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Unilateral renal agenesis in chick embryos: a model for chronic renal insufficiency

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ABSTRACT   Although renal agenesis and dysgenesis are relatively common and significant birth defects, no animal model to date has been utilized to adequately study these developmental pathologies. Blockage of the migration of the mesonephric duct in Day 2 chick embryos results in unilateral renal agenesis (URA) on the operated side, thus providing a model of chronic renal insufficiency. Embryos with URA respond with an increase in the rate of growth of the remaining meso- and metanephric kidney. The allometric scaling of single (left) kidney weight to total body weight in control embryos is $KM= 3.48M^{0.98}$ compared to $KM= 3.02M^{1.16}$ in embryos with URA. In addition, embryos with URA exhibit a progressively polycystic mesonephros with distinct glomerulonephritis and expansion of the renal tubules. These renal changes are insufficient for normal urine (allantoic fluid) production and oliguria persists throughout incubation. While mortality is unaffected by URA in embryos up to Day 14 of incubation, there is a steady increase in mortality after Day 14; no chick embryo with URA lives beyond Day 18 of the 21-day incubation period.

KEY WORDS: *kidney, embryo, fetus*

Adequate fetal urine production is essential for production of amniotic fluid in mammals and allantoic fluid and hydromineral balance in non-placental amniotes. Not surprisingly, the International Fetal Surgery Registry has reported that 93% of neonatal deaths are due to pulmonary hypoplasia with a significant number of these cases being attributed to decreased amniotic fluid during gestation, secondary to renal insufficiency (Elder, *et al.,* 1987). Renal insufficiency may occur through several mechanisms including bi- or unilateral renal agenesis (URA) or fetal obstructive uropathy (Elder, *et al.,* 1987; Morse *et al.,* 1987; Chevalier, 1990). In these cases, a mild to severe oligohydramnios follows with associated pulmonary hypoplasia and further renal damage (Harrison, 1982; Uchida *et al.,* 1990). Although acute experimental nephrectomy and urinary obstructive intervention of late-stage fetal mammals has been accomplished (for example, see Ward *et al.,* 1989), no animal model to date has been utilized which adequately mimics congenital, *chronic* renal insufficiency. The current data, therefore, do not usually encompass first or second trimester amniote developmental stages. Hence, available models of renal insufficiency do not include information about the response(s) of the mesonephroi or even the early metanephroi.

Interestingly, avian embryos are easily altered (by blockage of the migration of the mesonephric duct at Day 2) to produce embryos with true bi- or unilateral renal agenesis (Boyden, 1927; Bishop-Calamé, 1965, 1966; Merchant-Larios *et al.,* 1984). Although this technique clearly results in embryos with chronic renal insufficiency (embryos thus altered fail to develop either a meso- or metanephric kidney on the operated side), this animal model has been used primarily to study the kidneys' role in gonad differentiation, but not renal pathology or physiology.

We, therefore, present an embryonic chick model of unilateral renal agenesis which is useful in examining the subsequent alterations in the contralateral meso- and metanephros. We further present data on the effect of URA on allantoic fluid (urine) formation, and embryo growth and survival.

Embryos undergoing sham-surgery wound-healed in several hours and exhibited no detectable abnormalities during development. Approximately 80% of the embryos receiving an eggshell membrane implant survived this procedure and were typically observed to lack the right vitelline artery and vein and associated vascular branchings (Fig. 1A,B). The remaining anterior and posterior vessels were shifted to a slightly right-lateral position. These vessels, as well as the left vitelline artery and vein, appeared dilated in comparison with controls, but no quantitative measurements were made of this phenomenon. Earlier workers have noted such vascular anomalies in «naturally-occurring» unilateral renal agenesis (Boyden, 1927). There were no measurable differences in mortality between control and URA embryos through Day 14 of incubation. After Day 14, however, mortality of URA embryos significantly increased com-

