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Renal macrophage activation and Th2 polarization precedes the development of nephrotic syndrome in Buffalo/Mna rats

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Background. At 8 weeks, Buffalo/Mna rats spontaneously develop a nephrotic syndrome associated with focal segmental glomerulosclerosis (FSGS). We have previously demonstrated that this glomerulopathy recurs after renal transplantation, thus supporting the relevance of this rat model to human idiopathic nephrotic syndrome [1]. In this study, we describe renal immune abnormalities which appear in parallel to the initiation and progression of the spontaneous Buffalo/Mna nephropathy.

Methods. Buffalo/Mna rat kidney samples were harvested before (4 weeks) and after the occurrence of proteinuria (at 10, 18, and 24 weeks, and at 12, 15, 18, and 24 months). Renal immune cell populations [total lymphocytes, macrophages, T, B, and natural killer (NK) cells] and the expression kinetics of various related cytokine [transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-1, IL-2, IL-4, IL-6, IL-10, IL-12, and IL-13], chemokine [regulated upon activation, normal T cell expressed and secreted (RANTES) and monocyte chemoattractant protein-l (MCP-1)] and T-cell receptor β (TCR β) chain transcripts were studied serially during the course of the disease.

Results. In the Buffalo/Mna kidneys, in parallel to the proteinuria, the focal and segmental glomerular lesions began to develop at 10 weeks (affecting 2.4 ± 0.8% of glomeruli), increased in number, then in intensity (10.4 ± 0.8% at 24 weeks, 14.6 ± 2.3% at 12 months, and 28.9 ± 7.4% at 18 months). Before the onset of the disease, at a nonproteinuric stage, the transcript expression analysis revealed a strong production of some macrophage-associated cytokines, particularly TNF-α (350-fold higher than control levels), which was corroborated by monocyte infiltration. A minor T-cell infiltrate (associated with an increase in Cβ TCR transcripts), with a predominantly Th2 profile and the down-regulation of Th1 cytokines was also observed. These abnormal macrophage and T-cell patterns remained stable after the onset of the disease. No changes in chemokine and TGF-β transcripts were observed during the initial stages of the disease.

Conclusion. Our data suggest that the Buffalo/Mna rat disease may be the result of an immunologic disorder, involving macrophages and Th2 lymphocytes. We hypothesize that this modified environment could result in the production of a factor deleterious to the glomeruli. Thus, this rat strain could provide a new model for the study of human nephrotic syndrome.

Key words: FSGS, idiopathic nephrotic syndrome, rat, Buffalo/Mna, immunologic disorders.

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Idiopathic nephrotic syndrome with primary focal segmental glomerular sclerosis (FSGS) is a disease of unknown etiology, whose symptoms include a selective proteinuria and nonspecific lesions with a hyalinosis and synechia between the floculus and Bowman's capsule. Immunosuppressive regimens such as corticoids, cyclosporine A, and cyclophosphamide can influence the disease outcome to some extent (for review see [2]) but at least 20% of patients ultimately require hemodialysis and/or kidney transplantation for end-stage renal failure (ESRF) [3]. In addition, in 25% to 40% of transplanted patients, the initial disease immediately relapses, leading to graft loss in 50% of cases [4]. This immediate recurrence strongly suggests the presence of an albuminuric plasmatic factor(s), a hypothesis that has been strengthened by the beneficial effect of plasmatic exchanges [5–7] and immunoadsorptions [8, 9].

Despite significant recent progress in the understanding of the genetic abnormalities associated with idiopathic nephrotic syndrome [10], the disease mechanisms, particularly those at the disease onset, are unknown. Several animal models of proteinuria have been described, including age-associated nephropathy [11], nephron reduction [12, 13], and toxic-induced nephrosis [14, 15]. However, although these experimental models can help to identify the mechanisms involved in glomerular sclerosis progression, they are not pertinent as models for the initial stages of the human disease. For this reason, we have been studying the Buffalo/Mna rat strain which, at 8 weeks of age, spontaneously develops a selective

proteinuria associated with hypoalbuminemia, hyperlipidemia, and glomerular epithelial cell alterations with foot process flattening and cytoplasmic vacuolization at the ultrastructural level [16, 17]. The Buffalo/Mna rats were first reported because they present a spontaneous thymoma [18] regulated by an autosomal-dominant gene [19] associated with muscular weakness and demonstrated to be linked to plasmatic antiryanodine receptor antibodies [20]. Following analysis of genetic segregation, two autosomal-recessive genes were proposed to determine susceptibility to glomerular sclerotic lesions [21]. Both genes are located on chromosome 13, but are separate from the proteinuria gene Pur 1 located on the long arm of human chromosome 1 [22]. Moreover, neonatal thymectomy experiments have shown that nephrotic syndrome is functionally unrelated to the thymic disease [23]. In the same way, in our hands, thymectomy in adult Buffalo/Mna rats has no effect on their proteinuria (unpublished personal data), suggesting the absence of a direct interaction between the renal disease and that of the thymoma.

In addition to a genetic susceptibility to develop proteinuria [21, 22], the involvement of an extrarenal factor has been demonstrated by our group in a previous study [1]. We showed that the disease recurs on normal kidneys after renal transplantation into Buffalo/Mna recipients whereas the glomerulopathy regresses when a Buffalo/Mna kidney is transplanted into a normal recipient [1].

In this study, we have attempted to characterize the development of Buffalo/Mna nephropathy by analyzing the histologic lesions, the cells infiltrating the diseased kidneys, and the renal cytokine transcript accumulation. We report here that the prealbuminuric stage of the disease is characterized by an early macrophage infiltration and a Th2 polarization. We suggest that these immunologic disorders could be involved in the Buffalo/Mna nephropathy and that these observations may help to understand this rat disease. Such observations may also be related to the human disease and used to elucidate the human pathologic mechanisms.

METHODS

Animals

The Buffalo/Mna rat line, maintained in our laboratory, was originally kindly provided by Dr Saito (Central Experimental Institute, Nokawa, Kawasaki, Japan). All animals were born from a unique couple and bred for at least 10 generations. Five Buffalo/Mna rats were sacrificed at each time point: 4, 10, 18, and 24 weeks and 12, 15, and 18 months. Five inbred, age-matched Wistar-Furth rats [with the same major histocompability complex background (MHC) as Buffalo/Mna], obtained from an established colony (Janvier, Le Genest Saint Isle, France), were used as controls and sacrificed at 4, 10, 18, and 24 weeks. All animals were fed with standard laboratory food. The animal care was in strict accordance with our institutional guidelines.

Proteinuria measurement

The animals were placed in metabolic cages for 24 hours before measurement with free access to drink but without food to avoid contamination of urinary samples. The total urinary protein concentration (g/L) was measured by a colorimetric method using a Hitachi autoanalyzer (Boehringer Mannheim, Grenoble, France). Urinary creatinine (mmol/L) was measured by the Jaffé method. Proteinuria was expressed according to the following formula: proteinuria (g/mmol) = (urinary protein) (g/L)/(urinary creatinine) (mmol/L).

