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Original Article

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In mice, proteinuria and renal inflammatory responses to albumin overload are strain-dependent

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Abstract

Background. The availability of genetically modified mice has increased the need for relevant mouse models of renal disease, but widely used C57BL/6 mice often show resistance to proteinuria. 129/Sv mice are considered more sensitive to certain renal models. Albumin overload, an important model of proteinuric disease, induces marked proteinuria in rats but barely in C57BL/6 mice. We hypothesized that albumin overload would induce more proteinuria in 129S2/Sv than C57BL/6J mice.

Methods. Male and female C57BL/6J and 129S2/Sv mice received bovine serum albumin (BSA) for 11 days. Control groups received saline injections. Injected BSA was immunohistochemically localized to study intrarenal handling of overloaded protein. Renal macrophage infiltration (F4/80 immuno-staining) and glomerular ultrastructure (electron microscopy) were assessed.

Results. The BSA-treated groups were similarly hyperproteinemic at Day 11 (D11). Proteinuria differed widely. In C57BL/6J mice, it remained unchanged in females but significantly, though mildly, increased in males (from 3 ± 1 to 8 ± 2 mg/day, $P < 0.05$). In 129S2/Sv, proteinuria was marked in both males and females (4 ± 1 to 59 ± 14, and 0.6 ± 0.2 to 29 ± 9 mg/day, respectively, both $P < 0.01$). Proteinuria was accompanied by tubulo-interstitial macrophage infiltration in 129S2/Sv mice. Injected BSA was visualized within glomeruli in both strains and in the urinary space and tubules of 129S2/Sv but not C57BL/6J mice, indicating much greater glomerular leakage in the former. No glomerular macrophages or ultra-structural differences were detected.

Conclusion. There are major strain differences in the proteinuria and renal inflammatory response of mice to albumin overload, which are not due to structural variation in the filtration barrier but possibly to functional differences in glomerular protein permeability.

Keywords: albumin; gender; glomerular permeability; mouse; proteinuria

Introduction

Albumin overload is a well-known model of renal tubulointerstitial disease in rats [1]. Strain and gender related differences in magnitude of the proteinuria response to albumin overload have been reported, but females and males of all tested rat strains (Wistar, Sprague–Dawley, DA and PVG) became significantly proteinuric [2]. Given the increasing recognition of an independent causal role of proteinuria in tubulointerstitial inflammation and ultimately in renal fibrosis [3], the albumin overload model represents an outstanding platform for *in vivo* investigation of the impact of excess protein load on tubular cells in a primarily non-haemodynamic and non-immunologic setting. The replication of this model in mice, with the attendant possibility to employ genetically modified strains, would be very useful in characterizing the specific impacts of defined genetic traits on the pathophysiologic processes involved. However, in recent reports of protein overload in C57BL/6 mice [4,5], although effects such as gene expression alterations were observed, the proteinuric response was not impressive, which may significantly limit the potential for exploitation of aspects of the model. This is especially disappointing because of the popularity of the C57BL/6 strain as a background for transgenic and knockout mouse models.

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In contrast to the C57BL/6, the 129S2/Sv mouse strain appears to be remarkably responsive to inducement of chronic renal disease. Proteinuria and marked glomerulosclerosis are seen in male 129S2/Sv mice 9 weeks after 5/6 nephrectomy, while male C57BL/6J mice show no proteinuria and only mild sclerosis 24 weeks after the same degree of renal ablation [6]. Similarly, male 129/Sv mice are more susceptible to development of hypertension and renal failure after deoxycorticosterone acetate (DOCA) plus salt than C57BL/6 mice [7]. In rats differences in proteinuria are noted between strains after protein overload [2], and we hypothesized that this is true also for mice. Such differences would have potentially important implications in the development of genetically modified strains, as identical genetic modifications can result in mice with massive phenotypic differences depending on their original genetic background [8]. Thus, we conducted experiments to determine whether 129S2/Sv mice would show more proteinuria in response to albumin overload than C57BL/6J mice. Because male gender is generally associated with more proteinuria than female gender, and chronic renal disease progresses more rapidly in males [9], we expected a more pronounced strain difference in males. We found, as expected, that 129S2/Sv had much more proteinuria than C57BL/6J despite similar elevations in plasma albumin levels. We conducted electron microscopic examination of kidneys from both strains, to seek possible glomerular structural differences that could help explain the disparity in proteinuria, but we found, none. Finally, we analysed renal interstitial macrophage infiltration to determine whether the proteinuria difference is of functional pathologic significance in terms of susceptibility to renal inflammation after protein overload.