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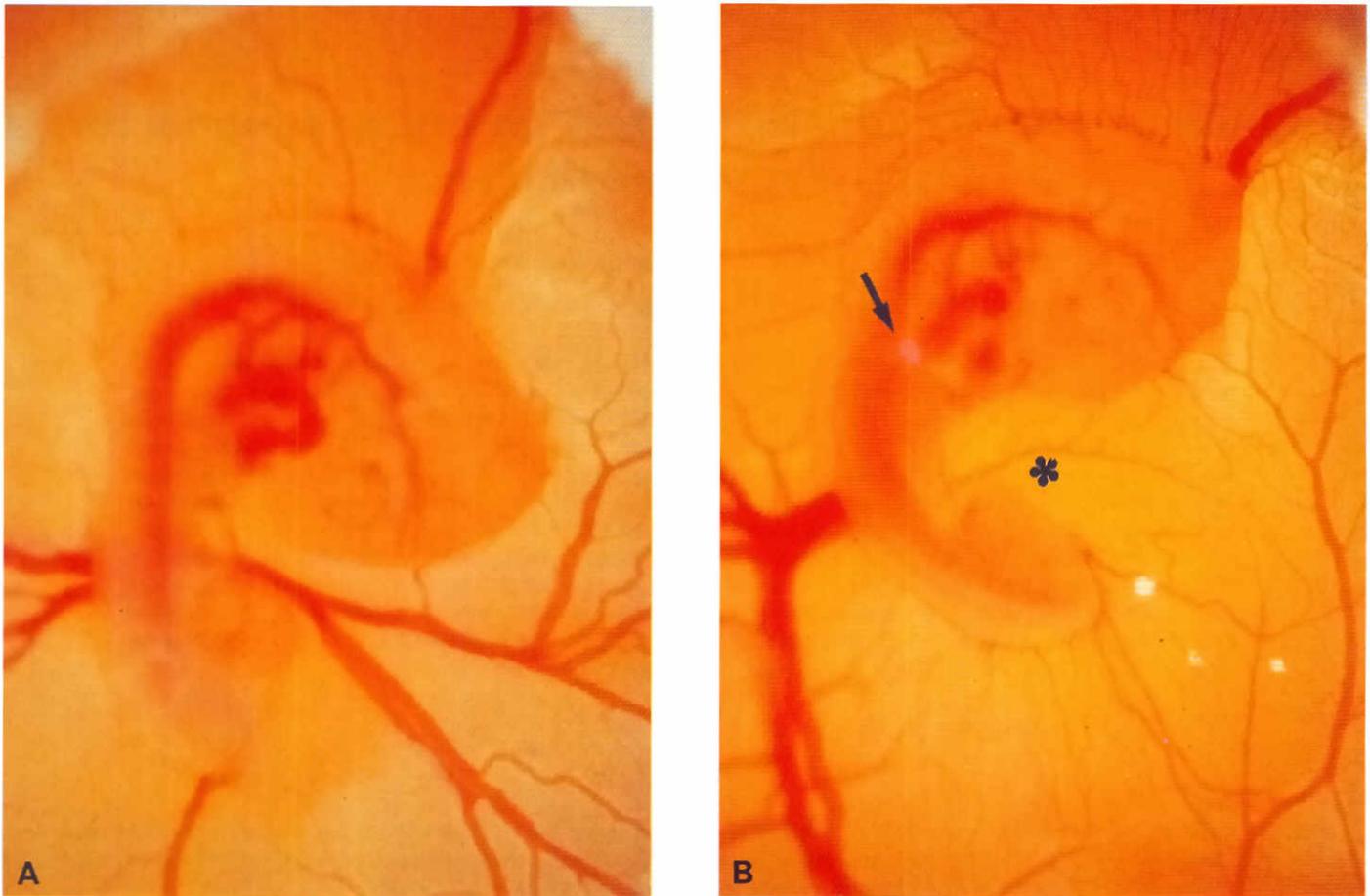**Fig. 1. Embryos of 4 days of incubation. (A)** *Control embryo.* **(B)** *Embryo in which a sterile eggshell membrane (arrow) has been inserted to prevent the migration of the mesonephric (Wolffian) duct. Note the absence of the right vitelline artery and vein and the poor expansion of the allantois (asterisk).*

pared to control animals and no URA embryo survived beyond Day 18.

