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TECHNICAL NOTE

Can murine diabetic nephropathy be separated from superimposed acute renal failure?

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Can murine diabetic nephropathy be separated from superimposed acute renal failure?

Background. Streptozotocin (STZ) is commonly used to induce diabetes in experimental animal models, but not without accompanying cytotoxic effects. This study was undertaken to (*1*) determine an optimal dose and administration route of STZ to induce diabetic nephropathy in wild-type mice but without the concurrent acute renal injury resulting from cytotoxic effects of STZ and (*2*) evaluate the pattern of tubular injury and interstitial inflammation in this model.

Methods. Male Balb/c mice received either (*1*) STZ (225 mg/kg by intraperitoneal injection.); or (*2*) two doses of STZ 5 days apart (150 mg/150 mg/kg; 75 mg/150 mg/kg; 75 mg/75 mg/kg; and 100 mg/100 mg/kg by intravenous injection). Another strain of mice, C57BL/6J, also received STZ (200 mg/kg intravenously or intraperitoneally). Renal function and histology were examined at weeks 1, 2, 4, and 8 after induction of diabetes. In initial optimization studies, animals were sacrificed at week 1 or week 2 and histology examined for acute renal injury.

Results. Following a single intraperitoneal injection of 225 mg/kg of STZ, only two thirds of animals developed hyperglycemia, yet the model was associated with focal areas of acute tubular necrosis (ATN) at week 2. ATN was also observed in C57BL/6J mice given a single intravenous or intraperitoneal dose of STZ (200 mg/kg), at week 2 post-diabetes. At an optimal diabetogenic dose and route (75 mg/150 mg/kg by intravenous injection 5 days apart), all mice developed diabetes and no ATN was observed histologically. However, even with this regimen, glomerular filtration rate (GFR) was significantly impaired from week 2. This regimen was accompanied by progressive histologic changes, including tubular and glomerular hypertrophy, mesangial area expansion, as well as interstitial macrophage, CD4+ and CD8+ T-cell accumulation.

Conclusion. By careful optimization of STZ dose, a stable and reproducible diabetic murine model was established. However, even in this optimized model, renal functional impairment was observed. The frequency of ATN and functional impairment casts doubt on conclusions about experimental diabetic nephropathy drawn from reports in which ATN has not been excluded rigorously.

Key words: murine, diabetic nephropathy, acute renal failure, interstitial inflammation.

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Streptozotocin (STZ)-induced diabetes is a commonly used experimental model in rats and mice to investigate the pathogenesis of human diabetic nephropathy. Mice are more resistant to the diabetogenic effect of STZ than rats. In mice, other investigators have used either, in excess of two consecutive daily intraperitoneal injections of 200 mg/kg [1]; a single intravenous injection of 300 mg/kg [2]; or intraperitoneal administration at lower doses of 50 mg/kg for 5 consecutive days [3].The possible superimposition of acute tubular necrosis (ATN) was not considered in these studies [1–3], and as a consequence the interpretation of findings is confounded. The aim of this study was therefore to identify a nonnephrotoxic dose of STZ in wild-type mice and then determine characteristics of renal interstitial inflammation and tubular injury in this optimized model.

METHODS

Animals

Inbred male BALB/c and C57BL/6J mice, 6 to 8 weeks old and weighing 20 to 25 g (Animal Resource Center, Perth, Australia) were housed at the Animal Care Facility, Westmead Hospital, and allowed free access to standard food pellets and water. All experimental protocols were approved by the Animal Care and Ethics Committee, Westmead Hospital.

Experimental design

In the initial study**,** animals (regimen A) received a single intraperitoneal dose (225 mg/kg) of STZ (Sigma Chemical Co., St. Louis, MO, USA) (S-0130) dissolved in sterile saline. Lower doses of STZ were examined when this regimen was found to cause ATN. In a second study, STZ was administered intravenously twice, 5 days apart. The doses used were (regimen B) 75 mg/kg/

150 mg/kg; (regimen C) 150 mg/kg/150 mg/kg; (regimen D) 100 mg/kg/100 mg/kg; or (regimen E) 75 mg/kg/75 mg/kg. In a supplementary study, C57BL/6J mice received a single intravenous(regimen F) or intraperitoneal (regimen G) dose (200 mg/kg) of STZ. All animals were nonfasted at the time of STZ administration.

