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Nephrotoxic serum nephritis in nude rats: the roles of host immune reactions

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SUMMARY

A description is made of renal lesions in rats induced by heterologous (rabbit) nephrotoxic serum with or without subsequent host immune reactions against it and the effects of immune reactions on the course of classical nephrotoxic serum (Masugi) nephritis are discussed. The disease was induced by injecting congenitally athymic ACI nude rats (rnu/rnu) and their normal heterozygous littermates (rnu/+) with rabbit anti-rat glomerular basement membrane (GBM) antiserum. In the autologous phase, rat IgG and immunoglobulins were localized in a linear pattern along capillary walls only in nephritic heterozygous rats. In the indirect plaque-forming cell (PFC) assay against rabbit immunoglobulins in the autologous phase, significantly more PFC could be detected in nephritic heterozygous rats than in nephritic nude rats. The nude and heterozygous rats were essentially the same with respect to the amount of urinary protein, histological change and clinical course. At least in classical nephrotoxic serum nephritis in rats, host immune reactions against GBM bound heterologous nephrotoxic serum were concluded to have no effect on the course of the disease.

Keywords nude rats nephrotoxic serum nephritis host immune reaction

INTRODUCTION

The immune mechanism is considered to be an important factor responsible for the progress of chronic stages in human glomerulonephritis (Unanue & Dixon, 1967). The data of Hammer & Dixon (1963), that rats tolerant to the heterologous immunoglobulins show no progressive nephrotoxic serum nephritis regardless of the severity of initial injury appear to support this view. The importance of the autologous phase in occurrence or chronic progression has also been shown by Unanue & Dixon (1965) in accelerated types of nephrotoxic serum nephritis with the active immunization of rats with heterologous (rabbit) immunoglobulins. Immunologic events, however, may not necessarily be essential for the chronic progression of glomerulonephritis. Shimizu (1974) and Oite (1979), using tolerant and thymectomized rats, respectively, demonstrated that the absence or marked suppression of humoral response to injected rabbit IgG failed to make any difference in the course of nephrotoxic serum nephritis. Nahas *et al.* (1985) noted that host antibody titre did not influence progression to renal failure following nephrotoxic serum nephritis, by using the transplantation technique. Non-immunological factors, including haemodynamics, genetic and metabolic factors, have also been shown to be important in chronic progression (Brenner, 1985; Yoshida *et al.*, 1988). Thus, immune reactions should be re-examined for their involvement in the course of the disease. For this purpose, congenitally athymic experimental animals would be ideal, since artificial immunological tolerance or suppression may be incomplete or terminated, and transplantation technique itself may affect the course of nephritis. Two such studies have been conducted (Kusuyama, Nishihara & Saito, 1981; Okada *et al.*, 1982) using nude mice. However, in nephritis the rat is used most frequently, and many features in nephrotoxic serum nephritis in rats have been found to differ from those in mice (Unanue, Mardiney & Dixon, 1967).

The present study was conducted to examine renal lesions in rats induced by heterologous (rabbit) nephrotoxic serum alone with or without subsequent host immune reactions against it and determine the effects of these reactions on the course of classical nephrotoxic serum nephritis.

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MATERIALS AND METHODS

Animals

Female nude (rnu/rnu) and heterozygous (rnu/+) rats with ACI background were provided by Dr M. Matsuyama (Aichi Cancer Centre) and bred in our laboratory. All experiments were done with 7-week-old female rats weighing 70–135 g.

The animals were housed in a clean room. Their food, bedding cages and drinking water were autoclaved before use. Nude rats thus did not suffer from wasting disease.

Nephrotoxic serum (NTS)

Rat glomerular basement membrane (GBM) antigen was a prepared with DNAse and deoxycholate according to the method of Meezan, Hjelle & Brendel (1975).

Two rabbits were injected subcutaneously four times at 2-week intervals with the GBM preparation suspended in an equal volume of Freund's complete adjuvant. The rabbits were bled four times after three immunizations. These antisera and normal rabbit sera (NRS) were decomplemented by heating at 56°C for 30 min and absorbed with excess packed Wistar rats erythrocytes. NTS and NRS were passed through the Acrodisc (Gelman Sciences, Ann Arbor, MI) before use.