Methods

Animal treatments

We employed male and female C57BL/6J and 129S2/Sv mice, age 14–18 weeks (Harlan Nederland, Horst, The Netherlands). The mice were maintained on a standard diet (RMH-TM; Hope Farms, Woerden, The Netherlands) and tap water *ad libitum*. For experiments, animals were housed in pairs in cages in a room maintained at 22°C, 60% humidity with a 12/12-h light/dark cycle. The Utrecht University board for studies on experimental animals approved the protocol. Male and female mice of both strains received intraperitoneal (i.p.) injections of low-endotoxin bovine serum albumin (BSA, A-9430) (Sigma Chemical Co., St. Louis, MO) (dissolved in saline) for 9 days over an 11 day period. BSA was administered 5 days/week on a stepwise incremental dose regimen [4], rising from 2 mg/g body weight on the first day (D1) to the maximum dose of 10 mg/g on D5, which was thereafter maintained. Mice in strain, gender and age matched control groups received i.p. saline injections of corresponding volumes. Proteinuria was measured at baseline (D0), and subsequently at D4 and D11 of treatment, in 16-h urine samples collected during overnight placement in metabolic cages. At termination (D11), mice were anaesthetized with an IP ketamine/xylazine/atropine cocktail and blood samples collected by venacava cannulation. Kidneys were harvested and samples either fixed in 4% formaldehyde, or snap-frozen in liquid nitrogen and stored at –80°C until analysed. In urine and plasma, total protein was measured by the Bradford method (Bio-Rad Laboratories, Munich, Germany), and BSA and mouse serum albumin (MSA) by immunoelectrophoresis using rabbit anti-bovine or anti-mouse antibodies (Nordic Immunological Laboratories, Tilburg, The Netherlands). Plasma urea was measured enzymatically (Elitech, Sees, France), and plasma oncotic pressure by membrane osmometry (Amicon 10PK, Millipore, Billerica, MA, USA). Systolic blood pressure was measured in conscious mice by the tail-cuff method (IITC, San Diego, CA, USA).

Renal histology and immunohistochemistry

Formaldehyde-fixed, paraffin-embedded kidney tissue was stained using standard procedures with haematoxylin and eosin and periodic acid-schiff for light microscopic examination by a renal pathologist in blinded fashion. For intrarenal visualization of injected BSA, kidney sections were deparaffinized, blocked with endogenous buffer/hydrogen peroxide, boiled in 10 mM citrate buffer (pH 6.0), and incubated with rabbit anti-BSA antibody (Nordic Immunological Laboratories, Tilburg, The Netherlands, 1:3000 in 5% NGS/PBS), followed by horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (Powervision) (Klinipath, Leiden, The Netherlands). They were developed with Nova Red (Vector, Burlingame, CA, USA) and counterstained with haematoxylin. Semi-quantitative assessment of the degree of renal cortical BSA staining was carried out in blinded fashion, using a cumulative scoring system with points awarded for the presence of BSA within Bowman's space or in the parietal epithelium ('evidence of BSA leakage' - 2 points); presence of BSA in normal tubules ('weak tubular staining' - 4 points); and BSA in dilated/damaged tubules ('strong tubular staining' - 8 points). To assess renal macrophage infiltration, frozen kidney sections were dried, fixed with acetone, blocked and incubated with a rat antibody against the mouse macrophage antigen F4/80 [10] (Serotec Benelux, Oxford, UK). Sections were further incubated with horseradish peroxidase-conjugated rabbit anti-rat and swine anti-rabbit antibodies (DakoCytomation BV, Herverlee, Belgium). They were developed with Nova Red and counterstained with haematoxylin. F4/80-positive cells per high power field were counted in blinded fashion.