Following STZ, morning blood glucose was measured daily by tail-vein sampling using a glucometer (Roche, Basel, Switzerland). Only animals with two consecutive morning nonfasting blood glucose levels of >16 mmol/L were included in the study. Long-acting insulin (Humulin Lente, Aza Research, Castle Hill, NSW, Australia) was given subcutaneously to prevent weight loss and maintain hyperglycemia (blood glucose levels between 15 and 30 mmol/L) or normoglycemia (blood glucose levels <10 mmol/L). Diabetic and control animals were sacrificed under anesthesia at time points detailed under respective figure/table legends.

Renal function

Animals were placed in metabolic cages for 16 hours with food and water, a day prior to sacrifice. Total urinary protein was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Urinary glucose was measured using a glucometer (Roche), and diluted where necessary to fit within the linearity range of the glucometer. Urinary creatinine was measured by the Vitros Crea slide method, as the high glucose content in urine of diabetic animals interfered with measurements using the Jaffé method [4]. Blood samples for serum creatinine were obtained at the time of sacrifice by cardiac puncture, and measured with a commercial kit (Sigma Chemical Co.) (555-A) using an improved colorimetric assay based on the Jaffé method [5, 6]. Creatinine clearance was calculated as creatinine excreted in the urine (16-hour period) divided by the creatinine concentration in serum, and the results were expressed as mL/min.

Histology and morphometric analysis

At the time of sacrifice, after blood samples had been taken, the animals were exsanguinated and kidney samples were removed and weighed. Coronal slices were immersion-fixed in 10% neutral-buffered formalin for 24 hours, then dehydrated in graded alcohols, and embedded in paraffin. Sections, 4 μm in thickness, were stained with periodic acid-Schiff (PAS). A comparison of histologic markers of tissue injury was made among animal subgroups using an image analysis system (Optimas 5.23) (Optimas Corporation, Seattle, WA, USA) as described previously [7]. Mean values were calculated from each of 20 glomeruli, 50 tubules, or 10 random cortical regions per section.

Table 1. Average blood glucose level (mmol/L) control and diabetic mice induced with different streptozotocin (STZ) dose regimen

STZ regimen	Group	Control	Diabetic			
			Week 1	Week 2	Week 4	Week 6	Week 8
A 225 mg/kg intraperitoneally	1	5.7 ± 0.25 ($N = 4$)		17.7 ± 0.18 ($N = 4$)	22.3 ± 0.17 ($N = 4$)	15.1 ± 0.25 ($N = 4$)	
	2 (NG)			7.5 ± 1.65 ($N = 4$)			
	3	6.5 ± 0.40 ($N = 6$)	23.8 ± 2.17 ($N = 4$)	19.9 ± 3.59 ($N = 6$)			
B 75 mg, 150 mg/kg intravenously	1	5.5 ± 0.35 ($N = 4$)	19.2 ± 6.81 ($N = 4$)	26.0 ± 4.79 ($N = 5$)	23.0 ± 2.12 ($N = 5$)		21.2 ± 2.54 ($N = 8$)
C 150 mg, 150 mg/kg intravenously	1			18.8 ± 1.43 ($N = 3$)			
D 100 mg, 100 mg/kg intraperitoneally	1			10.9 ± 2.74 ($N = 3$)			
E 75 mg, 75 mg/kg intravenously	1			7.17 ± 0.56 ($N = 3$)			
F 200 mg/kg intravenously	1	7.6 ± 0.49 ($N = 4$)	20.0 ± 2.58 ($N = 5$)	25.2 ± 2.51 ($N = 4$)			
G 200 mg/kg intraperitoneally	1			20.2 ± 3.35 ($N = 4$)			

Dose regimens A to E, BALB/c male mice were injected at doses indicated; regimen A was a single intraperitoneal injection; regimen B to E were intravenous injections of two doses of STZ, given 5 days apart.
Dose regimen F and G, C57Bl/6J male mice were injected at doses indicated. Both regimens were single-dose injections given intravenously and intraperitoneally, respectively.
Animals were sacrificed at: weeks 2, 4, and 6 (regimen A, group 1); week 1, 2, 4, and 8 (regimen B). For all other regimens (C to G), animals were sacrificed at week 1 and/or week 2. Control animals were sacrificed at the same time as the last time point of the diabetic animals. All animals were maintained moderately hyperglycemic (HG, 15 to 30 mmol/L), except in regimen A, group 2, where the animals were maintained at normoglycemia (NG). All data are expressed as mean ± SD. Blood glucose levels of diabetic (HG) animals were significantly different from control animals ($P < 0.001$). Blood glucose levels of normoglycemic diabetic animals (NG) were not different from control groups ($P > 0.5$).