Experimental protocol

The rats were divided to the six groups. In the nephrotoxic groups (A, rnu/+; B, rnu/rnu), three rats from each group were killed on the days 1, 3, 7, 14 or 28 after injection of 0·5 ml/100 g body weight of NTS, respectively. In two other nephrotoxic groups (C, rnu/+; D, rnu/rnu), three rats from each group were killed on days 7, 14 or 28 after injection of 1·0 ml/100 g body weight of NTS, respectively. In two control groups (E, rnu/+; F, rnu/rnu), two rats from each group were killed on days 1, 14 or 28 after injection of 0·5/100 g body weight of NRS, respectively.

All rnu/rnu rats were confirmed to be athymic at the time of killing.

Histological studies

Kidneys were divided into three parts. Tissue for light microscopy was fixed in 10% buffered formalin, embedded in paraffin, cut in 4-μm sections, and stained with haematoxylin and eosin (H-E), periodic acid–Schiff (PAS) and periodic acid–silver methanamine (PAM). For ultrastructural examination, the renal cortex was fixed in 2% glutaraldehyde in phosphate buffer (0·2 M, pH 7·2) for 2 h and post-osmicated for 1 h. Then, the sample was dehydrated in alcohol series and embedded in Quetol 810. Ultra-thin sections were prepared on ultratome (Reichert Ultracut, Vienna, Austria). The ultra-thin section was double-stained by uranyl acetate and lead nitrate and examined by a Jeol 100 CX microscope. Specimens for immunofluorescence were snap-frozen in precooled *N*-hexane and kept at −70°C until use. The specimens were cut at 3-μm with a cryostat (Sakura, Tokyo, Japan) and stained with FITC-labelled anti-rat immunoglobulins (Dakopatts, Glostrup, Denmark), anti-rat IgG (Nordic Immunology Laboratory, Tilburg, The Netherlands), anti-rat C3 (Cappel, Cochranville, PA) and anti-rabbit IgG (Dakopatts). They were examined with a Zeiss fluorescence microscope equipped with epi-illumination.

Measurement of urinary protein excretion

At 1, 3, 7, 14 and 28 days after injection rats were housed in metabolic cages to allow collection of 24-h urinary samples. Urinary protein excretion was measured by the biuret method (Weichselbaum, 1946).

Estimation of serum antibody titre

Rat anti-rabbit immunoglobulin antibody titre was determined by ELISA. Microtitre plates were coated with 100 μl of 50 μg/ml solution of normal rabbit globulin in carbonate buffer for 2 h at 37°C. After extensive washing, the plates were blocked with 0·5% bovine serum albumin overnight at 4°C. The following reagents were added sequentially, with washing following each addition: 100 μl of test serum (1/100 dilution), 100 μl of peroxidase-conjugated anti-rat immunoglobulins (1/4000 dilution; Dako), 100 μl of substrate solution (0·1 M sodium citrate, pH 4·8, containing 0·4 mg/ml *o*-phenylenediamine and 0·0033% H_2O_2). Reaction was stopped by adding 6 *N* H_2SO_4 and absorbance was measured at 492 nm with an ELISA autoreader (Bio-Rad, Richmond, CA). Reagent blanks and normal ACI rat serum were used as negative controls. Sera from three heterozygous (rnu/+) ACI rats, killed 7 days after immunization with 500 μg rabbit immunoglobulins with Freund's complete adjuvant, were used as positive control.

Plaque-forming cell (PFC) assay

The number of anti-rabbit immunoglobulin PFC in the spleen was determined by the modified method of Cunningham & Szenberg (1968), using sheep erythrocytes coupled with rabbit immunoglobulins by ECDI (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride) (Sigma, St Louis, MO) as the target, and adding rabbit anti-rat immunoglobulins for indirect PFC assay.

Statistical analysis

Data were analysed using Student's *t*-test.