Electron microscopy

Male and female C57BL/6J and 129S2/Sv mice were treated with saline or BSA, anaesthetized as described, and perfused for 3 min at 200 mmHg with a solution of 3% glutaraldehyde and 0.1% picric acid in 0.1 M cacodylate buffer. Kidney slices were immersed overnight in the same solution, and then transferred into 0.1 M cacodylate buffer until further processing. Kidney tissue blocks were dehydrated and embedded by standard procedures. Semithin sections (1 μm) were cut on an ultramicrotome, then stained and examined by light microscopy. From areas of interest selected in the semithin sections, ultrathin sections were cut using a diamond knife and studied with a Philips 301 electron microscope.

Statistics

Results are expressed as mean ± SEM. For multiple group comparisons, data were analysed by ANOVA, Kruskal-Wallis ANOVA on ranks, or repeated measures ANOVA as appropriate, followed by the Student–Newman–Keuls or Dunn's *post hoc* test as applicable. For comparisons of two groups, Student's *t*-test was used (rank sum test or *t*-test with Welch correction were employed where the data were not normally distributed). Statistical significance was accepted at P-values < 0.05.

Results

Strain and gender differences in proteinuria

At D0, in both strains proteinuria was higher in males than in females; C57BL/6J 2.7 ± 0.4 *vs* 0.6 ± 0.1 mg/day ($P < 0.05$) and 129S2/Sv 3.4 ± 0.5 *vs* 0.6 ± 0.1 mg/day ($P < 0.05$). Within each gender, there were no differences between strains. At the end-point, all BSA-treated groups were similarly hyperproteinemic (Figure 1A and B, and Table 1), but proteinuria differed widely between the two strains. In response to BSA, proteinuria massively increased in male 129S2/Sv but not significantly in C57BL/6J mice (Figure 1C); and while female 129S2/Sv mice developed highly significant proteinuria, in their C57BL/6J counterparts protein excretion completely failed to increase (Figure 1D). Proteinuria in both male and female 129S2/Sv was already significant by D4 and was sustained or slightly increased thereafter. Urine BSA and MSA were measured in BSA-treated males. In 129S2/Sv male mice albumin accounted for 23% of total urine protein (BSA 32 ± 7, MSA 14 ± 3, total protein 59 ± 13 mg/day; $n = 10$). In C57BL/6J males mouse albumin accounted for only 3% of total urine protein (BSA 5.6 ± 0.3, MSA 0.4 ± 0.1, total protein 10.4 ± 1.2 mg/day; $n = 4$).

No strain effect on renal function and blood pressure

Albumin treatment for 11 days had no major effect on renal function (plasma urea, Table 1).