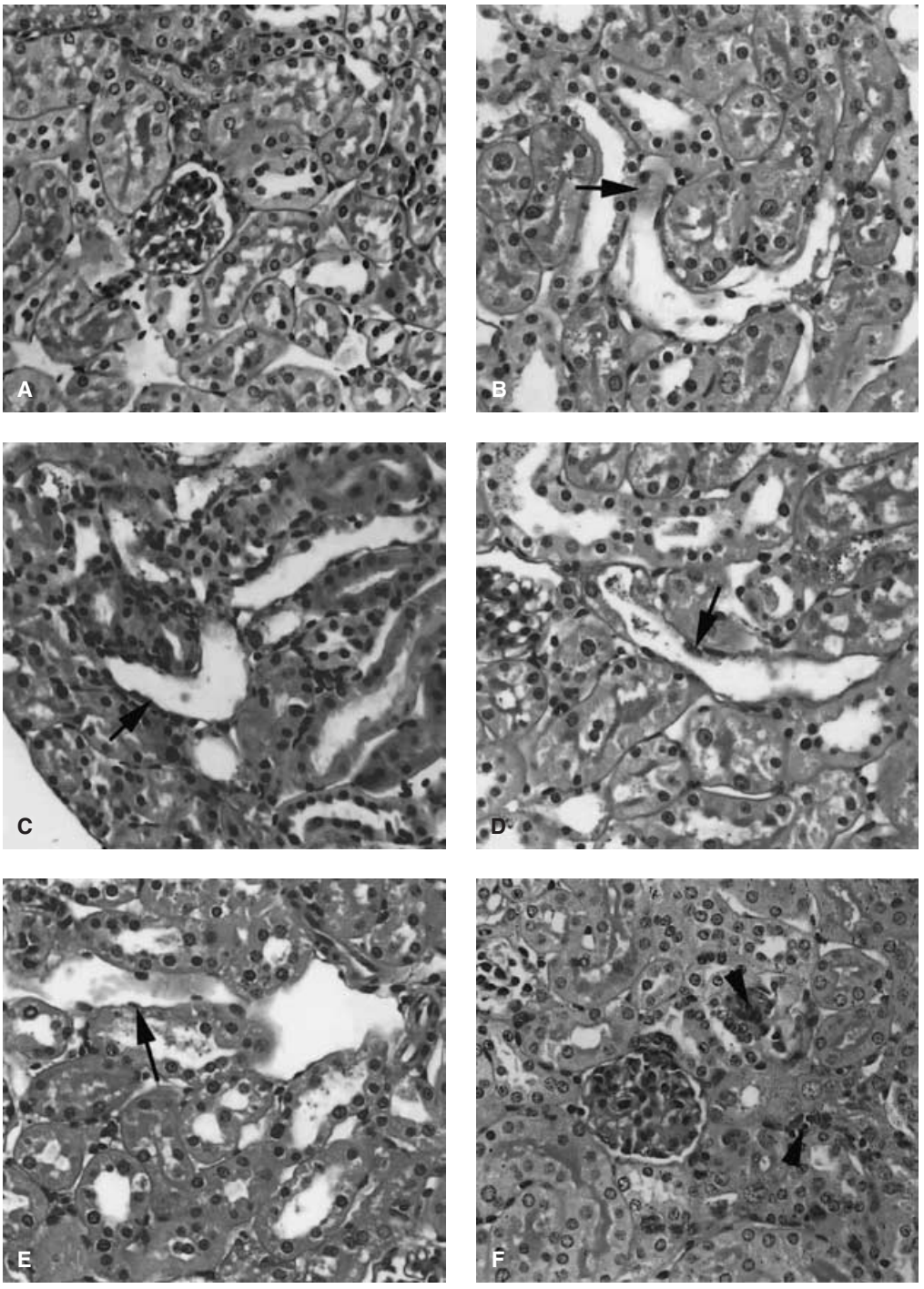

Fig. 1. Comparison of histologic changes in periodic acid-Schiff (PAS)–stained renal cortical sections from BALB/c mice (*A* to *D* and *F*) and C57BL/6J mice (*E*). (A) Without any injection. (B to D) with a single high dose of streptozotocin (STZ) (225 mg/kg intraperitoneally, (F) with two low doses of STZ (75 mg, 150 mg/kg intravenously, and (E) with a single high dose of STZ (200 mg/kg) intravenously. Diabetic animals maintained at normoglycemia were sacrificed at week 2 (D), while those maintained at hyperglycemia were sacrificed at week 1 (B) and week 2 (C, E, and F). The arrowed tubules in (B to E) show denudation of the epithelial cells consistent with acute tubular necrosis (ATN). Arrowheads (F) denote interstitial cell infiltrates (magnification 400×).