Unilateral renal agenesis resulted in severely reduced allantoic fluid volume on both Days 8 and 14. Allantoic fluid is reduced by 39% in Day 8 URA embryos compared to controls (2.45 ± 0.36 ml *vs* 1.50 ± 0.16 ml, C *vs* URA, respectively; p= 0.03). The observed reduction of allantoic fluid in URA embryos persists at Day 14 (C= 11.4 ± 0.7ml, URA= 9.3 ± 0.3ml; p <0.05), but the differences between groups are not as striking.

Embryos with URA exhibited marked edema at each developmental age examined. With the exception of Day 6 embryos, however, there existed no significant differences between the wet weights of URA and control embryos or total tissue mass (i.e., embryo+ extraembryonic membranes).

URA significantly affects kidney mass. The allometric scaling of *left* kidney weights to total body weights in control embryos (log transformed data, base 10) is KM= $3.43M^{0.98}$ (r^2= 0.941) compared to KM= $3.02M^{1.16}$ (r^2= 0.928) in URA embryos. When *total* kidney mass (i.e., left and right meso- and metanephroi) are scaled to total body mass, control embryos exhibit the allometric relationship, KM= $6.90M^{0.96}$ (r^2= 0.921) compared to $3.02M^{1.16}$ (r^2= 0.928) in URA embryos; the differences between these slopes is significant

at the p <0.01 level. Interestingly, the only other study to examine the importance of the mesonephros in the allometric scaling of kidney mass utilized the post-hatch, juvenile lizard, *Sceloporus jarrovi* (Beauchat and Braun, 1988). In these animals, the mesonephros persists for some time after hatching. The allometric relationship of the post-hatch *Sceloporus* kidney (meso- + metanephros) to body mass is KM= $5.73M^{0.86}$ compared to our control, pre-hatched *Gallus gallus*.

Embryos of ten days of incubation typically present two well-differentiated mesonephroi which stain well with Trypan Blue (Fig. 2A). Several studies have shown that this supravital dye must first pass through the glomerular vessels before entering the proximal tubules (for example, see Linshaw *et al.*, 1986). By comparison, blockage of the migration of the mesonephric duct results in embryos with a single meso- and metanephros. The mesonephros of animals with URA routinely stains with Trypan Blue with much more intensity than in control embryos (Fig. 2B). The metanephroi of both control and URA embryos are not well differentiated by Day 10 and hence, rarely pick up the stain. However, the metanephros of embryos with unilateral renal agenesis appears to pick up Trypan Blue at an earlier developmental stage than control embryos.