Light microscopic examination

Kidney samples were fixed for 20 minutes in Carnoy solution and then in 10% buffered formalin and embedded in paraffin. Three micro meter sections were stained with hematoxylin and eosin, periodic acid-Schiff (PAS), Masson trichrome, or periodic acid-silver methenamine (PAM). Slides were analyzed in a blind fashion by an independent pathologist. Lesions were estimated and counted in five fields at a 100× magnification.

Immunohistology and quantitative analysis of cellular populations

Kidney pieces from Buffalo/Mna and Wistar-Furth rats were embedded in optimal cutting tissue compound (Tissue Tek) (Miles, Elkhart, IN, USA), snap-frozen in precooled isopenthane and stored at −80° C until use. Frozen 6 μm tissue sections were fixed in acetone, permeabilized with a solution of methanol 10% H_2O_2, incubated with a Biotin Blocking System (Dako Corporation, Carpinteria, CA, USA), then saturated with rat serum diluted 1/10 in phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA) and stained using a three-step indirect immunoperoxidase technique [20]. The primary antibodies were mouse IgG antirat monoclonal antibodies: Ox1-Ox30 (a mix of two anti-CD45 antibodies, a pan-leukocyte marker), R7.3 [anti-T-cell receptor αβ (anti-TCRαβ)], Ox33 [anti-CD45 receptor antagonist (anti-CD45 RA)], ED-1 (anti-CD68), or 3.2.3 [(anti-CD161 or natural killer receptor (NKR)]. All of these monoclonal antibodies were obtained from the European Collection of Animal Cell Cultures (ECACC), then purified in our laboratory and pretested on healthy rat splenocytes to assess their optimal dilution. Nonspecific staining was taken into account by omission of the first antibody. The secondary antibody used was a rat adsorbed (negligible cross-reactivity) horse biotinylated antimouse IgG (Vector Laboratories, Burlingame,

CA, USA). Finally, tissues sections were incubated with horseradish peroxidase (HRP) streptavidin and developed with "Very Intense Purple" (VIP Kit) (Vector Laboratories).

The cell-infiltrated area was determined by quantitative morphometric analysis [24]. Briefly, positively stained cells in each section were counted by morphometric analysis using a point counting method with a 121 intersection square grid in the eyepiece of the microscope. Results were expressed as the percentage of the area of each renal section occupied by cells of a particular antigenic specificity (± SEM). The percentage of area infiltrate was calculated as follows: [(number of positive cells under grid intersections) ÷ (total number of grid intersections = 121)] × 100. The sections were examined at a ×400 magnification. The accuracy of the technique is proportional to the number of points counted. Thus, 15 fields were counted for each labeled section of high density and 40 fields for sections of low density (<10%). We chose a random start point on a border section and moved from field to field. Each counted field was adjacent to the previous and subsequent field [24]. Counting was scored in a blind fashion by two observers.

RNA extraction and cDNA synthesis

The decapsulated kidneys of each animal were ligatured, cut, immediately snap-frozen in liquid nitrogen, and stored at −80°C until used for RNA extraction. Total RNA from kidney pieces was isolated by the guanidium isothiocyanate procedure and purified on a Cesium chloride gradient [25]. Ten micrograms of RNA were reverse-transcribed into cDNA using 14 μg/mL of oligo $(dT)_{25-30}$, 10 mmol/L dithiothreitol (DTT), 0.5 mmol/L of each deoxynucleosidetriphosphate (dNTP), 40 U RNAsin (Promega, Madison, WI, USA), and 200 U Maloney-murine leukemia virus (M-MLV) reverse transcriptase in 5× first-strand buffer (Life Technologies, Gaithersburg, MD, USA). The cDNA synthesis reaction was brought to a final reaction volume of 100 μL.

Relative quantification of mRNA transcripts

The principle of quantitative reverse transcription-polymerase chain reaction (RT-PCR) using the SYBR Green® method has been described previously [26]. Direct detection of PCR products was monitored by measuring the increase in fluorescence due to the binding of the dye labeler SYBR Green® to double-stranded DNA. The level of fluorescence, monitored by the ABI PRISM® 7700 Sequence Detection Application program (Applied Biosystems, Foster City, CA, USA), was thus directly proportional to the level of PCR product.

Oligonucleotides and standard construction. Standards were amplified from samples known to contain the given mRNA sequences using specific primers (Table 1). The amplification products were separated electrophoretically and purified using a gel extraction kit (QIAquick Gel Extraction kit) (Qiagen, Hilden, Germany). The absorbance of each standard at 260 nm and the molecular weight of the cDNA enabled the calculation of the number of copies per milliliter and the preparation of a serial dilution from 10^7 to 10^2 copies per well.

Table 1. Sequences of amplification primers

mRNA	Sequences 5′-3′	Length of amplification product
5′ r HPRT	gcgaaagtggaaaagccaagt	76
3′ r HPRT	gccacatcaacaggactcttgtag	
5′ r IL-1α	agtcactcgcatggcatgtg	100
3′ r IL-1α	atatgtcgggctggttccac	
5′ r IL-2	ccttgtcaacagcgcaccc	399
3′ r IL-2	gctttgacagatggctatcc	
5′ r IL-4	ccaccttgctgtcaccctgt	390
3′ r IL-4	aggatgctttttaggctttc	
5′ r IL-10	tcagcactgctatgttgcc	403
3′ r IL-10	ccttgcttttattctcacagg	
5′ r IL-12 p40	gaaacagtgaacctcacctg	261
3′ r IL-12 p40	tgcttcacacttcaggaaagt	
5′ r IL-13	agcaacatcacacaagaccag	320
3′ r IL-13	cacaactgaggtccacagct	
5′ r IFN-γ	tggatgctatggaaggaaaga	314
3′ r IFN-γ	gattctggtgacagctggtg	
5′ r TGF-β	ctactgcttcagctccacagaga	279
3′ r TGF-β	accttgggcttgcgacc	
5′ r TNF-α	cttatctactcccaggttctcttcaa	204
3′ r TNF-α	gagactcctcccaggtacatgg	
5′ r MCP-1	atgcaggtctctgtcacgct	341
3′ r MCP-1	ggtgctgaagtccttagggt	
3′ r RANTES	gcatccctcaccgtcatcct	260
5′ r RANTES	tagctcatctccaaatagttgat	
5′ r Cβ	tctgtgctgaccccattgc	69
3′ r Cβ	ttccctgaccatgtggagct	

Abbreviations are: HPRT, hypoxanthine guanine phosphoribosyl transferase; IL, interleukin; INF-γ, interferon-γ; TGF-β, transforming growth factor-β; TNF-αtumor necrosis factor-α; MCP-1, monocyte chemoattractant protein-1; RANTES, regulated upon activation, normal T cell expressed and secreted; Cβ, constant part of TGF-β chain.