RESULTS

Proteinuria

The course of urinary protein excretion is shown in Fig. 1. In rats injected with 0·5 ml NTS/100 g body weight (groups A, B), the amounts of urinary protein excretion on days 1 ($n=15$), 3 ($n=12$), 7 ($n=9$), 14 ($n=6$) or 28 ($n=3$) after injection in group A (rnu/+) were essentially the same as those in group B (rnu/rnu). In rats injected with 1·0 ml NTS/100 g body weight, no

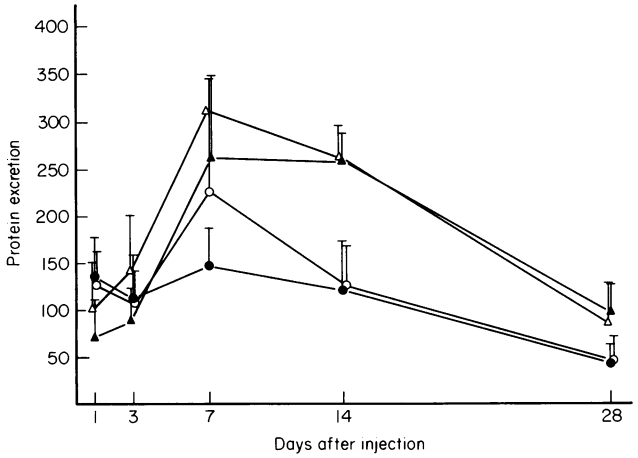

Fig. 1. Urinary protein excretion (mg/100 g) body weight per 24 h). Mean ± s.d. ●, Group A; ○, group B; ▲, group C; △, group D (see Materials and Methods for groups).

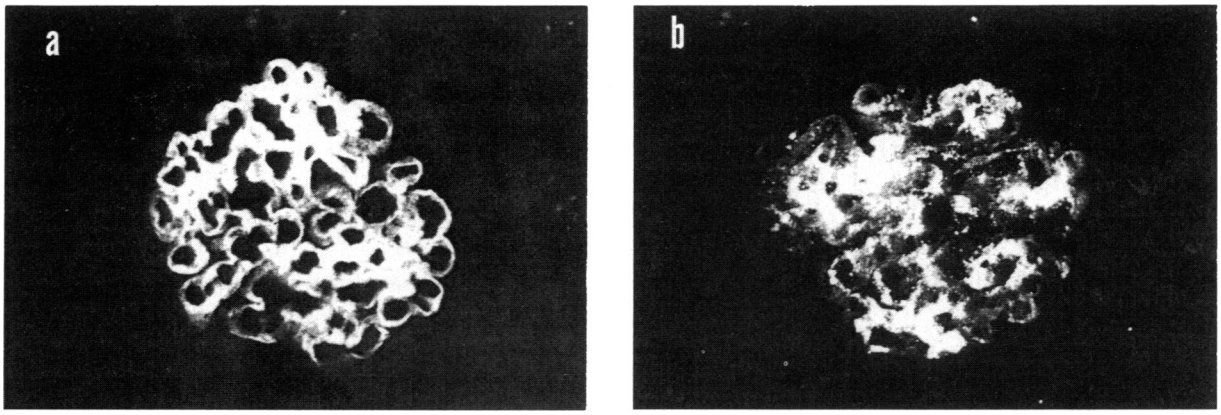

Fig. 2. Photomicrographs demonstrating rat immunoglobulins by direct immunofluorescence in glomeruli from (a) heterozygous rats in group C; and (b) nude rats in group D, killed 7 days after NTS injection. (a) linear capillary deposits of rat immunoglobulins; and (b) irregular and interrupted capillary deposits of rat immunoglobulins. Magnification × 280.

significant differences could be detected between groups C (rnu/+) and D (rnu/rnu), although three rats died due to the weight loss in group D. In rats injected with 0·5 ml NRS/100 g body weight (groups E, F), there was no abnormal proteinuria.

Immunofluorescence (IF) findings

The results of immunofluorescence findings are summarized in Table 1. In the rats from groups A, B, C and D, the patterns of rabbit IgG were linear along the capillary loop during the period of observation. In the control rats from groups E and F, staining for rabbit IgG was not observed in the glomeruli.

On day 1, granular mesangial deposits of rat immunoglobulins and IgG were found in groups A and B, as also in the control rats (groups E, F). Linear capillary deposits of rat immunoglobulins and IgG were seen from day 7 to 14 in group A and from day 7 to 28 in group C. Irregular and interrupted capillary deposits of rat immunoglobulins and IgG were noted from day 7 to 14 in group B and from day 7 to 28 in group D (Fig. 2). These patterns, however, were not linear and differed from those in groups A and C.