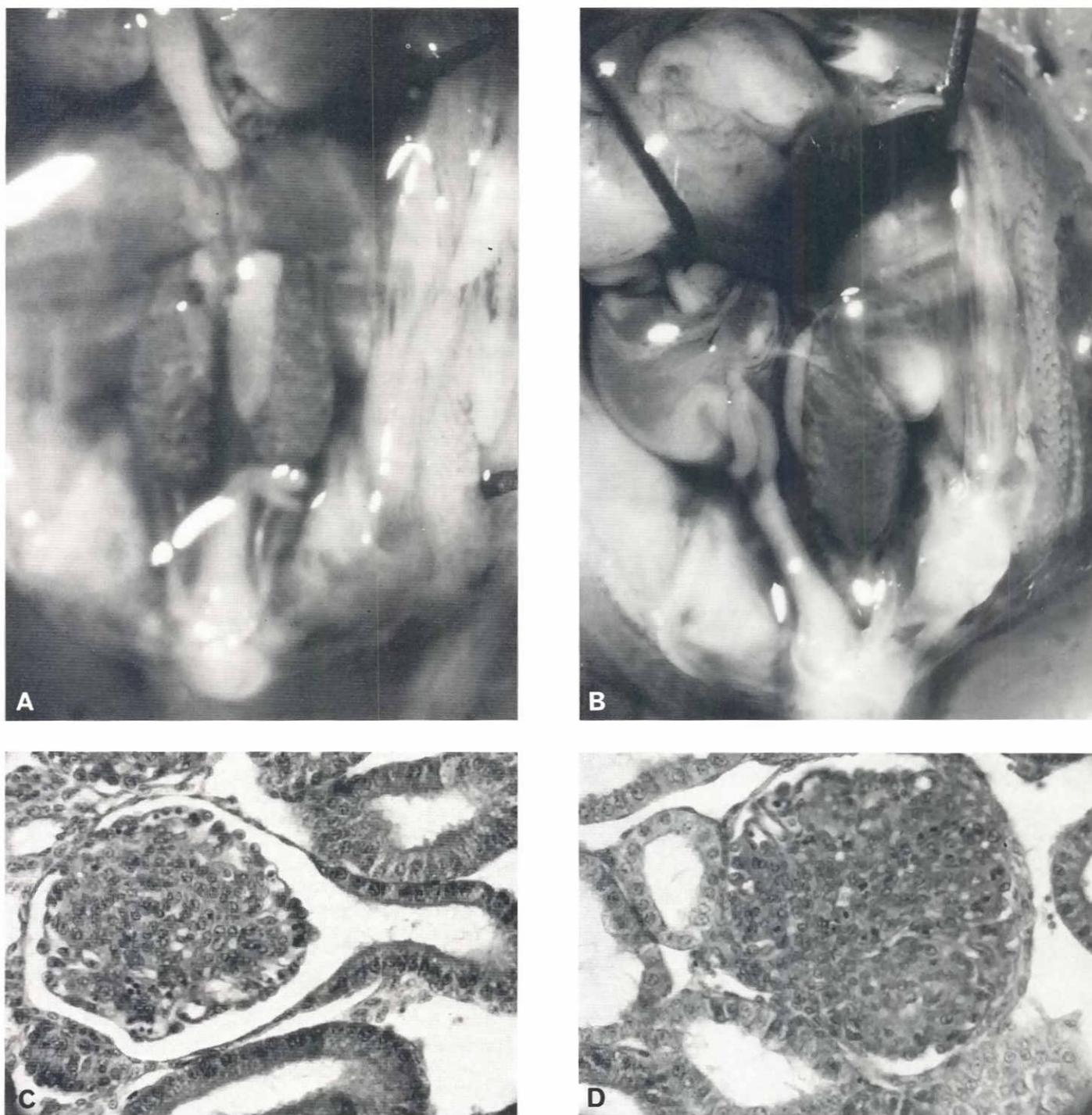Histological sections through the mesonephroi of Day 10 animals

Fig. 2. Glomerulonephritus in response to URA. (A) *Mesonephros of Day 10 control or* **(B)** *URA embryos stained with Trypan Blue (overlying gonads are white).* **(C)** *Histological section through typical mesonephric glomerulus of Day 10 control or* **(D)** *URA embryos. Note the expansion of the glomerular tuft and mesangium in the animal with URA.*

reveal a marked expansion of the glomerular capillaries and mesangiam in URA animals compared to controls, often obliterating Bowman's space (Fig. 2C,D).