PCR amplification and analysis. Ten microliters of 1/10-diluted cDNA sample were amplified in 25 μL of SYBR Green® PCR Core Reagent (Applied Biosystem) with 0.6 U of AmpliTaq Gold polymerase, 0.25 U of Amperase uracyl-H-glycosylase, 200 nmol/L of each dNTP, 300 nmol/L of each primer and 3 mmol/L of $MgCl_2$, in 10× SYBR Green® PCR Buffer (1× final concentration). Amplifications were performed in an ABI Prism® 7700 Sequence Detector (TaqMan) (Perkin-Elmer, Wellesley, MA, USA). Each sample was analyzed in duplicate. The number of copies of the cDNA target sequence was deduced from a comparison of the measured fluorescence with the standard curve. To normalize the levels of the target sequences, the quantity of each given transcript was divided by the quantity of housekeeping gene (HPRT) transcripts obtained for each sample.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. The nonparametric Mann-Whitney test was used to compare ratio values between groups for

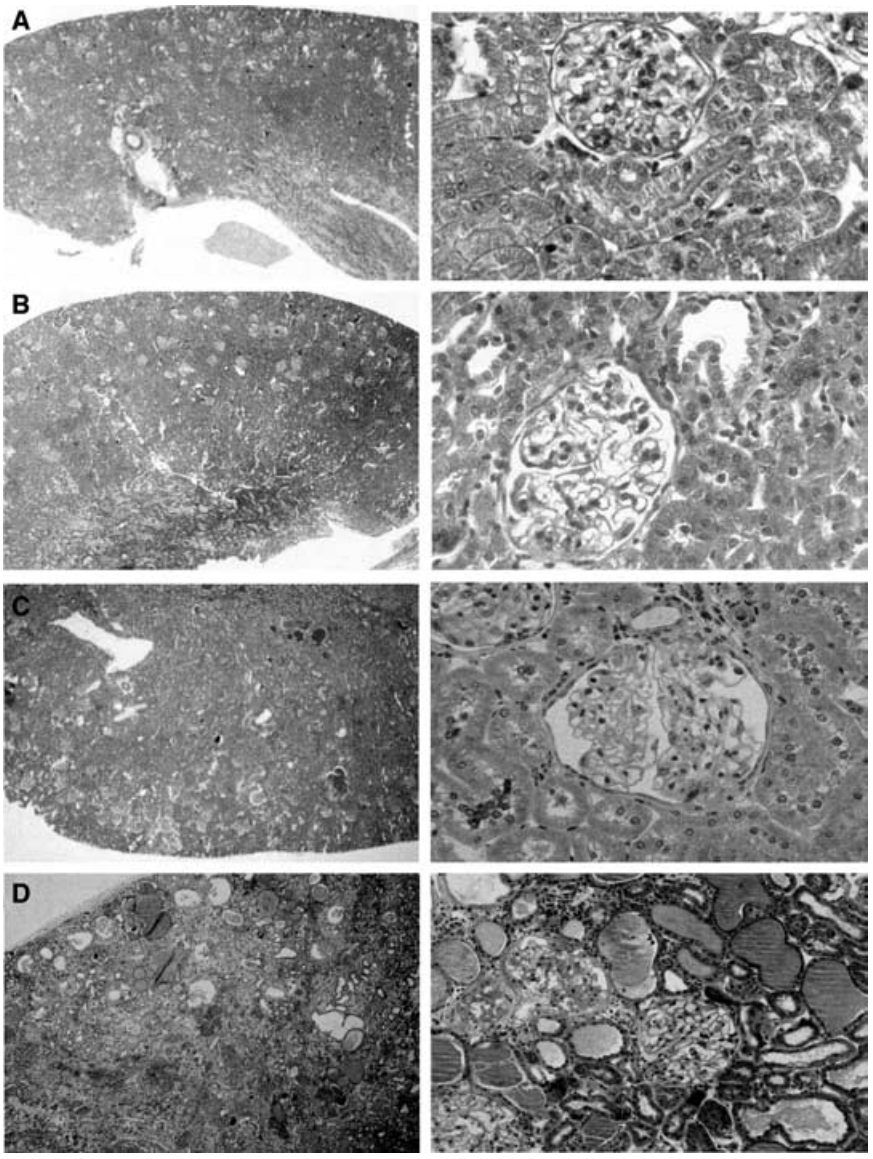


Fig. 1. Light microscopic examination of Buffalo/Mna kidneys during the course of initial disease development ($N = 3$). (*A*) At 10 weeks, onset of glomerular lesions (×25 and ×400). (*B*) At 18 weeks, increase in glomerular lesions and development of tubular lesions (×25 and ×400). (*C*) At 12 months, damaged glomeruli were numerous (with the onset of flocculocapsular synechia), sclerotic lesions and tubular dilatations were extensive (×25 and ×400). (*D*) At 18 months, microcystic tubular dilatations were numerous. Some glomeruli were completely sclerotic and atrophic, whereas others presented segmental lesions with flocculocapsular synechia or had a subnormal appearance (×25 and ×200).

each time point and to compare the% of surface area between groups for each point. $P < 0.05$ was considered as significant.

RESULTS

Histologic examination of Buffalo/Mna kidneys during the course of the disease

At 4 weeks, the Buffalo/Mna kidneys showed no glomerular lesions but marked podocyte swelling was apparent. At 10 weeks, glomerular lesions began to appear but remained scarce (affecting only 2.4 ± 0.8% of the glomeruli) and were not extensive in the flocculi. In addition, dilated tubules were rare and isolated. From 18 weeks to 12 months, glomerular and tubular lesions increased in number and intensity, damaged glomeruli represented successively 6.4 ± 4.1%, 10.4 ± 0.8% (24 weeks),

Table 2. Histologic evaluation of Buffalo/Mna kidneys at different stages during the course of the disease

Buffalo/Mna	% of damaged glomeruli	Tubular dilatation	Interstitial fibrosis
4 weeks	0 ± 0	0.3 ± 0.6	–
10 weeks	2.4 ± 1.4	3.3 ± 1.2	–
18 weeks	6.4 ± 7.1	2 ± 2	–
24 weeks	10.4 ± 1.4	22 ± 14.9	–
12 months	14.6 ± 4	19.7 ± 7.2	–
15 months	16.3 ± 5.9	42.3 ± 15.6	+/–
18 months	28.9 ± 12.9	56.3 ± 20.5	+
24 months	43.2 ± 27	54.7 ± 30.1	+

Slides were analyzed in a blinded fashion by an independent pathologist. Lesions were estimated and counted in five fields at a 100× magnification. Three series of samples were examined. Results were expressed as mean ± SE. Under the term "damaged glomeruli," we counted all glomeruli with focal segmental glomerulosclerosis (FSGS) lesions [i.e., segmental lesions of flocculus, sclerotic areas (± extensive), flocculocapsular synechia, or atrophic glomeruli].

