure 4. Time course of the urinary excretion of albumin (a) and fibronectin (b) in rats treated with ADR (open squares), with both ADR and styrene (closed squares), with styrene (closed circles), and fresh air (open circles).

to ADR and styrene showed a peak after one-month co-exposure to styrene. At the sixth week, the geometric mean in rats co-exposed to styrene was 4-fold higher than that in animals treated with ADR alone, whereas the geometric means in the two groups were overlapping by the end of the experiment. Plasma proteins with low M_r were one-two orders of magnitude higher in ADR-treated rats, whereas styrene exposure did not induce significant changes (Figure 5a and b).

The histopathologic score values are summarized in Table 2. The most striking result was the score for interstitial fibrosis, which was significantly higher in rats co-exposed to ADR and styrene as compared to ADR-treated rats. Similar changes were observed for cellular infiltrates, also being three-fold greater after combined exposure to ADR and styrene than in ADR-treated rats.

A close relationship was found between the urinary excretion of fibronectin at the sixth week (when the above mentioned pick was observed) and the histopathological score for interstitial fibrosis occurring two months later, the Spearman's rho being 0.8 (Figure 6). It is worth mentioning that urinary fibronectin in samples collected at the 13th week just prior to the sacrifice was less closely correlated with the histopathological score for interstitial fibrosis. Similar results were obtained correlating the histopathological score for fibrosis with albuminuria and, although less closely, with urinary RBP and CC16 (not shown).

DISCUSSION

The present study demonstrates that subchronic exposure to 300 ppm of styrene, an aromatic hydrocarbon widely used in the plastics industry, is associated with a small increase in the urinary excretion of plasma proteins, especially of those with high M_r, thus providing experimental support to epidemiological findings obtained in epidemiological studies (16–21). An interaction between ADR and styrene exposure was also apparent, as indicated by higher urinary protein excretion rates and higher histopathological scores for interstitial fibrosis and cellular infiltrates as compared to those obtained in rats treated with either ADR or styrene alone, thus supporting epidemiological evidence suggesting a role of solvent exposure in the progression of renal disease towards chronic renal failure (31,32).

Despite their preliminary nature, due to the limited observation period, these findings also support the starting hypothesis of an interaction between hydrocarbon exposure and renal disease from other causes, resulting in an accelerated evolution towards chronic renal failure. Hyperfiltration is often found in tubulo-interstitial nephropathies and is thought to precede an impairment in renal function, as shown in experimental models of lead-induced nephropathy (41). Also, hyperfiltration is known to characterize the early stages of diabetic nephropathy and to be forerunner of chronic renal failure (42). In diabetes, like in styrene exposure, there is an accumulation of ketoacids, which might play a role in renal dysfunction. Indeed, an increased kidney weight has been found in rats exposed to styrene (43) and phenylglyoxylic acid (44), the end-product of styrene biotransformation.