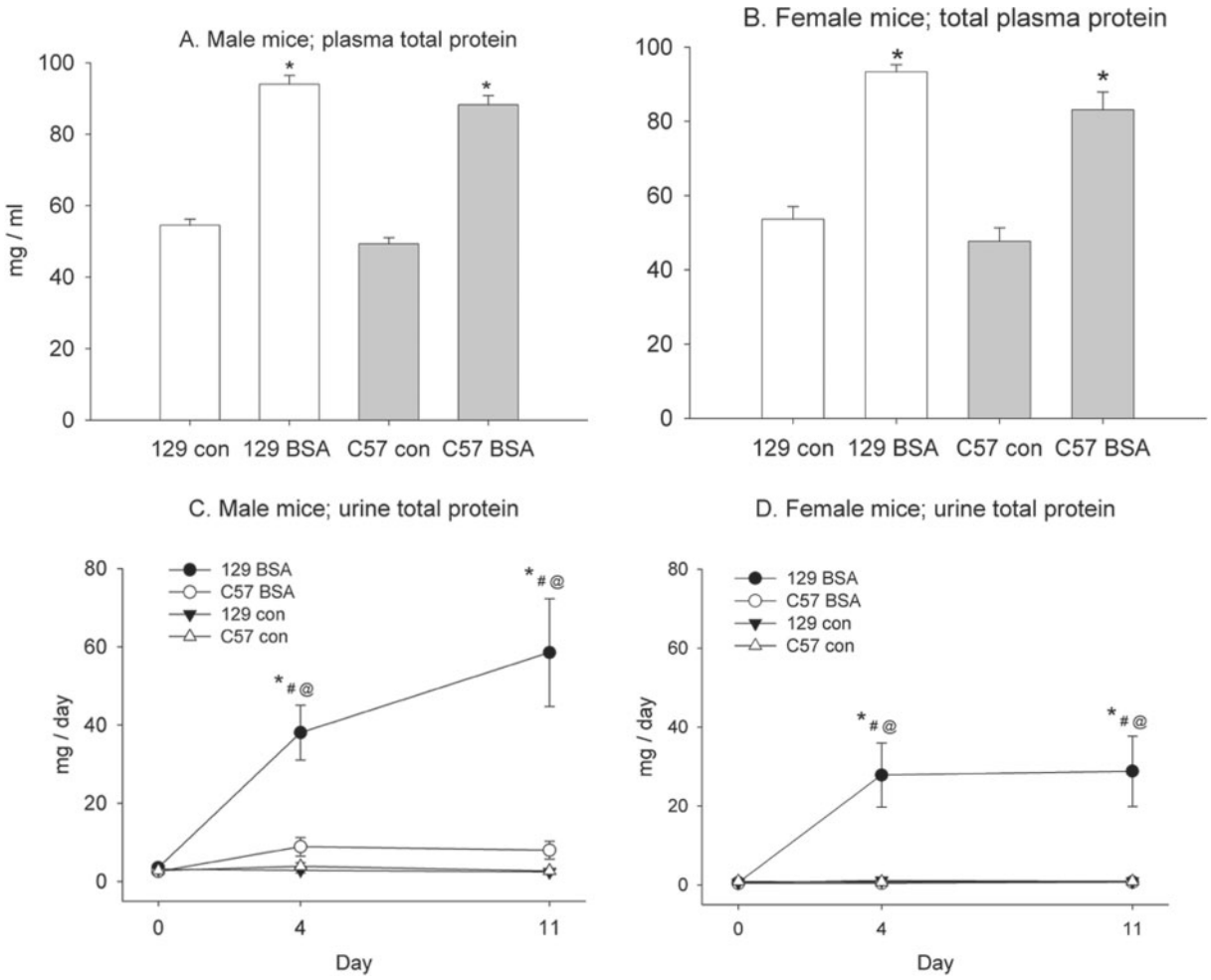

Fig. 1. The effect of BSA treatment on plasma and urine protein levels. Plasma total protein levels in male (**A**) and female (**B**) mice after 11 days of BSA; and proteinuria in the same groups (**C**, **D**) during the course of treatment. *$P < 0.001$, BSA *vs* Control (con); #$P < 0.001$, 129S2/Sv *vs* C57BL/6J mice; @$P < 0.001$ *vs* D0. Abbreviations: 129, 129S2/Sv mice; C57, C57BL/6J mice; con, control saline-injected mice.

Table 1. Plasma measurements following albumin treatment over 11 days

	129S2/Sv				C57BL/6			
	Male		Female		Male		Female	
	BSA	Saline	BSA	Saline	BSA	Saline	BSA	Saline
n	16	13	7	7	11	9	9	5
TP (g/l)	94 (2)*\$#	54 (2)	93 (2)*\$#	54 (3)	88 (3)*	49 (2)	83 (5)*	48 (4)
MSA (g/l)	29 (1)*\$#	33 (1)\$#	37 (2)*#	45 (2)	45 (4)	42 (3)	43 (1)	50 (2)
BSA (g/l)	34 (1)	–	39 (2)	–	29 (2)	–	35 (4)	–
Urea (mmol/l)	10.9 (0.7)	10.1 (1.4)	8.1 (1.2)	9.2 (0.9)	10.5 (0.6)*	8 (1)	ND	ND
COP (mmHg)	25.4 (0.7)*\$#	14 (0.7)	29.4 (2.6)*	14 (1)	28.6 (1.7)*	15 (1)	25 (2)*	18 (1)

Abbreviations: TP, total protein; BSA, bovine serum albumin; MSA, mouse serum albumin; COP, colloid oncotic pressure; ND, not determined.
Mean (SEM); *$P < 0.05$ BSA *vs* saline; #$P < 0.05$ C57BL/6J *vs* 129S2/Sv; \$$P < 0.05$ male *vs* female.