Diffuse capillary deposits of rat C3 (Fig. 3) were observed in groups A, B, C and D on days 1–14, but not in the rats of groups E and F.

Light microscopic study

No remarkable difference could be found in light microscopical findings for groups A and B or C and D during the period of observation.

On day 1, there had occurred no remarkable influx of polymorphonuclear cells (PMN) in glomeruli of groups A and B. Mild endocapillary proliferation developed. No severe proliferative lesions could be seen. On days 14 and 28, increased mesangial matrix was apparent. On day 28, there was greater increase of matrix in groups C and D (Fig. 4). Rats in groups E and F showed no significant abnormalities in histological study.

Electron microscopic study

The findings obtained by electron microscopy were essentially the same with those by light microscopy. On day 1, the small number of PMN were noted within glomeruli, some of which were in intimate contact with GBM through endothelial fenestra.

As the days elapsed after NTS injection, PMN were replaced by monocytes. Focal flattening and fusion of podocytes were also noted beginning on day 1 and lasted thereafter until the end of the experiment. No abnormality in GBM ultrastructure was noted, nor were there any electron-dense deposits in all the glomeruli examined. Mild swelling of endothelial cells appeared on day 1, peaked at day 7 and gradually subsided toward day 28. Those glomerular changes were found in both rats injected with 0·5 ml and 1·0 ml NTS. No essential difference was noted between these two groups of rats, but somewhat more severe in group C and D. Also, no significant difference was noted between rnu/rnu and rnu/+ rats in terms of the severity of glomerular abnormalities.

Serum antibody levels

There seemed to be no remarkable differences in anti-rabbit immunoglobulin titres for groups A, B, C and D, although the small number of rats that survived did not permit adequate statistical analysis (Table 2).

In the PFC assay, clear-cut differences could be seen in the numbers of PFC in rnu/rnu and rnu/+ spleens injected with 1·0 ml of NTS. In rnu/+ rats, 2164 ± 528 ($n=3$) and 1515 ± 367 ($n=3$) PFC/spleen were observed days 7 and 14, respectively. Virtually no PFC (422 ± 41 ($n=3$) on day 7, and 227 ($n=1$) on day 14) could be detected in rnu/rnu rats.

DISCUSSION

This is the first report, to our knowledge, in which an athymic nude rats have been used as an experimental model to clarify T cell-associated immune mechanisms in the induction and progression of classical nephrotoxic serum nephritis induced by one-shot i.v. injection of NTS.

No marked differences could be found in the severity of nephrotoxic serum nephritis between heterozygous (rnu/+) and athymic nude (rnu/rnu) ACI rats, although host immune reactions against the rabbit IgG were weakly but clearly demonstrated in rnu/+ rats by means of IF and PFC assay.

The typical linear pattern of rat IgG along GBM in IF is observed in a section from rnu/+ rats provided direct evidence of host autologous antibody reaction against rabbit IgG bound to GBM. Interrupted capillary deposits of rat immunoglobulins

Table 1. Immunofluorescent findings in nephrotoxic nephritis

	Rabbit IgG						Rat immunoglobulins						Rat IgG						Rat C3					
Days	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F
1	+++	+++	ND	ND	−	−	M+	M+	ND	ND	M+	M+	M+	M+	ND	ND	M+	M+	+++	+++	ND	ND	−	−
3	+++	+++	ND	ND	ND	ND	M+ C+	M+ I+	ND	ND	ND	ND	M+ C+	M+ I+	ND	ND	ND	ND	++	++	ND	ND	ND	ND
7	+++	+++	+++	+++	ND	ND	M+ C++	M+ I++	M+ C++	M+ I++	ND	ND	M+ C++	M+ I++	M+ C++	M+ I++	ND	ND	+	+	++	++	ND	ND
14	+++	+++	+++	+++	−	−	M+ C+	M+ I+	M+ C+++	M+ I+	M+	M+	M+ C+	M+ I+	M+ C+++	M+ I+	M+	M+	+	+	+	+	−	−
28	+++	+++	+++	+++	−	−	M+	M+	M+ C++	M+ I+	M+	M+	M+	M+	M+ C++	M+ I+	M+	M+	−	−	−	−	−	−

C, capillary, linear; M, mesangial, granular; I, capillary, interrupted.
ND, not done.
Immunofluorescence intensity: +, definite; ++, strong; +++, very strong.