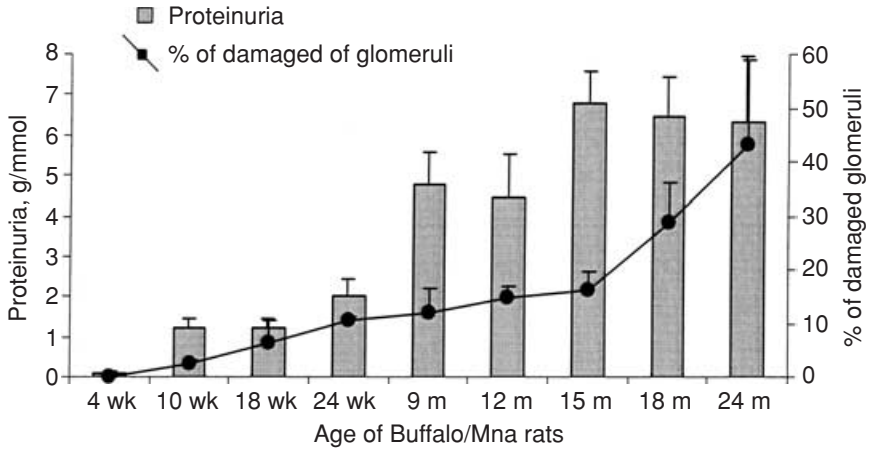


Fig. 2. Parallel evolution of the proteinuria during the course of the disease. Results expressed as grams of urinary proteins/mmol of urinary creatinine ± SEM ($N = 6$ to 21) and progression of glomerular lesions (results expressed as% of damaged glomeruli ± SEM) ($N = 3$) (see Table 2).

and 14.6 ± 2.3% of the total glomeruli (Fig. 1) (Table 2) with the appearance of flocculo-capsular synechia and an increase in the sclerotic area. At 12 months, tubular dilatations were substantial and were either isolated or in clusters. From 15 to 24 months, sclerohyalinosis spread to the flocculi (16.3 ± 3.4% of affected glomeruli at 15 months, then 28.9 ± 7.4% at 18 months and 43.2 ± 15.6% at 24 months) and was associated with a global glomerular atrophy. Moreover, the onset of a moderate mononuclear lymphoid infiltration was observed at this point (Table 2). Tubular dilatations continued to expand and their disposition in clusters was maximal around 18 to 24 months. In parallel, proteinuria began at 10 weeks, increased regularly until 15 months, then increased more rapidly when the glomerular sclerosis spread throughout the kidney (Fig. 2).

Initially, segmental glomerular lesions result from partial densification of the flocculus. At this level, the basal membranes appeared less defined, then pleated. The capillary lumina became very reduced or even nonexistent, without any endo- or extracapillary hypercellularity. The development of these lesions was associated with flocculocapsular synechia. These lesions spread to the entire flocculus, leading to small atrophic glomeruli. Basal capillary membranes were completely pleated and squeezed together, leading to a total disappearance of the capillary lumen.

In parallel, proximal tubules, which were only dilated at the beginning, became highly microcystic. These dilatations were clustered. The epithelium of these tubules was flat and their lumen contained voluminous cylinders with a protein aspect.

Quantitative assessment of Buffalo/Mna kidney infiltration

Buffalo/Mna kidneys displayed a global but moderate increase in their total leukocyte population during the course of the disease. In comparison with age-matched, healthy rat kidneys, these values were significantly different at 4, 18, and 24 weeks (4.6 ± 0.4% vs. 3.5 ± 0.3%; 6 ± 0.4% vs. 4.2 ± 0.2%, and 5.9 ± 0.3% vs. 4.8 ± 0.15%, respectively) ($P < 0.05$) (Figs. 3A and 4, upper panels).

The predominant infiltrating population was the monocyte-macrophage lineage which was significantly higher ($P < 0.01$) than in the controls at 4 weeks (0.95 ± 0.23% vs. 0.55 ± 0.03%), 10 weeks (1.45 ± 0.16% vs. 0.6 ± 0.12%), 18 weeks (1.3 ± 0.2% vs. 0.6 ± 0.08%), and 24 weeks (1.56 ± 0.33% vs. 0.6 ± 0.03%) (Figs. 3B and 4, middle panels). On the other hand, the T-cell population present in the Buffalo/Mna kidneys increased and was significantly more represented than in control kidneys from the first month to 10 weeks (0.45 ± 0.06% vs. 0.28 ± 0.01%; 0.5 ± 0.07 % vs. 0.3 ± 0.05%; respectively) ($P < 0.05$) (Figs. 3C and 4, lower panels). The levels of B lymphocytes and NK cell populations did not significantly differ between Buffalo/Mna and control rats (data not shown). A trend (albeit non significant) toward a periglomerular and glomerular staining of infiltrating cells was also noted (Fig. 4).

Quantitative analysis of mRNA transcripts in Buffalo/Mna kidneys during the course of the disease

In accordance with the increase in the monocyte population in the Buffalo/Mna kidneys, tumor necrosis factor-α (TNF-α) mRNA showed an early and strong accumulation in Buffalo/Mna kidneys at 4 weeks, before the onset of the disease (1.7 ± 0.5% vs. 0.05 ± 0.006%) ($P < 0.01$) (Fig. 5). Interleukin (IL)-12 (Fig. 5), IL-1, and IL-6 transcripts (data not shown) were also significantly increased at this time ($P < 0.01$). Transcript accumulations decreased thereafter, albeit remaining significantly elevated at 10 weeks for TNF-α and at 18 weeks for IL-12.

To evaluate the global T-lymphocyte infiltrate and/or activation in the Buffalo/Mna kidney, β chain accumulation was also measured. TCR β chain mRNA was strongly increased as early as 4 weeks (0.62 ± 0.17% vs. 0.018 ± 0.002%) ($P < 0.005$) and remained elevated throughout