Immunohistochemistry

Coronal slices of kidneys were embedded in 22-oxacalcitriol (OCT) compound (Tissue-Tek, Sakura Finetek, Torrance, CA, USA), frozen in liquid nitrogen and then stored at −70°C. Frozen sections, 5 μm in thickness, were fixed with acetone at 4°C for 15 minutes. Sections were incubated with 5% normal rabbit serum followed by antibodies against macrophages

Table 2. Comparison of functional and histologic markers in study where a single high dose of streptozotocin (STZ) (225 mg/kg) was administered intraperitoneally

	Control	Week 2 post-STZ	Week 4 post-STZ	Week 6 post-STZ
Serum creatinine μmol/L	18.0 ± 4.9	29.3 ± 2.9[a]	18.6 ± 1.2	37.4 ± 7.6[a]
Glomerular tuft area μm^2	2373 ± 43	2308 ± 47	2471 ± 46	2666 ± 44[a]
Glomerulosclerosis index (%PAS material in tuft)	28.2 ± 0.6	27.8 ± 0.7	30.5 ± 0.7	29.1 ± 0.7
Proximal tubular cell height μm	16.3 ± 2.6	NA	NA	15.3 ± 2.6[b]
Proximal tubular diameter μm	32.7 ± 0.7	NA	NA	31.4 ± 0.6
Interstitial area (% of cortex)	13.9 ± 4.7	18.3 ± 3.5[a]	13.1 ± 3.4	16.6 ± 5.6[a]

Tubular cell ultrastructure was not assessed (NA) at 2 and 4 weeks after streptozotocin (STZ) because of the presence of acute tubular necrosis (ATN).
All data expressed as mean ± SD.
[a] $P \leq 0.01$ when compared to control group.
[b] $P \leq 0.05$ when compared to control group.

(rat antimouse F4/80 antigen) (Serotec)(MCA-497), CD4 (purified antimouse CD4) (L3T4)(RM4-5) (Pharmigen) (553043), and CD8 (purified antimouse CD8a) (Ly-2) (53-6.7) (Pharmigen) (553027) for 60 minutes at room temperature; biotinylated rabbit anti-IgG (1:200); and then avidin-biotin-horseradish peroxidase complex (30 minutes each at room temperature). The reaction was visualized by 3,3-diaminobenzidine tetrahydrochloride (DAB).

The number of macrophages, CD4+ and CD8+ cells was quantitated in 10 nonoverlapping cortical fields (×400, measuring 0.075 mm^2 each). Using image analysis of cells as described above, the mean number of positive cells per interstitial field was calculated for each section and expressed as cells per mm^2. Similarly, the number of positive cells per glomerulus was counted (×400), and the mean for 20 randomly selected glomeruli was determined.

Statistical analysis

The results are expressed as mean ± standard deviation. Statistical comparisons between animal groups were performed using a computer-based statistical package (SPSS version 8.0, Chicago, IL, USA). A one-way analysis of variance (ANOVA) was used and subgroups were then compared by post hoc analysis using the least squares method. A P value of < 0.05 was considered to represent statistical significance.

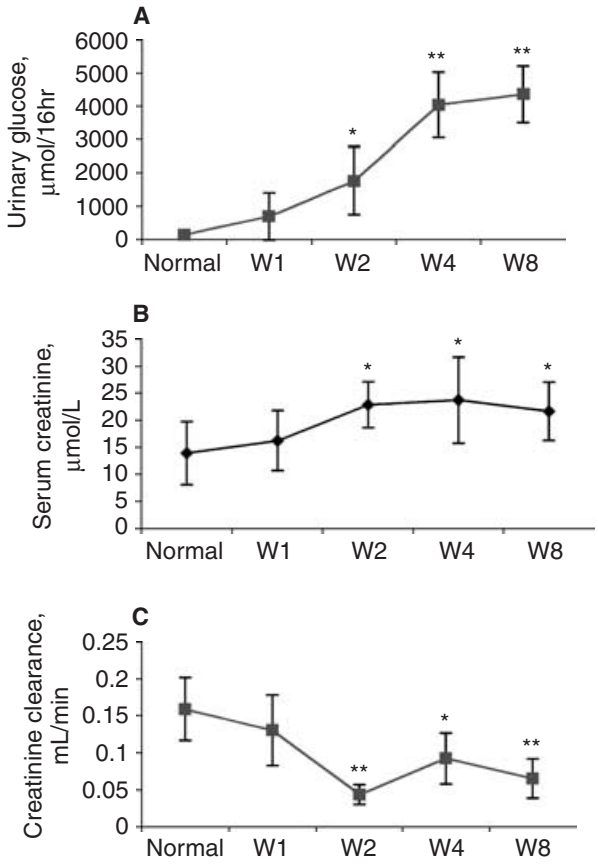

Fig. 2. Urinary glucose, μmol/16 hours (A), serum creatinine, μmol/L (B), creatinine clearance, mL/minute (C), for normal and diabetic BALB/c mice (regimen B, 75 mg, 150 mg/kg streptozocin (STZ) intravenously sacrificed at weeks (w) 1, 2, 4, and 8 post-STZ. Values are expressed as mean ± SD. *P <.05; **$P < 0.001$ when compared to normal animals.