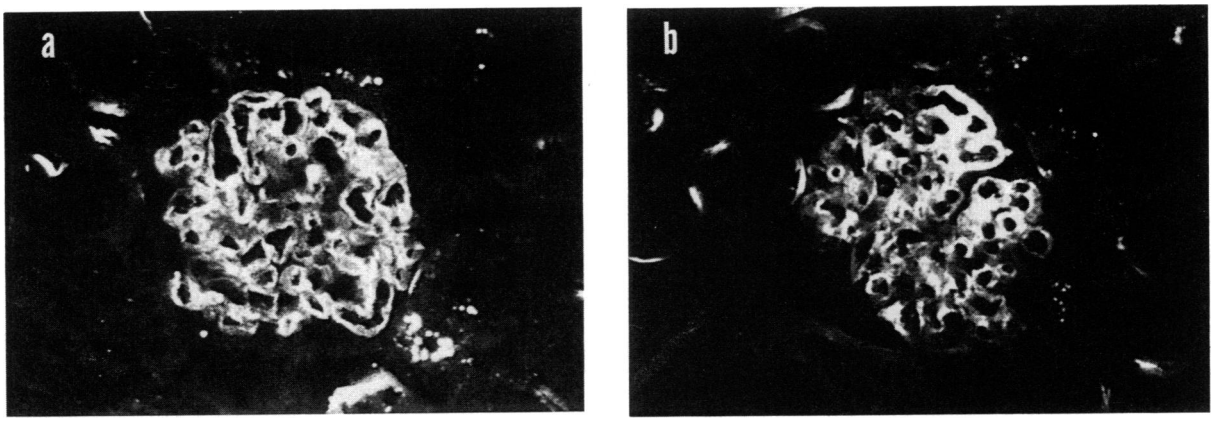

Fig. 3. Photomicrographs demonstrating rat-C3 by direct immunofluorescence in glomeruli from (a) heterozygous rats in groups A; and (b) nude rats in group B, killed 1 day after NTS injection. Magnification × 280.

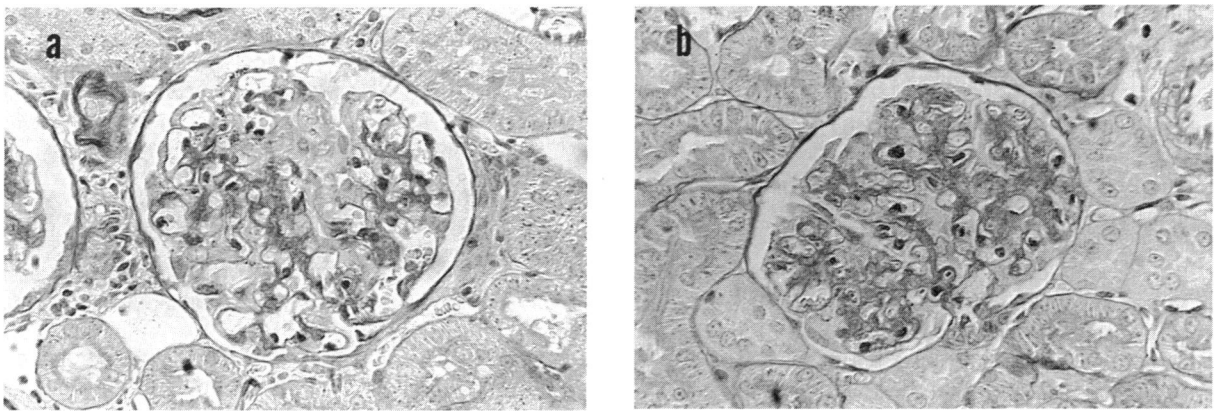

Fig. 4. Light microscope findings on the glomerulus from (a) heterozygous rats in group C; and (b) nude rats in group D, killed 28 days after NTS injection. Periodic acid–Schiff staining, magnification × 280.