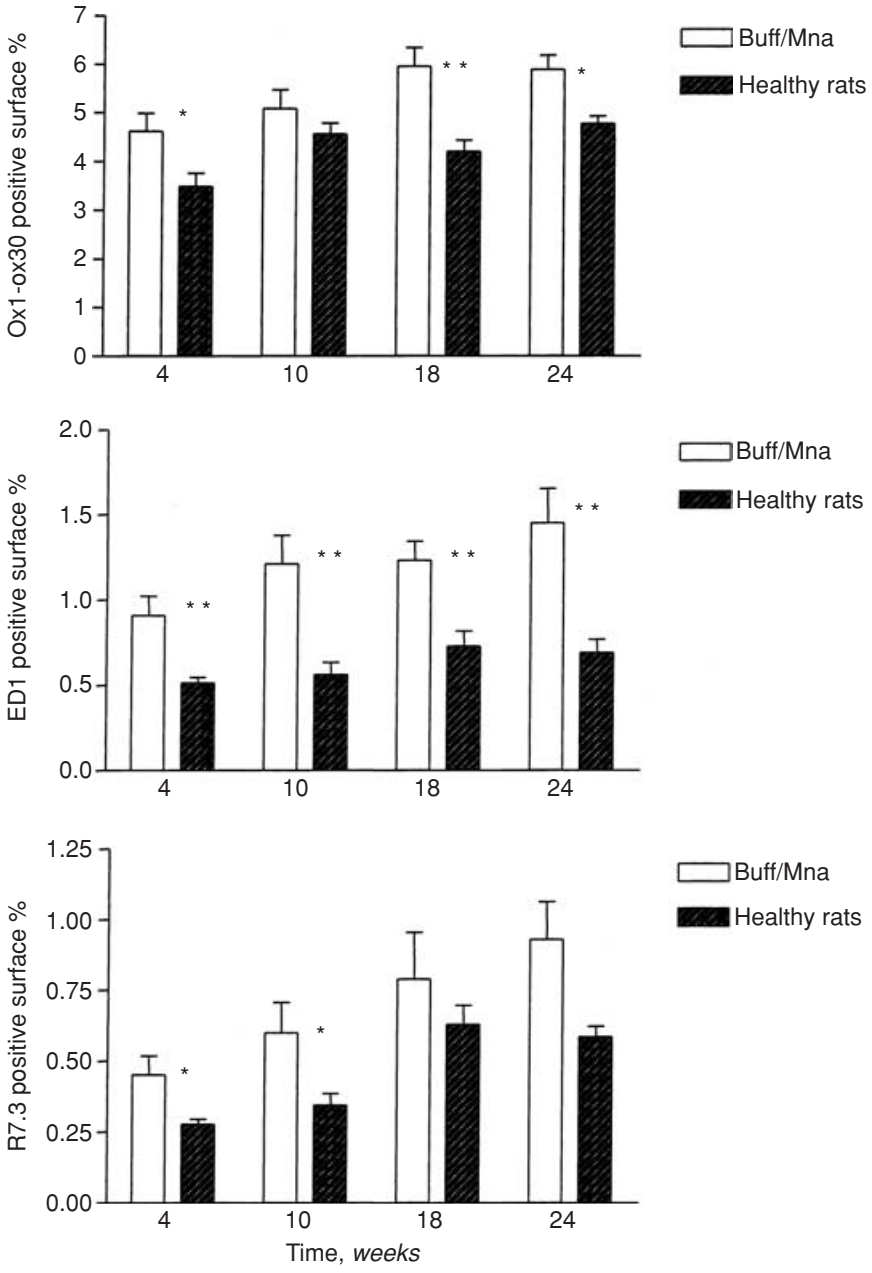


Fig. 3. Kinetics of infiltration by the cellular populations during the course of the initial disease in Buffalo/Mna (Buff/Mna) rats versus control kidneys. Results are expressed as the% (mean ± SEM) of surface area occupied by positive cells. At each time point, each group consisted of five animals. The control group consisted of age-matched healthy rats. (*A*) Pan-leukocyte (Ox1-Ox30 antibody) populations were examined. (*B*) Monocyte-macrophage ED-1 antibody populations were examined. (*C*) T lymphocytes (R7.3 antibody) were examined. *Significant difference <0.05 between the two groups; ** Significant difference <0.01.

the course of the disease (at 10 weeks 0.2 ± 0.08% vs. 0.04 ± 0.003% and at 18 weeks 0.243 ±0.03% vs. 0.035 ± 0.002%) ($P < 0.005$) (Fig. 6). The activation profile of the T-cell population present in the kidney sections was then measured for Th1 [IL-2 and interferon-γ (IFN-γ)] and Th2 cytokine transcripts (IL-4, IL-10, and IL-13). Interestingly, IL-2 and IFN-γ mRNA was weak until 24 weeks (and inferior to controls), indicating no Th1 polarization during the onset of the disease (data not shown). In contrast, at 4 weeks, Th2-related IL-10 and IL-13 transcripts were significantly higher in the Buffalo/Mna rats than in the controls (IL-10 0.075 ± 0.001% vs. 0.002 ± 0.0004%) ($P < 0.005$) (IL13 0.006 ± 0.002% vs. 0.001 ± 0.0002%) ($P < 0.05$) while IL-10 mRNA remained significantly more accumulated in Buffalo/Mna at 18 weeks ($P < 0.005$) and 24 weeks ($P < 0.01$) (Fig. 7). However, despite being significant, the level of IL-10 and IL-13 transcript accumulation remained low. In addition, as is often the case in rats, the level of IL-4 mRNA was at the technical limit of the SYBR Green® method and thus considered as negligible in both groups.

Regulated upon activation, normal T cell expressed and secreted (RANTES) and monocyte chemoattractant protein-l (MCP-1) chemokine transcripts were also measured. Both types of mRNA were decreased at 4 weeks (0.01 ± 0.004% vs. 0.145 ± 0.01%; 0.0001 ± 0.0002%

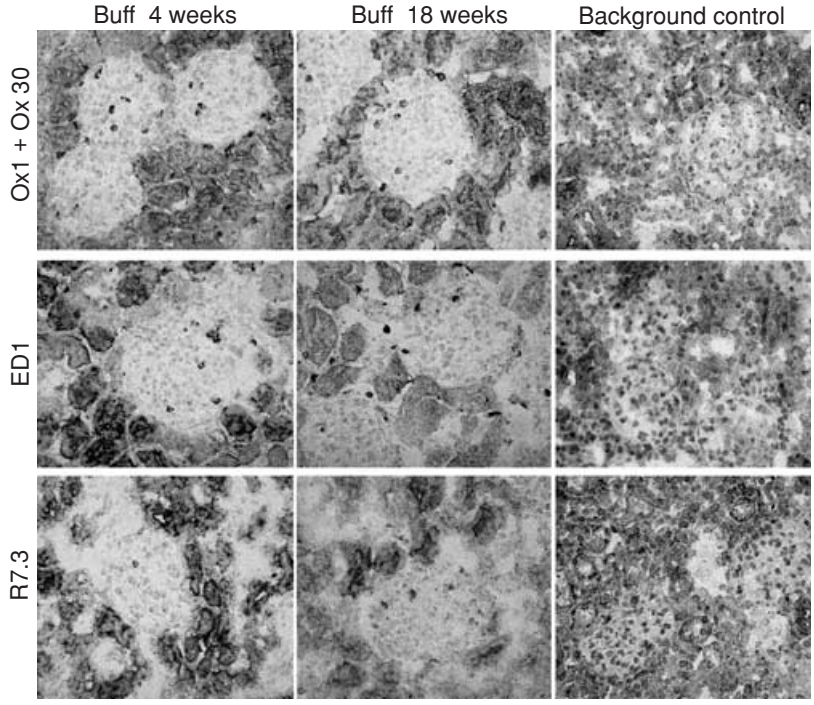


Fig. 4. Representative immunostaining of renal cellular infiltration during the course of the Buffalo/Mna (Buff/Mna) disease ($N = 5$). Upper panels show OX1 + OX30 positive staining. Middle panels show ED-1+ cells. Lower panels show R7.3$^+$ populations, at 4 and 18 weeks. Background control pictures are of nonspecific staining taken into account by omission of the first antibody (magnification for each picture was ×400).