By Day 16 of incubation, necrosis of the mesonephroi is already well advanced in most control embryos (Fig. 3A). Mesonephric tissue in these animals is white to yellow in appearance with little vascularization; no evidence of cyst formation is observed. In contrast, the majority of embryos with URA exhibit a polycystic mesonephric kidney and an expansion of the underlying metanephros (Fig. 3B). In addition, we have observed a small but significant
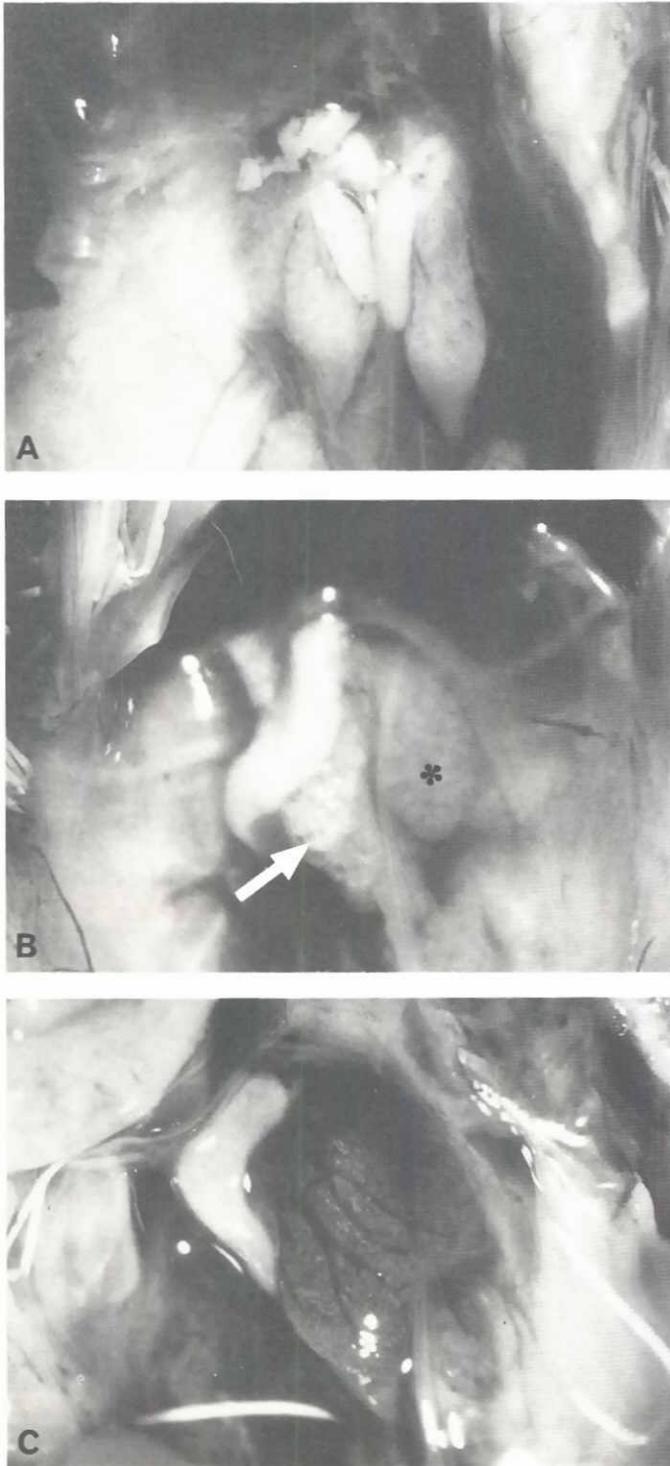
Fig. 3. Day 16 animals: (A) *control embryo with normal degeneration of the mesonephros.* **(B)** *Polycystic mesonephros (arrow) with expanding dorsal metanephros (asterisk) in embryo with URA.* **(C)** *Expanded renal vasculature and delayed regression of mesonephros in embryo with URA. Overlying gonads are white.*

number of these embryos which appear to have a temporal delay in the regression of the mesonephros. In these cases, a gross expansion of the mesonephric vasculature is readily apparent (Fig. 3C).

Few URA embryos survived to Day 18 of incubation and mesonephroi of both control and experimental groups are in advanced stages of degeneration. However, the mesonephros of URA animals contain greater numbers of histologically distinct glomeruli compared to controls.

The metanephroi of Day 18 embryos with unilateral renal agenesis contain numerous differentiated glomeruli as well as embryonic glomerular foci undergoing differentiation. In addition, there is a significant infiltration of granular leukocytes into the connective interstitium of the metanephros in animals with URA (Fig. 4B). By comparison, control embryos contain glomeruli which are routinely smaller and lack any evidence of the massive leukocyte infiltration (Fig. 4A).

In summary, it is estimated that unilateral renal agenesis (URA) affects 1 in every 1,000 births in humans (Elder et al., 1987) and that congenital anomalies are responsible for nearly 40% of all pathologic lesions involving the kidneys (Gorvoy et al., 1962). In addition, there is evidence that renal agenesis and dysgenesis may be increasing in human populations (Stroup et al., 1990).

Students of developmental or renal pathology have long been able to study late fetal or post-natal results of unilateral renal agenesis or obstructive uropathy. However, the ability to examine the *in situ*, developmental aspects of unilateral renal agenesis have been restricted due to the inaccessibility of early mammalian embryos and a general lack of an acceptable animal model. Our experiments clearly demonstrate that an elegantly simple and useful model of URA is available for long-term studies of URA involving both the mesonephric as well as the metanephric kidney.