Systolic blood pressures were measured by the tail-cuff method in male BSA-treated mice and did not differ between strains (mmHg, 97.5 ± 3.8 in 129S2/Sv *vs* 103.8 ± 4.4 in C57BL/6J, $P = 0.322$).

Intrarenal visualization of injected BSA

Immunohistochemical staining permitted us to visualize the injected BSA and determine its location within the nephron at the experimental endpoint, giving an insight into renal handling of the exogenous protein (Figure 2). In 129S2/Sv mice, BSA was prominent within the Bowman's space and the renal tubules (Figure 2A–B). However, in C57BL/6J mice (Figure 2C–D), while the intravascular presence of BSA within the glomerular tufts was obvious, very little of it was seen elsewhere including the urinary space and tubular lumina. Assessment of intrarenal BSA load by semi-quantitative estimation of cortical BSA staining revealed a significant strain difference, with staining much higher among 129S2/Sv mice (arbitrary units, 40.9 ± 11.5 *vs* 4.6 ± 1.6 in C57BL/6J mice, $P < 0.01$).

Renal morphology

Routine histology revealed no remarkable glomerular or tubular injury (not shown). On electron microscopy, glomerular ultrastructure was similar between strains after BSA treatment (Figure 3). In addition, tubular and glomerular appearances after BSA injections were not different from saline-treated animals, and there were no gender differences (not shown). Immunohistochemistry for the mouse macrophage antigen F4/80 revealed that BSA treatment induced significant tubulointerstitial macrophage infiltration in 129S2/Sv but not C57BL/6J mice, even though baseline F4/80 counts were slightly higher in C57BL/6J mice (Table 2 and Figure 4). Glomerular macrophages were not observed in any mice.

Discussion

Protein-overload proteinuria eventually leads to tubulointerstitial injury in rats [1]. We investigated this model in mice to probe the feasibility of protein-overload studies in genetically modified strains. Daily i.p. administration of endotoxin-free BSA in two mouse strains, C57BL/6J and 129S2/Sv, increased plasma protein concentration similarly from about 55 g/l to more than 90 g/l. Nevertheless, only in 129S2/Sv mice did this lead to severe proteinuria, accompanied by significant tubulointerstitial macrophage influx. This strain difference was observed in males and females, with little gender effect. Electron microscopic appearances in mice treated or not treated with BSA did not differ between strains. Immuno-histochemistry revealed that in 129S2/Sv mice much more BSA crossed the glomerular basement membrane (GBM), resulting in prominent staining in Bowman's space and protein-filled tubule sections. Besides having practical implications, these findings suggest major differences in the sieving coefficient of the GBM for albumin within a single species.

Many experimental models of progressive renal injury are associated with proteinuria. However, in C57BL/6J mice it is notoriously difficult to induce proteinuria in such models. For example, in a remnant kidney model practically no increase in proteinuria as well as blood pressure was observed in C57BL/6J mice [6]. In contrast, in the same model in rats, marked proteinuria accompanied uremia and hypertension [11]. Similarly, C57BL/6J mice on DOCA-salt only had mild hypertension and failed to develop proteinuria [7], while NOS inhibition with LNNA also failed to induce proteinuria or much renal injury in C57BL/6J wild type [12] or transgenic mice on C57BL/6J background [13]. Given the increasingly apparent independent role of proteinuria in renal inflammation and fibrosis [3], it is plausible that the marked resistance to proteinuria in the C57BL/6J mice is a key factor in its comparative insensitivity to well-defined models of progressive renal injury. This notion is supported