RESULTS

Only two-thirds of 18 BALB/c mice injected with 225 mg/kg (intraperitoneal injection) developed diabetes (blood glucose le vel >16 mmol/L) by day 7 (Table 1). At this dose, focal areas of ATN with tubular vacuolization were noted at week 1 and 2 in diabetic mice (Fig. 1B and C), and were associated with interstitial expansion and a rise in serum creatinine (Table 2). These changes were absent at week 4 but were seen again at week 6. Normalization of bblood glucose level (<10 mmol/L) up to the week 2 time point did not prevent ATN (Fig. 1D). The nephrotoxicity of STZ was not only confined to BALB/c mice. C57BL/6J mice injected with a dosage commonly used to induce diabetes in the study of diabetic nephropathy (≥200 mg/kg intravenously or intraperitoneally) exhibited morphologic evidence of ATN at week 2 (Fig. 1E), with a milder degree at week 1.

In contrast, two lower doses of STZ injected intravenously through the tail vein of BALB/c mice [75

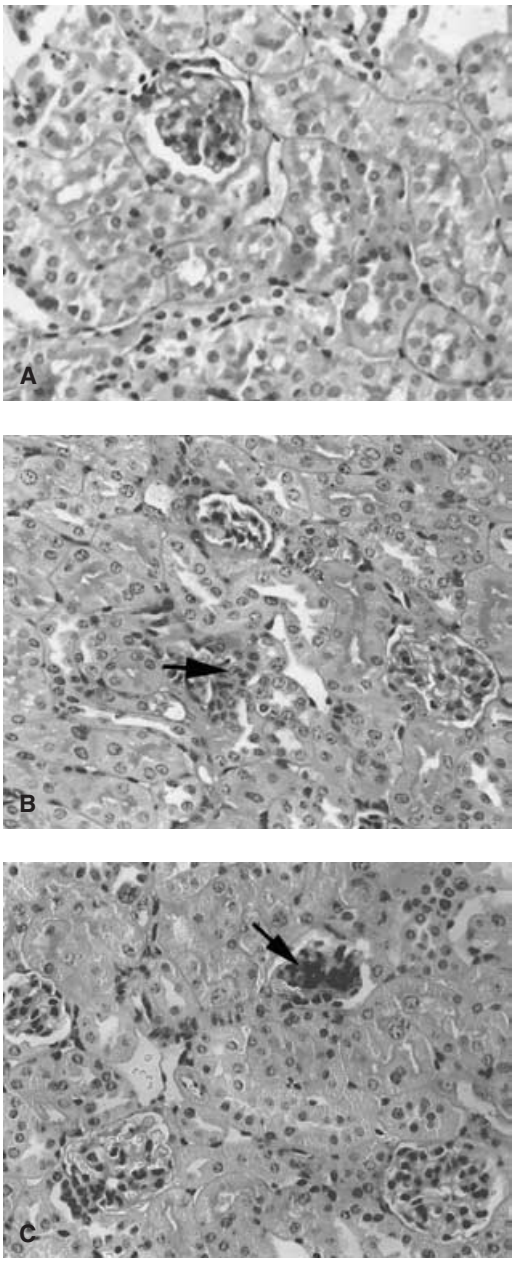

Fig. 3. Comparison of histologic changes in periodic acid-Schiff (PAS)–stained renal sections from BALB/c mice (A) without any injection, (B and C) injected with two low doses of streptozotocin (STZ) (75 mg, 150 mg/kg intravenously). Diabetic mice were maintained at hyperglycemia. Arrows denote interstitial cell infiltrates at week 2 (B) and glomerulosclerosis at week 8 (C) (magnification 400×).