Interestingly, the delay in the degeneration of the mesonephros in some URA embryos is accompanied by a gross expansion of the mesonephric vasculature. Our studies with Trypan Blue showing a more intense staining of kidneys in URA embryos may indicate an increase in single-kidney GFR at earlier developmental stages. While there have been some few animal models of renal hydronephrosis and compensatory hypertrophy utilizing an avian animal model (Hartenbower and Coburn, 1972; Berman and Maizels, 1982), these authors examined only the metanephric kidney of the late embryo or post-hatched chick. Thus, our model provides the first information available for examining the effects of true renal agenesis and chronic renal insufficiency in amniotes over extended developmental periods.

Experimental Procedures

Embryoculture

Fertile White Leghorn chicken eggs were obtained from the poultry facility at the University of Connecticut, Storrs, CT. All eggs were incubated at 37.5°C and constant high humidity in a Forma tissue culture incubator. At 33-40 h of incubation, eggs were removed from the incubator and fenestrated as described elsewhere (Murphy et al., 1986). This procedure creates a window in the eggshell through which observations, photography, fluid samplings, injections and surgery are easily performed.

Surgery

Our procedures for producing embryos with unilateral renal agenesis are only slightly modified from those of Bishop-Calamé (1965, 1966) utilizing embryos incubated for 33-36 h. Briefly, a small incision was made with a
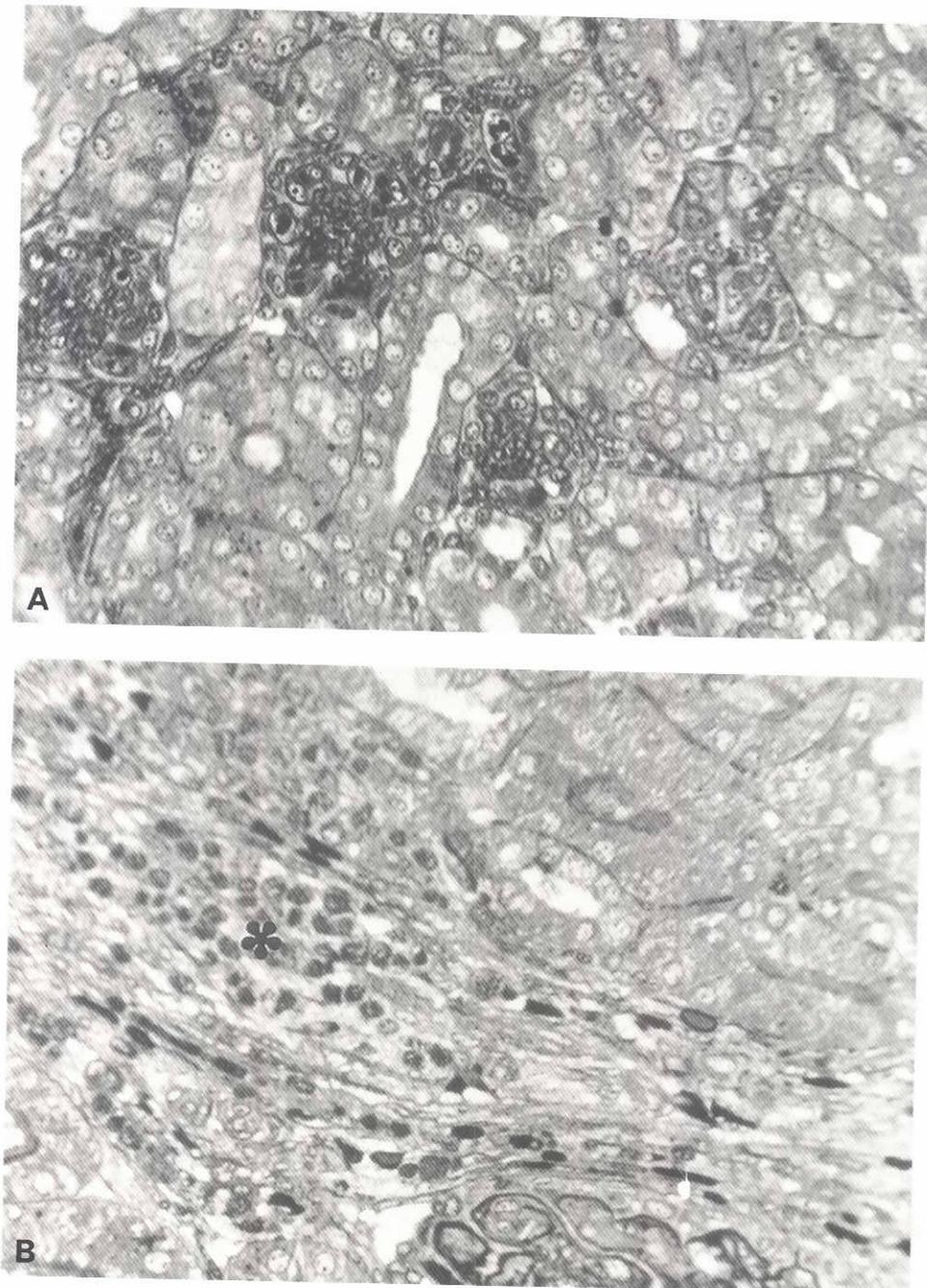
Fig. 4. Histological section through Day 18 metanephros: (A) *control and* **(B)** *URA. Note the infiltration of leukocytes into the interstitium of embryos with URA (asterisk).*