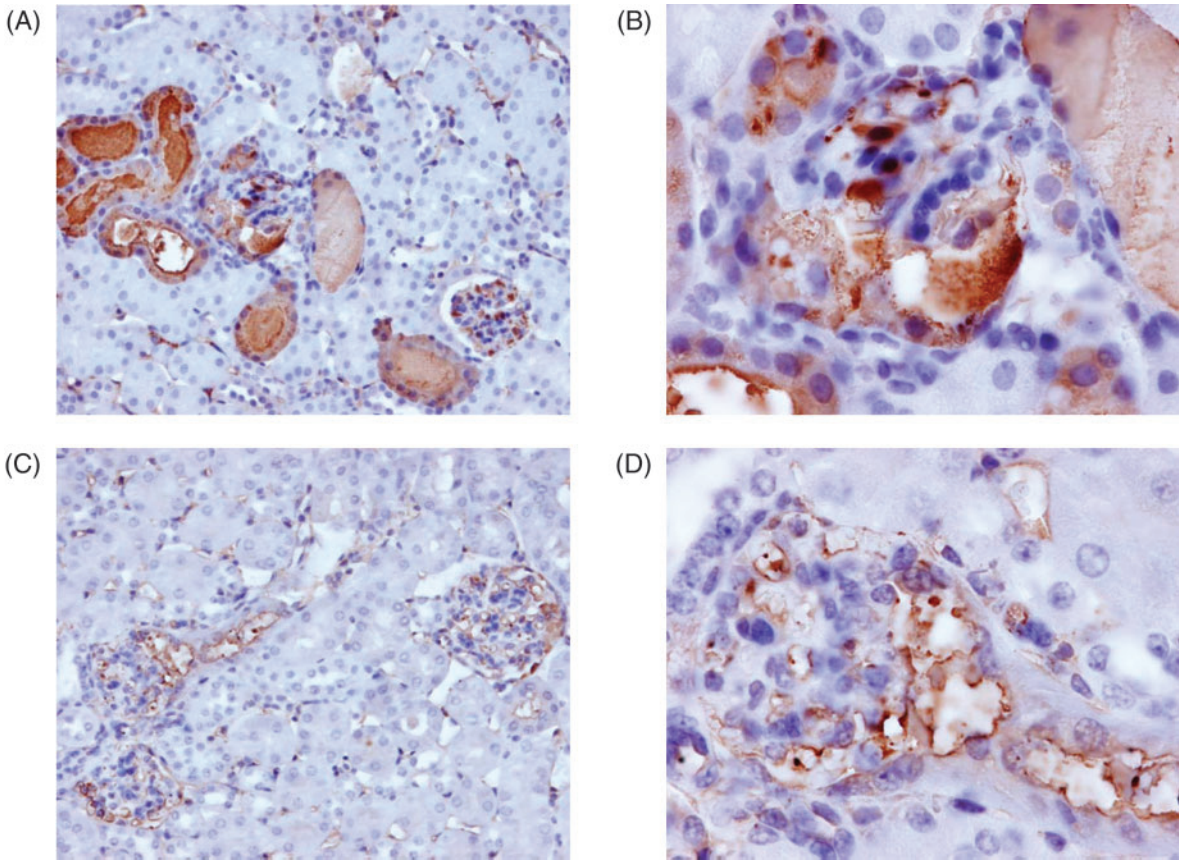

Fig. 2. Visualization of renal handling of injected BSA. (**A**) 129S2/Sv mice. Apparently normal and nearby leaking glomeruli with contiguous BSA-filled tubules (200×). (**B**) 129S2/Sv mice. Detail of a leaky glomerulus with a 'pool' of BSA in the urinary space (600×). **(C)** C57BL/6J mice. Tubules are largely BSA-free (200×). (**D**) C57BL/6J mice. Detail of positively stained glomerular tuft with little or no BSA visualized in the urinary space (600×).