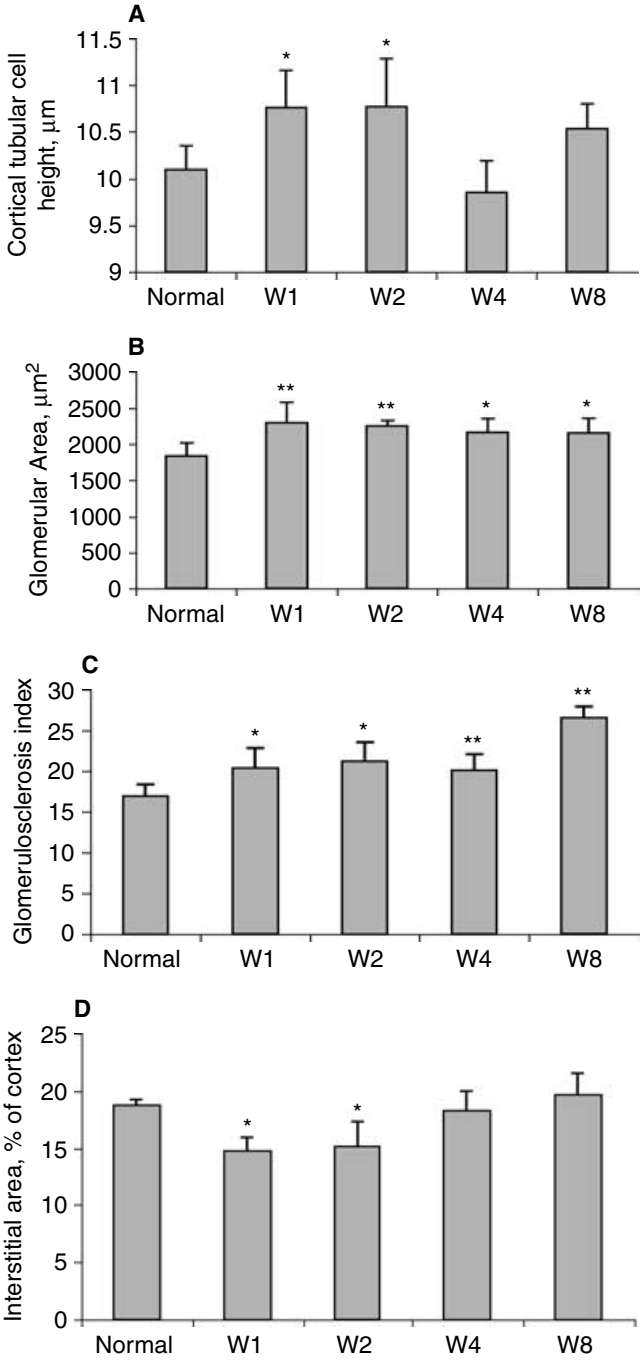

Fig. 4. Morphometric analysis of normal and diabetic BALB/c mice sacrificed at weeks (w) 1, 2, 4 and 8 after induction of diabetes with 75 mg, 150 mg/kg streptozotocin (STZ). Diabetic mice were maintained at hyperglycemia prior to sacrifice. (*A*) Cortical tubular cell height, μm. (*B*) Glomerular area, μm^2. (*C*) Glomerulosclerosis index [% of periodic acid-Schiff (PAS) material in tuft]. (*D*) Interstitial area (% of cortex). Values are expressed as mean ± SD. $^*P < 0.05$; $^{**}P < 0.005$.

mg/kg/150 mg/kg or 150 mg/kg/150 mg/kg (5 days after the first injection)], produced a more consistent model of diabetes, where all animals developed diabetes (Table 1) (Fig. 2A) Lower doses of 100 mg/kg/100 mg/kg or 75 mg/kg/75 mg/kg did not result in diabetes even up to 2 weeks later. There was no histologic evidence of ATN in the two-injection model (Fig. 1F). However, diabetic animals (regimen B, 75 mg, 150 mg/kg) developed progressive renal functional impairment from week 2, and this persisted until week 8 (Fig. 2B).

Proteinuria did not increase in diabetic animals expressed either as an absolute value (week 1, 6 ± 2; week