glass knife in the lateral body wall immediately posterior the most recently differentiated, caudal somite. A small piece of sterile eggshell membrane was inserted into this site to block the migration of the mesonephric (Wolffian) duct. Control embryos were either sham-operated and received no implant or served as unoperated controls for comparison.

Allantoic fluid

Determination of allantoic fluid volume was performed by inulin or polyethylene glycol (PEG) dilution methods on Day 8 and Day 14 embryos (Murphy et al., 1991). Ten or 20 µl (depending on embryo size/age) of ^{14}C-inulin (50 µCi/ml in avian saline) or tritiated PEG was injected directly into the allantoic cavity and allowed to equilibrate for 1 h. Mixing of the inulin is facilitated by the muscular contractions of the adjacent amnion; serial sampling of allantoic fluid indicates complete distribution of the inulin marker by 45 min post-injection. Allantoic fluid (10 µl) used for volume determinations was mixed with 10 ml of scintillation cocktail (Solvent-Free LSC Media, Isolab, Inc., Akron, OH). Radioactivity of allantoic fluid was determined in a Beckman LS-100C liquid scintillation counter using preset ^{14}C or tritium windows for 10 min and 0.5% preset error. Allantoic volume (AV) was calculated by dividing total ^{14}C-inulin or tritiated PEG injected into the allantois (CPM of standard) by the concentration of allantoic marker (CPM/ml of fluid).

Euthenasia and tissue processing

Twenty-four hours before the termination of each experiment, embryos received a bolus application of 0.4% Trypan Blue directly to the surface of the chorioallantoic membrane (CAM). This supravital dye is absorbed into the CAM capillaries and, by the following day, it is picked up by the proximal tubules of the kidneys only if the glomeruli are sufficiently differentiated and are filtering plasma (Linshaw *et al.*, 1986). At the end of each experiment, embryos were quickly removed from the eggshell and euthenized by immediate decapitation. Embryos and extraembryonic membranes were weighed after rinsing and blotting; the meso- and metanephroi were dissected free of surrounding tissues, weighed and placed into buffered formalin. Kidneys were processed and embedded in either plastic (glycomethacrylate; JB-4 kit, Polysciences, Inc.) or paraffin (TissuePrep, Fisher Scientific Co.) and sectioned between 1 and 6 μm. Tissue sections were stained with hematoxylin and eosin, PAS, or silver stain (Jones).

Statistics

Mean, standard deviation and standard error were calculated for Control *vs* URA comparisons. Significance was determined by Student's *t* test or appropriate analysis of variance (ANOVA). Linear regression was calculated by least squares linear regression methods on raw or transformed (log, base 10) data, depending on expected linear or allometric relationship. For the allometric relationship between kidney mass and embryo mass ($KM = aM^b$), KM is the kidney mass in mg, a is the intercept, M is the total embryonic tissue mass (i.e., embryo+membranes), and b is the slope (exponent).

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