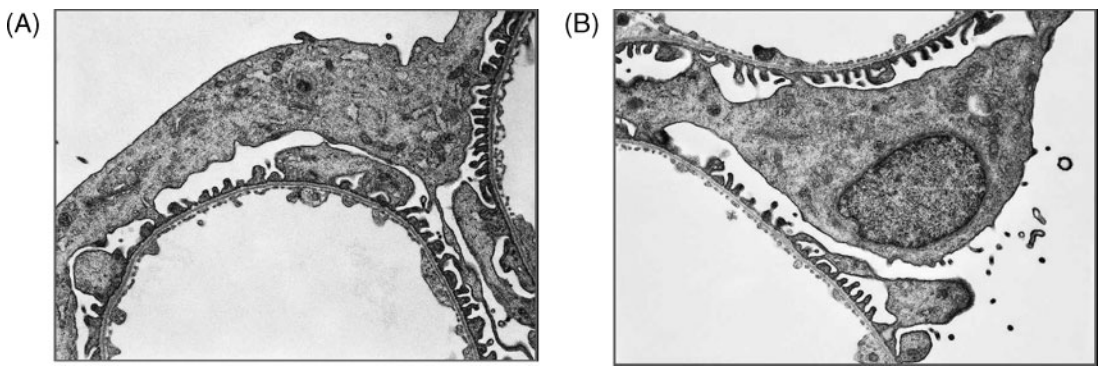

Fig. 3. Electron microscopy. Micrographs showing detailed glomerular ultrastructure including podocyte cell body, podocyte foot processes, glomerular basement membrane and endothelium, from male mice injected with BSA for 11 days. (**A**) 129S2/Sv (**B**) C57BL/6J. There are no remarkable structural variations that could account for the marked difference in proteinuria response. The final magnification for both micrographs is 15 000×.

by our finding that, unlike in 129S2/Sv, BSA treatment in C57BL/6J mice not only failed to provoke proteinuria, but also did not induce substantial interstitial macrophage infiltration.

Our BSA staining studies revealed that despite marked and similar increases in plasma protein concentrations, leaked protein was prominently stained in 129S2/Sv mice, but practically absent in C57BL/6J mice. Any substantial rise in plasma albumin level, irrespective of whether it increases or has little effect on the sieving coefficient, should result in at least a proportional increase in tubular albumin reabsorption

Table 2. Tubulointerstitial macrophage count (F4/80 positive cells)

		129S2/Sv	C57BL/6J
Male	BSA	63 (5)#	53 (3)
	Saline	21 (3)	44 (4)*
Female	BSA	62 (5)#	57 (4)#
	Saline	21 (5)	36 (4)

Mean (SEM); *$P < 0.05$ 129S2/Sv *vs* C57BL/6J, #$P < 0.05$ BSA *vs* saline.
There were no significant gender differences.

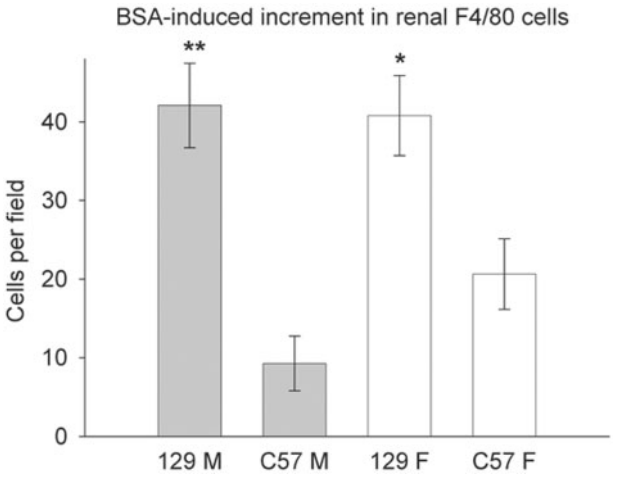

Fig. 4. Net influx of tubulointerstitial macrophages after BSA treatment (corrected for mean values found after saline treatment in each group). F4/80 positive cells were counted per 0.24 mm^2 field. *$P < 0.05$, **$P < 0.01$, 129S2/Sv *vs* C57BL/6J. Abbreviations: 129, 129S2/Sv mice; C57, C57BL/6J mice; M, male; F, female.