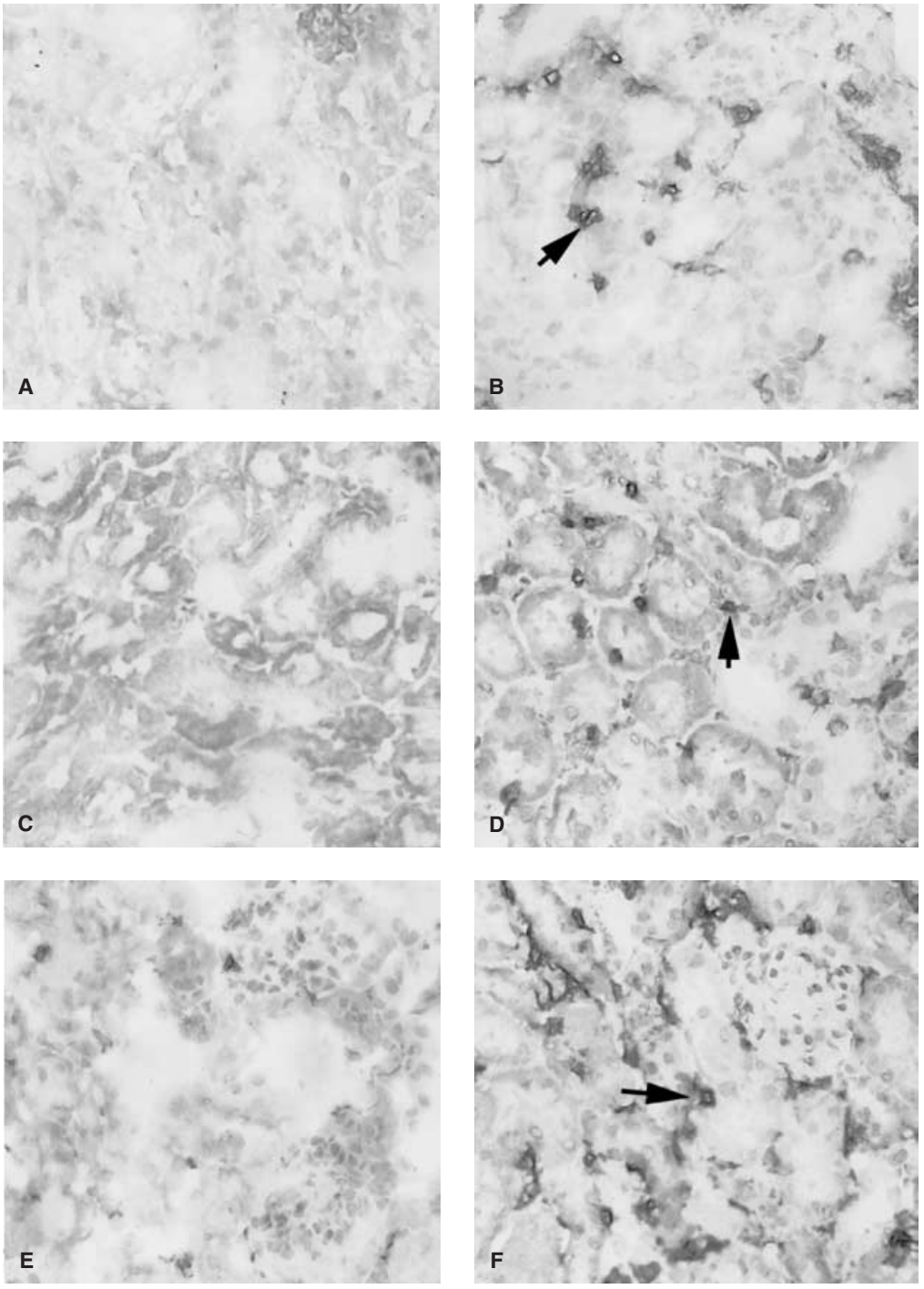

Fig. 5. Stained renal cryosections from BALB/c mice, sacrificed at week 2, (A, C, and E) without any injection; (B, D, and F) with two low doses of streptozotocin (STZ) (75 mg, 150 mg/kg intravenously) and maintained at hyperglycemia. (A and B) Stained for CD4. (C and D) Stained for CD8. (E and F) Stained for macrophage. Arrows denote respective cell surface markers (magnification 400×).

2, 3 ± 1; week 4, 7 ± 3; and week 8, 6 ± 3 vs. control, 10 ± 6 mg/16 hours) ($P > 0.1$), or as urinary protein-creatinine ratio (week 1, 3.59 ± 0.94; week 2, 3.15 ± 1.23; week 4, 3.65 ± 1.89; and week 8, 4.45 ± 1.55 vs. control, 3.80 ± 1.42 mg/μmol) ($P > 0.5$).