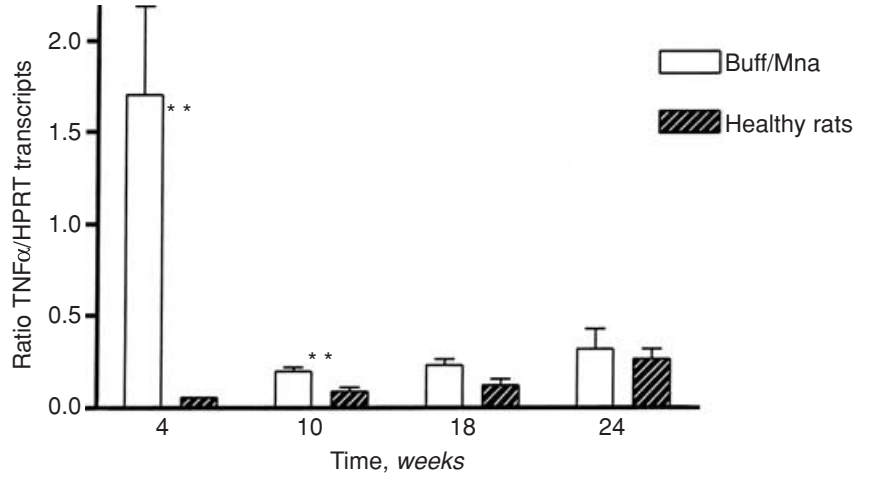
vs. 0.17 ± 0.03%; respectively) ($P < 0.005$). Whereas RANTES mRNA was lower only at the early time point (4 weeks), MCP-1 mRNA remained lower at all times ($P < 0.05$) (Fig. 8). Similarly, transforming growth factor-β1 (TGF-β1) mRNA levels were low in the early phases of the disease (at 4 weeks 0.04 ± 0.0008% vs. 6.26 ± 0.65%; at 10 weeks 1.4 ± 0.67% vs. 6.6 ± 0.45%) ($P < 0.005$), but reverted to normal levels at 18 weeks (Fig. 9).

Finally, we performed immunohistology to define which cell types were responsible for the production of the cytokines measured in the Buffalo/Mna kidneys. Unfortunately, the staining revealed by this technique was very weak, thus making it impossible to identify the origin of the cytokine production. Furthermore, we were unable to demonstrate that certain cytokines (such as IL-1, IL-6, IL-8, TNF-α, and TGF-β) were produced by resident renal cells, which would expect to be easier to detect.

DISCUSSION

In this study, we have shown that initial glomerular lesions of the spontaneous Buffalo/Mna disease, as assessed by proteinuria and histologic examination, developed progressively from 10 weeks until 12 months (Fig. 1) (Table 2). After 1 year, the sclerohyalinosis spread inside the floculi. These glomerular changes were paralleled by the development of proteinuria, which began at 10 weeks, increased until 15 months, and then increased rapidly when glomerular sclerosis spread throughout the kidney (Fig. 2). In addition, the number of total leukocytes of Buffalo/Mna kidneys was increased compared to that in the controls. This increase was principally due to an early rise in monocyte infiltration and a minor T-cell infiltrate, which preceded the occurrence of proteinuria and the ultrastructural podocyte alterations (data not shown). Moreover, cytokines typically produced by monocytes/macrophages, TNF-α, IL-12, IL-6, and IL-1, were significantly increased at the very onset of the disease (4 weeks), before the appearance of proteinuria and histologic lesions. The subsequent levels of these cytokines remained higher than in the control group. Similarly, an increase in TCR Cβ chain transcripts was observed early-on during the course of the disease. This increase could be related to T-cell activation, rather than an increase in the number of these cells (with regards to the mild T-cell infiltrate). Finally, despite this T-cell infiltrate, IL-2 and IFN-γ (Th1) transcripts were only barely detectable and were even lower than in the control group. In contrast, Th2-related cytokines (IL-10 and IL-13) were increased.

This finding of an early increase in TNF-α and Th2-related cytokines, in addition to our previous demonstration of the involvement of an extrarenal factor in this model of glomerulonephritis, highlights the Buffalo/Mna nephropathy as a relevant model for human idiopathic nephrotic syndrome. Indeed, in humans, TNF-α has been reported to be increased in the blood and urine of FSGS patients [27, 28] and TNF-α mRNA levels were elevated in the monocytes of these patients [28]. TNF-α has also been found to be increased in monocyte supernatants in puromycin aminonucleoside nephropathy (PAN) [29], and the injection of TNF-α into rats can trigger glomerular injury [30, 31]. In the Buffalo/Mna rats, the peak of



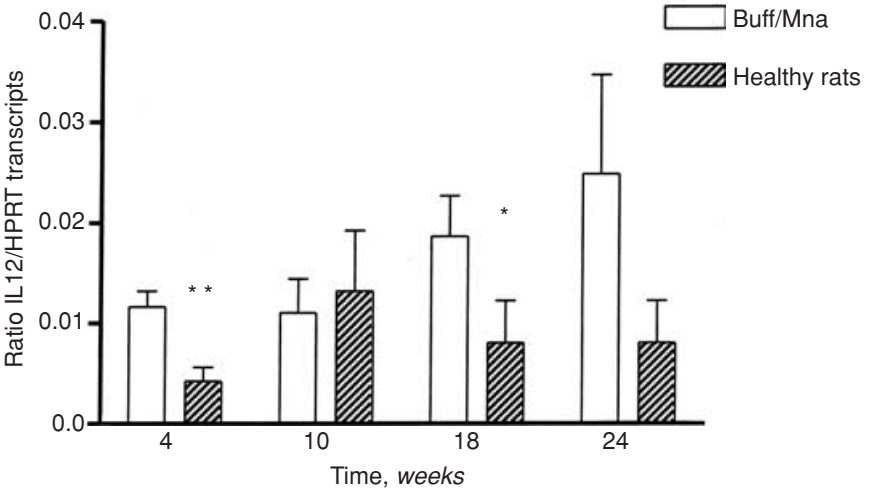


Fig. 5. Kinetics of tumor necrosis factor-α (TNF-α) and interleukin (IL)-12 mRNA expression in Buffalo/Mna (Buff/Mna) kidneys versus healthy kidneys during the initial course of the disease. Results are expressed as means ± SEM of the ratio of the number of cytokine transcripts to the number of HPRT transcripts for five animals at each time point. *Significant difference <0.05 between the two groups; **Significant difference <0.01; ***Significant difference <0.005.