Table 2. Serum anti-rabbit immunoglobulins antibody levels in nephrotoxic nephritis

	ELISA (OD_{492}) day				
Group	1	3	7	14	28
A (rnu/+) 0·5 ml NTS/100 g	0·056 ± 0·011 ($n=3$)	0·076 ± 0·026 ($n=3$)	0·095 ± 0·029 ($n=3$)	0·148 ± 0·080 ($n=3$)	0·111 ± 0·068 ($n=3$)
B (rnu/rnu) 0·5 ml NTS/100 g	0·054 ± 0·009 ($n=3$)	0·106 ± 0·035 ($n=3$)	0·146 ± 0·070 ($n=3$)	0·080 ± 0·007 ($n=3$)	0·056 ± 0·002 ($n=3$)
C (rnu/+) 1·0 ml NTS/100 g	ND	ND	0·014 ± 0·006 ($n=3$)	0·024 ± 0·004 ($n=3$)	0·152 ± 0·108 ($n=3$)
D (rnu/rnu) 1·0 ml NTS/100 g	ND	ND	0·045 ± 0·023 ($n=3$)	0·071 ($n=1$)	0·058 ± 0·018 ($n=2$)
E (rnu/+) 0·5 ml NTS/100 g	0·054 ± 0·011 ($n=2$)	ND	ND	0·173 ± 0·085 ($n=2$)	0·172 ± 0·201 ($n=2$)
F (rnu/rnu) 0·5 ml NTS/100 g	0·050 ± 0·002 ($n=2$)	ND	ND	0·096 ± 0·014 ($n=2$)	0·032 ± 0·002 ($n=2$)

Results are expressed as mean ± s.d. (negative control, 0·093 ± 0·016 ($n=3$); positive control: 1·181 ± 0·028 ($n=3$)).
ND, not done.

and IgG were present in rnu/rnu rats in proteinuric state and appeared to be due to the unspecific trapping of host immunoglobulins as a serum protein as a result of increased permeability of glomerular capillary wall. This increase was indicated by the large amount of urinary protein excretion.

In ELISA, no detectable antibody activity could be detected in the sera from any group of rats injected with NTS or NRS. The one-shot i.v. injection of small amounts of heterologous proteins is not a particularly good immunizing procedure. In the case of rnu/+ rats injected with NTS, the anti-rabbit IgG antibody (assuming it to have been produced) would have been absorbed with rabbit IgG bound to GBM, as suggested by IF findings. This may be one reason why no antibody activity could be detected in the sera from the groups of rnu/+ rats.

During the period of observation, there was no difference in the urinary protein/100 g body weight excretion following injection with 1·0 ml of NTS between rnu/rnu and rnu/+ rats.

We found no marked differences between rnu/rnu and rnu/+ rats in morphological findings by light microscopy.

The glomerular injury in the heterologous phase of nephrotoxic serum nephritis is usually considered to be mediated by complement and PMN (Unanue & Dixon, 1967). In this study the degree of rat C3 deposition in glomeruli and of PMN infiltration in rnu/rnu rats were similar to those in rnu/+ rats. From these results, the function of mediators in rnu/rnu rats seemed to be not different from that in rnu/+ rats.

Exudative changes observed in nude mice (Okada *et al.*, 1982) could not be seen in nude rats.

Nephritis of greater severity was induced by injection of 1·0 ml/100 g body weight NTS in both rnu/rnu and rnu/+ groups, than by 0·5 ml/100 g body weight. Our results, obtained using athymic nude rats, indicate that the severity and course of typical classical nephrotoxic serum nephritis by a one-shot i.v. injection of NTS may depend on the amount of injected NTS rather than the subsequent host immune reactions against it. However, based on these results, no general conclusion could be drawn as to the effect of the host immune reactions on the severity and course of this disease, due to the limited immune reactions in classical nephrotoxic serum nephritis as demonstrated in this paper by small number of PFC and absence of any detectable antibody titre in sera with ELISA.

At least on the classical nephrotoxic nephritis, we conclude that the severity of heterologous immune stimulus at the initial phase plays much more important roles in the persistence and progression of renal injury rather than host T cell-associated immune mechanisms. This conclusion was based on the adequate method used in this experiment, whose advantage is that, unlike methods used for suppressing the host immune reaction as in previous publications, such as irradiation (Kay, 1940), tolerance induction (Shimizu, 1974), thymectomy (Oite, 1979) and transplantation (Nahas *et al.*, 1985), it does not have an artificial influence on the course of nephritis itself.

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