Diabetic animals developed progressive histological changes (Fig. 3), including an increase in tubular cell height (Fig. 4A), glomerular hypertrophy (Fig. 4B), mesangial area expansion and increase in glomerulosclerosis index (Fig. 4C), and a reduction in interstitial area

Table 3. Comparison of interstitial inflammatory cell numbers between normal and diabetic BALB/c mice [diabetes was induced with 75 mg, 150 mg/kg streptozotocin (STZ) intravenous injection]

Interstitial inflammatory cell types (no. of cells/field)	Normal	Week 1 post-STZ	Week 2 post-STZ	Week 4 post-STZ	Week 8 post-STZ
CD4	3.3 ± 0.3	6.7 ± 2.3	16.1 ± 2.9[a]	10.1 ± 2.8[a]	10.4 ± 2.6[a]
CD8	1.8 ± 0.9	3.8 ± 1.2	18.3 ± 2.9[a]	10.3 ± 1.8[a]	9.8 ± 2.0[a]
Macrophage	8.7 ± 1.5	10.2 ± 2.9	53.0 ± 7.5[a]	38.4 ± 4.0[a]	23.2 ± 1.8[a]

[a] $P \leq 0.001$ vs. normal controls.

at weeks 1 and 2 (Fig. 4D). Despite the reduction in interstitial area, interstitial CD4+ and CD8+ T-cell and macrophage infiltration was detected as early as 1 to 2 weeks post-STZ injection (Fig. 5B, D, and F, respectively). Cell numbers reached a peak by week 2 ($P < 0.0001$ vs. control, for all three cell types), but decreased dramatically in later stages (Table 3). Glomerular CD4+, CD8+ T cell and macrophage numbers were not increased in STZ-mice.

DISCUSSION

The results of the present study demonstrate that, in male BALB/c mice, (*1*) a more stable and reproducible diabetic murine model could be established by intravenous, rather than intraperitoneal injection of streptozotocin; (*2*) the optimal diabetogenic dose of STZ that did not cause histologically evident ATN was 75 mg STZ/kg given intravenously, with a second dose of 150 mg/kg administered 5 days later; and (*3*) even at these apparently nonnephrotoxic doses, renal dysfunction was evident. Thus it is not possible to determine if the accompanying interstitial infiltration of lymphocytes and macrophages was a consequence of diabetes or superimposed ATN.

There are numerous regimens to induce STZ-induced diabetes in mice. Consistent with previous results we found that administration of STZ by the intraperitoneal route was a less reliable method of induction [3]. However, of greater importance, we found that a single high-dose of STZ intraperitoneally caused focal areas of ATN. Therefore, we examined lower doses of STZ and found that either 75 mg, 150 mg/kg and 150 mg, 150 mg/kg given intravenously 5 days apart, produced diabetes in all the mice without structural evidence of ATN, whereas lower doses did not induce diabetes. However, with optimally diabetogenic regimen, ATN could not be excluded as glomerular filtration rate (GFR) was significantly reduced. Utilization of STZ-diabetic model without ATN is important because superimposed ATN would confound the interpretation of results.

The occurrence of ATN is not specific to BALB/c mice when a nonoptimized dose of STZ is used to induce diabetes, neither is it as a result of hyperglycemia. In our supplementary study, C57BL/6J mice injected with a single high dose of STZ (200 mg/kg intravenous or intraperitoneal) also developed ATN at 1 to 2 weeks. Normalizing blood glucose level of diabetic BALB/c mice after STZ induction (225 mg/kg) did not alleviate ATN. Previous studies in mice [3] examined renal histology 8 weeks after STZ treatment, and did not take into account potential effects of ATN. There are numerous published studies examining a whole range of issues in STZ diabetic mice and other STZ diabetic rodent models. Rat islet cells are much more sensitive to the toxic effects of STZ so that much lower doses of STZ can be used (40 mg to 60 mg/kg). However optimal nonnephrotoxic STZ doses that do not cause acute renal injury histologically have not been assessed rigorously in rats either. In none of these has ATN been examined for adequately and so the conclusions must be questioned [8–14]. For example, renal bone morphogenic protein-7 (BMP-7) was reported to be decreased in rats with STZ diabetes [14]. Similar observations have been made in the setting of acute renal failure [15], and so it is quite possible that the reported changes in diabetic mice should be attributed to superimposed ATN rather than diabetes per se. The same argument applies to other observations in STZ-diabetic mice [8–13] where superimposed ATN has not been excluded rigorously.

At the optimal diabetogenic dose of STZ, diabetic animals developed progressive histologic changes typical of diabetic nephropathy, including tubular hypertrophy, glomerular hypertrophy, and mesangial area expansion. This was accompanied by an early interstitial inflammatory cell infiltration by macrophages and T lymphocytes, similar to human diabetic nephropathy as well as other murine models of chronic renal disease [16, 17]. The role of tubulointerstitial macrophages and T cells in this and other models of diabetic nephropathy remain unclear, as does their relationship to superimposed ATN.

CONCLUSION

Our study shows that even with careful optimization of the STZ dose, superimposed ATN cannot be separated from diabetic nephropathy. The possible effect of superimposed ATN must be considered when drawing conclusions from the model of STZ diabetes.

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