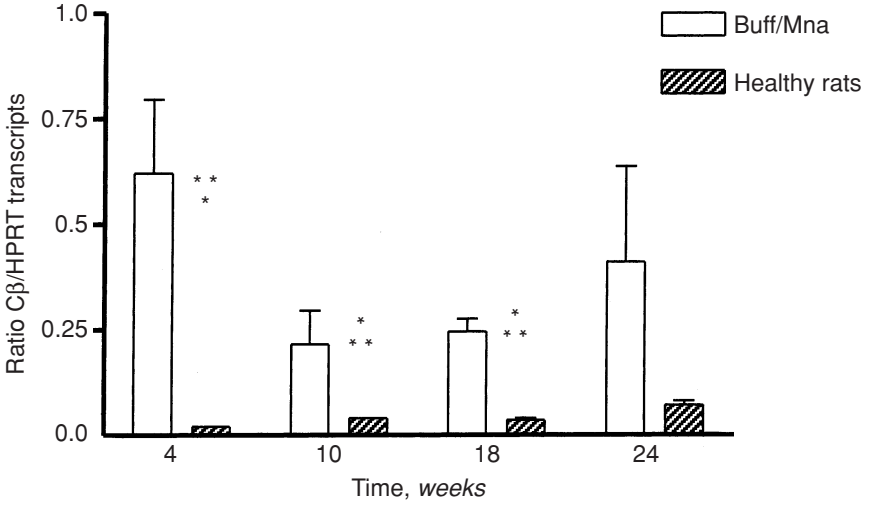


Fig. 6. Kinetics of Cβ mRNA expression in Buffalo/Mna (Buff/Mna) kidneys versus healthy kidneys during the initial course of the disease. Results are expressed as means ± SEM of the ratio of the number of cytokine transcripts to the number of HPRT transcripts for five animals at each time point. *Significant difference <0.05 between the two groups; **Significant difference <0.01; ***Significant difference <0.005.

TNF-α expression at 4 weeks occurred clearly before the onset of the nephropathy and could thus be an initiating factor of the glomerulonephritis. In the same manner, an increase in IL-12 production by the monocytes of idiopathic nephrotic syndrome patients has also been described [32], but this was not the case for IL-6 [33, 34]. However, IL-6 has been associated with glomerular damage in glomerulonephritis [33]. The role of macrophages

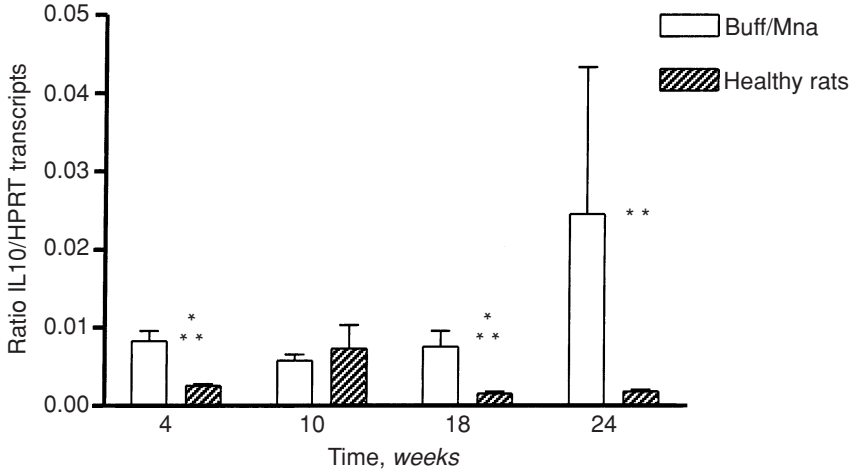
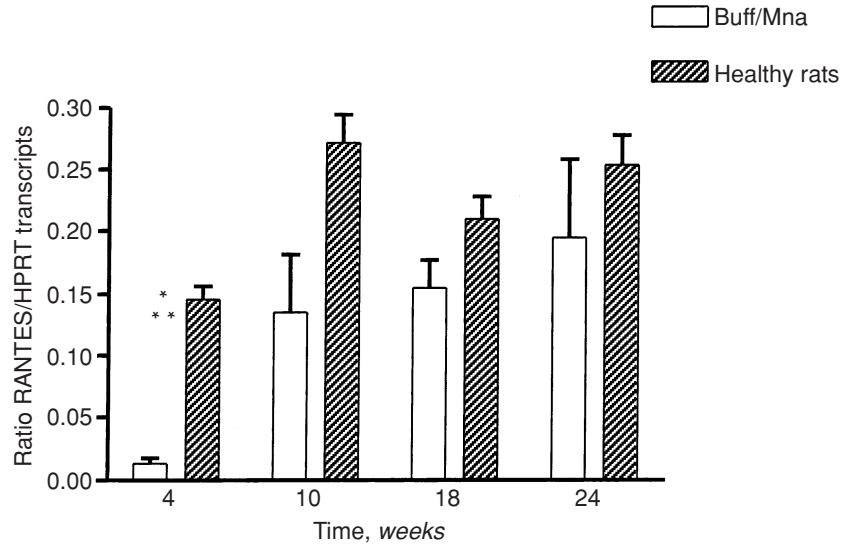


Fig. 7. Kinetics of interleukin (IL)-10 mRNA expression in Buffalo/Mna (Buff/Mna) kidneys versus controls kidneys during the initial course of the disease. Results are expressed as means ± SEM of the ratio of the number of cytokine transcripts to the number of HPRT transcripts for five animals at each time point. *Significant difference <0.05 between the two groups; **Significant difference <0.01; ***Significant difference <0.005.



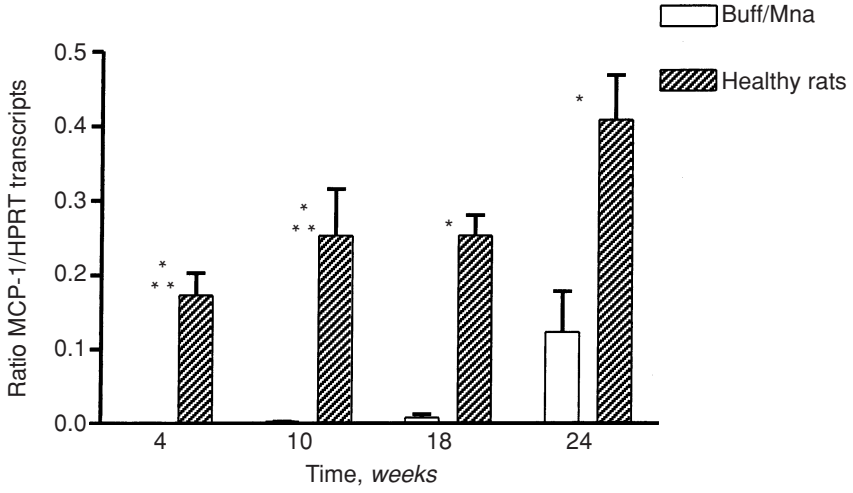


Fig. 8. Kinetics of regulated upon activation, normal T cell expressed and secreted (RANTES) and monocyte chemoattractant protein-l (MCP-1) chemokine mRNA expression: in Buffalo/Mna (Buff/Mna) kidneys versus controls kidneys during the initial course of the disease. Results are expressed as means ± SEM of the ratio of the number of cytokine transcripts to the number of HPRT transcripts for five animals at each time point. *Significant difference <0.05 between the two groups; **Significant difference <0.01; ***Significant difference <0.005.

that release these cytokines [35] can thus be suspected in the onset of the Buffalo/Mna disease. In the human disease, macrophages, named foam cells, were abundant in the lesion area [36], as well as in the PAN model. Their recruitment is favored by nephrotic syndrome–associated lipidic disorders and the macrophage-related production of TGF-β [37] may contribute to the late phases of glomerulosclerosis [38].