in order to avoid negative nitrogen balance. In rats tubular albumin reabsorption capacity is apparently operating close to its maximum because administration of BSA immediately results in a selective proteinuria that comprises both exogenous and endogenous albumins [14]. The absence of BSA in practically all nephrons in the C57BL/6J mice suggests that the strain difference we describe is related to differences in glomerular permselectivity and not to differences in tubular reabsorption. What factors could be responsible for such a difference? Electron microscopic analysis did not indicate any structural variations in the filtration barrier that could account for the wide disparity in proteinuria. Systemic blood pressure was similar in BSA-treated male mice, indicating no strain differences in systemic contribution to pro-filtration hydrostatic forces. Nevertheless, we cannot exclude the possibility of intrarenal angiotensin-dependency in explaining the proteinuria difference, because mice are polymorphic for renin genes [15]. While some strains, such as C57BL/6J and BalbC mice, possess only one renin gene, Ren-1, which is expressed in the kidneys, most other strains have an additional renin gene, Ren-2, which is mostly expressed in the submaxillary gland. Such strains include 129S2/Sv, the Swiss mouse, and various wild mice such as *M. musculus, M. hortulanus* and *M. domesticus*. In diabetic rats the decreased number of anionic sites on the GBM is corrected by ACE inhibition [16]. However, our pilot experiments with losartan at a high dose (150 mg/l) failed to abrogate the strain difference in glomerular permselectivity, suggesting that yet other factors are involved.

Since there are no obvious ultra-structural differences to explain the wide gap in proteinuria response, it is very likely that electrical or molecular factors within the GBM are at play. There may well be significant inter-strain variations in electrostatic forces within the membrane, because even subtle differences in composition may influence biophysical characteristics like 'fixed' charge and isolectric points (pI), causing disparities in albumin sieving coefficients that could underlie the observed difference in proteinuria. There is also the possibility of genetic heterogeneity between mouse strains in the molecular make-up of the slit diaphragm, which is one of the key determinants of glomerular permeability [17]. The slit diaphragm possesses a highly complex organization comprising structural as well as biochemical signalling properties, with structural components including the membrane proteins nephrin and podocin, the cytoplasmic 'adaptor' molecule CD2AP, and the adhesion proteins Neph-1 and FAT-1 [18]. Genetically modified mice lacking each of these proteins develop massive proteinuria, and mutations of genes coding for nephrin, podocin and CD2AP have been implicated in specific forms of human glomerular disease [18]. In rats, experimental disruption of the normal interaction between nephrin and Neph-1 led to proteinuria in the absence of foot process abnormalities [19]. Thus, it is attractive to speculate that differential expression of crucial slit diaphragm proteins, or genetically determined variations in important protein–protein interactions, may render certain strains of mice (such as the 129S2/Sv variety) more susceptible to proteinuria than others, even with preserved glomerular ultrastructure.

Judging by urinary composition, filtration of mouse albumin appears to be restricted even more efficiently than bovine albumin in glomeruli of the C57BL/6J strain. It is known that albumin variants having different pI differ in GBM permeability [20]. Using Web-based resources, we obtained the amino acid sequences of BSA (http://ca.expasy.org/cgi-bin/niceprot.pl?P02769) and MSA (http://ca.expasy.org/cgi-bin/niceprot.pl?P07724), then employed a sequence-based pI calculator (http://www.embl-heidelberg.de/cgi/pi-wrapper.pl) (all accessed September 2005) to determine that the approximate pI of MSA is 5.41 while that of BSA is 5.50. This implies that BSA will carry a weaker negative charge at physiologic pH, favouring its filtration and explaining at least in part, why it is more readily excreted than MSA in both strains.

The protein-overload model in 129S2/Sv mice offers the additional attraction of providing possibilities for studying proteinuric disease with or without tubular

injury, based on a range of severity levels. In our model of relatively brief BSA administration, we observed pronounced proteinuria with preserved renal function and absence of histological injury.

In summary, we have demonstrated a marked strain difference in the response of mice to albumin overload. In terms of development of proteinuria, and tubulointerstitial macrophage infiltration, 129S2/Sv mice are much more responsive than C57BL/6J mice. This difference is not accompanied by inter-strain variation in glomerular ultrastructure. Glomerular leakage of injected BSA in the 129S2/Sv strain indicates the importance of glomerular protein permeability as a key determinant of susceptibility to proteinuria.

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Conflict of interest statement. None declared.

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