Other mechanisms might account for styrene nephrotoxicity, which has recently been attributed to mercapturates resulting from glutathione conjugates of styrene oxide (45,46). However, both experimental and epidemiological investigations suggest that styrene is only weakly nephrotoxic (47,48). In agreement with these findings, styrene-exposed rats showed minimal pathological damage associated with proteinuria, with no
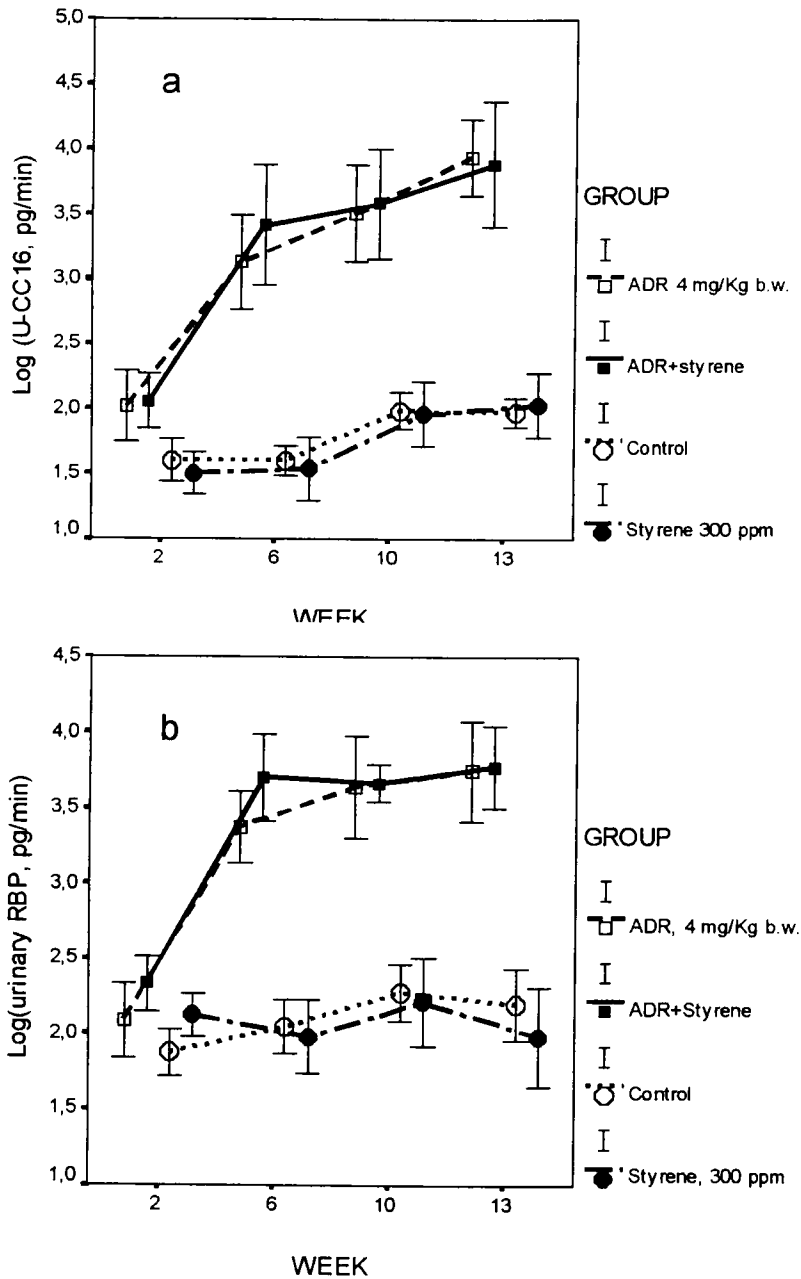
Figure 5. Time course of the urinary excretion of albumin (a) and fibronectin (b) in rats treated with ADR (open squares), with both ADR and styrene (closed squares), with styrene (closed circles), and fresh air (open circles).

Table 2

Summary of Morphometric Scoring (on a Scale From 0 To 3.0) of Renal Changes at the Time of Sacrifice (Week) 13). At This Time, No Glomerular Changes Were Apparent in Any Group. Units Are Arbitrary Scores, as Defined in Materials and Methods.

Experimental Group	Kidney Weight Mean (SEM)	Interstitial Fibrosis Median (Range)	Cellular Infiltrates Median (Range)	Cystic Dilatations Median (Range)	Hyaline Tubules Median (Range)
Controls	0.34 (0.02)	0.04 (0 – 0.1)	0.0 –	0.0 –	0.00 –
Styrene 300 ppm	0.36 (0.02)	0.14[a] (0.1 – 0.2)	0.0 –	0.4[a] 0 – 2.0	0.40[a] 0 – 2.0
ADR 4mg/Kg	0.44 (0.16)[a,b]	0.52[a,b] (0 – 1.0)	0.4[a,b] 0 – 1.0	2.0[a,b] 1.0 – 3.0	2.2[a,b] 1.0 – 3.0
ADR 4mg/Kg + Styrene 300 ppm	0.49 (0.22)[a,b,c]	1.30[a,b,c] (0.6 – 1.8)	1.4[a,b,c] 0 – 3.0	2.0[a,b,c] (1.0 – 3.0)	2.0[a,b] 1.0 – 3.0

[a] Significantly different from controls ($p < 0.05$, Mann-Whitney U Test)
[b] Significantly different from styrene ($p < 0.05$, Mann-Whitney U Test)
[c] Significantly different from ADR ($p < 0.05$, Mann-Whitney U Test)

WEEK: 6
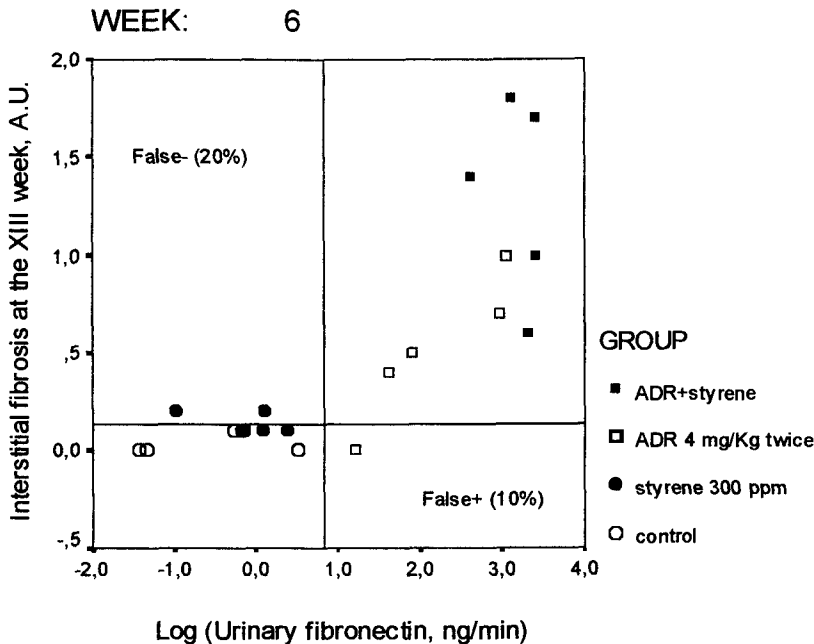
Figure 6. Relationship between the urinary excretion of fibronectin at the 6th week and the histopathological scoring for interstitial fibrosis at the end of the experiment.