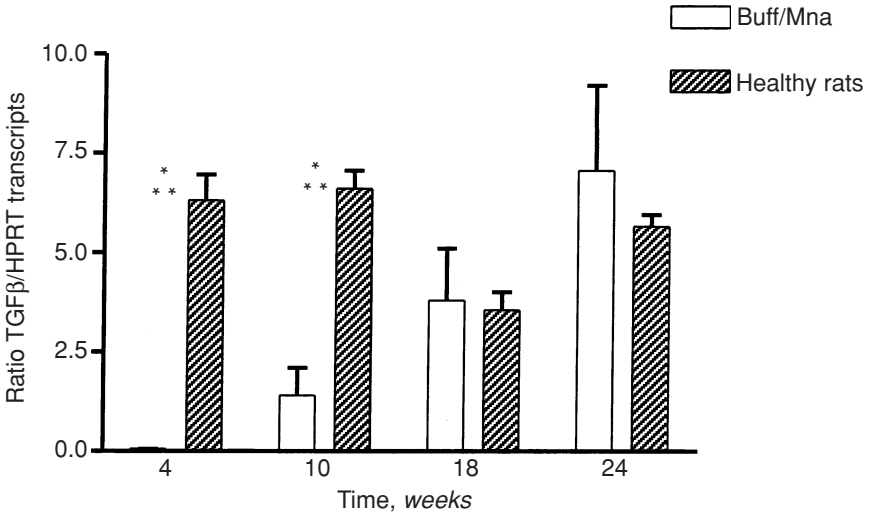


Fig. 9. Kinetics of transforming growth factor-β (TGF-β) mRNA expression in Buffalo/Mna (Buff/Mna) kidneys versus controls kidneys during the initial course of the disease. Results are expressed as means ± SEM of the ratio of the number of cytokine transcripts to the number of HPRT transcripts for five animals at each time point. *Significant difference <0.05 between the two groups; **Significant difference <0.01; ***Significant difference <0.005.

The cytokine profile in the peripheral blood lymphocytes (PBL) of idiopathic nephrotic syndrome patients has also been recently studied [39]. Despite initial reports showing an increase in plasmatic or urinary IL-2 and sIL-2R [34, 40–43], a Th2 profile appeared to predominate [44, 45], with a considerable rise in IL-13 [39, 46, 47] as well as IL-4 [47, 48]. These findings are in agreement with the known association of idiopathic nephrotic syndrome with atopic phenomena [49, 50] and an abnormal isotypic switch (high levels of IgE) [51]. Our study in the Buffalo/Mna rats revealed a very low Th1 profile and, despite a weak IL-4 expression (frequently described in rats, even in the context of Th2-mediated disease), an increase in IL-13 and IL-10. An active role for T cells in this model is suggested by the presence of this Th2 profile before the onset of proteinuria and podocyte injury. In addition, the down-regulation of the chemokines tested (RANTES and MCP-1) may be related to this cytokine environment (IL-4 and IL-10 inhibit while IFN-γ induces RANTES production) [52]. In humans, an increase in plasmatic and lymphocytic IL-8 has been reported in idiopathic nephrotic syndrome, but the rat IL-8 counterpart has never really been defined and therefore has not yet been tested [53]. Finally, our study showed that TGF-β mRNA does not accumulate in the early phase of the Buffalo/Mna disease but may promote the late accumulation of extracellular matrix. An increase in urinary TGF-β has been found in FSGS patients in the late phases of the disease, where sclerotic lesions were advanced [54]. However, whereas the role of TGF-β in the progression of various glomerular diseases is well known [55], its role at a very early stage of the disease remains elusive.

The role of thymic hyperplasia in the Buffalo/Mna renal disease is questionable. We measured the blood formula of 20 Buffalo/Mna rats and 20 healthy rats of other strains (Wistar-Furth, Lewis 1A, Lewis 1W, Sprague-Dawley). We found a roughly similar leukocyte number but a slight increase in the percentage of blood lymphocytes, as also reported by other groups [56]. This increase could partly explain the increase in T lymphocytes in Buffalo/Mna kidneys, but not the rise in monocytes or the activation of monocytes and T lymphocytes within the Buffalo/Mna kidneys. We cannot exclude the possibility that these expanded "thymic" lymphocytes, which have been described as being phenotypically and functionally unchanged [56, 57], explain the increased number of T cells in the Buffalo/Mna kidneys. Nevertheless, neonatal [23] or adult thymectomy (personal unpublished data) have no effect on proteinuria and Buffalo/Mna nephrotic syndrome, thus suggesting that this nephropathy is not secondary to or dependent on the thymic abnormalities.

Idiopathic nephrotic syndrome represents a heterogeneous group of glomerular disorders in humans, with different outcomes depending on the response to steroid therapy and the possibility of recurrence after transplantation. Furthermore, recent progress in understanding the putative mechanisms of acquired idiopathic nephrotic syndrome suggests two caricatural forms of steroid-resistant nephrotic syndrome: an "immune mediated" form and a form related to genetic changes and characterized by architectural podocyte cytoskeleton alterations. The Buffalo/Mna disease is resistant to steroid treatment (unpublished data) [16] and previous studies have shown a genetic predisposition to proteinuria in this strain regulated by two autosomal-recessive genes. One locus was identified as Pur1 on chromosome 13 that is syntenic to the long arm of chromosome 1 in humans (which contains the NPSH2 gene) [21]. However, the podocin sequence in the Buffalo/Mna rats is normal [abstract; Morita et al, ASN 2002, SU-PO297] and the role of an extrarenal factor in the development of proteinuria suggests that the genetic background represents rather a susceptibility factor for proteinuria development. In this study, we have shown that macrophages and activated T lymphocytes

are likely to be involved in Buffalo/Mna disease pathogenesis. The early infiltration by these cells and the simultaneous expression of their respective products did not enable the population responsible for the initiation of the injury process to be defined. The production of a factor toxic for the glomeruli, as suggested by the recurrence of the initial disease after transplantation, which is related to the presence of the mononuclear infiltrate, remains elusive.

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