clear tendency towards worsening during the 3-month observation period. Nevertheless, co-exposure to styrene in ADR-treated rats resulted in much higher proteinuria and more severe renal damage.

The choice of ADR among the available alternatives of model diseases, e.g., puromycin-induced nephropathy, is justified by the progressive nature of the ADR-induced renal changes and of the associated dysfunction. This response is often transient in other experimental models, making the demonstration of interactions with other risk factors highly susceptible to chance. Moreover, in ADR-nephropathy glomerular lesions appear at an advanced stage, despite the early occurrence of proteinuria in the nephrotic range (49). Such a marked proteinuria has been attributed to minimal glomerular changes, mainly the fusion of podocytes' pedicellar processes (50), while the progression of renal disease has mainly been attributed to CD4+ lymphocyte infiltration, with subsequent production of IL-2 and TGF-β, a key factor in the promotion of intersitial fibrosis (49). In styrene-exposed workers, changes in the proportion of lymphocyte subsets in peripheral blood have been reported (51), supporting the hypothesis of styrene-induced immunological disturbances, which could play a role in the progression of renal disease. However, peripheral lymphocytes represent less than 0.5% of total lymphocytes and therefore may not parallel their level and/or activity in the target organ.

Oxidative stress has been suggested as a mechanistic basis of ADR-induced nephropathy and the protective effects of glutathione-dependent processes its progression has been postulated (52,53), though results inconsistent with this hypothesis have also been reported (54). Decreased renal GSH levels associated with oxidative stress has been docu-

mented following styrene administration (55). This process may lower cellular defense mechanisms against ADR-generated reactive oxyradicals as a factor aggravating the ADR nephrotoxicity or promote the generation of free radicals due to activity of renal β-lyases, in analogy with mercapturates generated by the biotransformation of nephrotoxic organohalogenated compounds (56).

Changes in membrane fluidity and permeability to plasma proteins might represent a generic mechanism of action of organic solvents (57,58), which could explain the enhanced proteinuria and perhaps the associated acceleration in the progression of renal disease. Recent evidence suggests that a factor responsible for enhanced glomerular permeability is responsible for heavy proteinuria in focal segmental glomerulosclerosis (59), and that its removal from plasma prevent the progression towards chronic renal failure (60). Whether ADR-induced segmental glomerulosclerosis is also caused by a serum permeability factor is still unclear, but in any case both in the human and in the animal glomerular disorders, sustained heavy proteinuria seems to be essential in the progression of renal disease. Styrene and probably other hydrocarbons would act enhancing ADR-induced proteinuria and for this reason play a role in the progression of renal damage.

An additional objective of this study was the validation of markers of early renal damage assessed in terms of relationships with renal pathology. The values recorded for all such markers were closely related to the severity of interstitial fibrosis and cellular infiltration observed several weeks later. However, despite these relationships, a cut-off over which measured proteinuria could assume any diagnostic or prognostic value was not identified. In the present study, proteinuria was shown to occur as a dynamic process changing over time, whereas only a single evaluation of renal pathology was carried out at the end of the experiment (13th week). Thus, even small increases in urinary proteins at the second week would have been predictive of interstitial fibrosis, whereas the same values at a later stage would have been associated with normal renal histology.

It ought to be noted that a decrease in GFR is usually found in ADR nephropathy (49,50). However, GFR has been measured at the end of the experiment, which is usually longer by one month (49) or even one year (50) than the present study. Also, the doses we used were greater and were associated with 50% mortality rate in ADR-treated groups, but this did not result in more severe renal damage as compared to both the literature and to our previous findings in a pilot study, in which ADR had been administered in lower doses (2 mg/Kg b.w.). Therefore, a new experiment is going on with lower doses and prolonged observation period, which should conceivably enable us to draw more definite conclusions and to demonstrate the full range of ADR*styrene interactions.

ACKNOWLEDGMENT

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