Depletion of CD8 positive cells in nephrotoxic serum nephritis of WKY rats

Katsutoshi Kawasaki, Eishin Yaoita, Tadashi Yamamoto, and Itaru Kihara

Department of Pathology, Institute of Nephrology, Niigata University School of Medicine, Niigata, Japan

Depletion of CD8 positive cells in nephrotoxic serum nephritis of WKY rats. Following a small dose of nephrotoxic serum (NTS) WKY rats demonstrated crescentic glomerulonephritis, which was characterized by the early infiltration of CD8 positive cells in glomeruli. In vivo depletion of CD8 positive cells from WKY rats completely prevented proteinuria (4.6 ± 4.8 mg/day vs. 105.3 ± 11.6 mg/day on day 10; $N = 19$, $P < 0.001$) and crescent formation (2.7 ± 2.9% vs. 94.3 ± 2.6%; $P < 0.001$). Immunofluorescence revealed complete inhibition of the influx of CD8 positive cells and subsequent reduction of the infiltration of macrophages in the glomeruli. Glomerular binding of ^{125}I-anti-rat glomerular basement membrane antibodies, host anti-rabbit IgG production and the C_3 level in the circulation were the same as in the control. These data indicate that CD8 positive cells play a key role in glomerular injury and crescent formation. This model provides a useful system for studying the cellular mechanisms that lead to glomerular injury and subsequent crescent formation.

The importance of humoral immunity in the pathogenesis of glomerulonephritis has been well established [1]. However, the role of cell-mediated immunity has not been fully investigated. Bhan et al [2, 3] showed that the passive cell transfer of sensitized lymphocytes induced glomerulonephritis. Kreisberg et al [4] demonstrated lymphocyte infiltration in the capillary lumen by electron microscopic study. In 1984, Bolton et al [5] reported that proliferative nephritis with crescents was present in bursectomized (humoral immunity-independent) chickens and that nephritic eluates were incapable of transferring disease. Recently, they succeeded in inducing glomerulonephritis by the transfer of mononuclear cells [6]. Thus, it has been suggested that cell-mediated immunity may produce glomerular injury in experimental glomerulonephritis [reviewed in 7, 8].

In 1985, Tipping et al [9] reported that small numbers of T lymphocytes (helper phenotype) can be detected by monoclonal antibodies (mAbs) in glomeruli of rats with an accelerated model of nephrotoxic serum nephritis. These authors considered that T cells may play a role, especially helper T cells, during the influx of mononuclear phagocytes in glomerulonephritis.

In WKY rats, a very small dose of nephrotoxic sera (NTS) has been shown to induce severe proliferative and necrotizing

glomerulonephritis with crescent formation [10]. This strain demonstrated increased activities of natural killer (NK) cells and antibody-dependent cell-mediated cytotoxicity (ADCC) compared with BN rats [10]. Non-helper type lymphocytes take part in the pathogenesis of glomerulonephritis.

In this study, we examined a possible contribution of lymphocytes to the development of nephrotoxic serum nephritis in WKY rats and clarified the participation of CD8 positive cells, not of CD4 positive cells, in the development of glomerular lesions and crescent formation in this model.

Methods

Nephrotoxic serum nephritis in WKY rats

Animals. Inbred WKY rats (Charles River Japan Inc., Atsugi, Kanagawa, Japan) of both sexes, aged 12 weeks, were used. The other strains used, Lewis, F344, WKA and SHR rats, were bred and maintained in our laboratory by brother-sister mating. Animals were fed standard rat chow and given free access to water.

Rabbit anti-glomerular basement membrane (GBM) serum (NTS). Rat GBM was prepared by the method of Krakower and Greenspon [11]. GBM was digested with trypsin (Sigma Chemical Co., St. Louis, Missouri, USA) for three hours at 37°C. After heating at 60°C for 30 minutes, the mixture was centrifuged at 76,000 × g for one hour and the supernatant was lyophilized. Three albino rabbits were immunized subcutaneously with one mg each of the lyophilized sample emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Michigan, USA). Boosters were given in the second, fourth and sixth weeks using the same immunogen. Sera were drawn 10 days after the last booster. Before use the antisera were heat-decomplemented and absorbed with an equal volume of packed rat red blood cells overnight at 4°C and two times with five mg of lyophilized rat serum/ml of antiserum. The specificity was confirmed by in vitro indirect immunofluorescence using fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG (Seikagaku Kogyo Co., Tokyo, Japan) on frozen sections of normal Wistar rat kidney. Sharp linear immunofluorescence was observed only along the GBM and the tubular basement membranes.

A preliminary study showed that the amount of NTS necessary to induce proteinuria was more than 0.5 ml/100 g body weight at either the heterologous or autologous phase in Lewis, F344, WKA and SHR strains. With a dose of 0.025 ml NTS/100 g body weight, these rats produced no proteinuria and minimal

glomerular changes during a period of 14 days. In contrast, this dose was sufficient for overt proteinuria and glomerular lesion in WKY rats.

Experimental schedule. To obtain the NTS dose necessary to induce proteinuria, NTS of 0.2, 0.1, 0.05 and 0.025 ml per 100 g body weight was injected intravenously (i.v.) to four groups of WKY rats consisting of five rats each. With ether anesthesia, renal biopsies were done on day three, and all rats were sacrificed by axillar bleeding on day 14. The amount of protein excretion in urine per 24 hours was determined on days 0, 1, 4, 7, 11 and 14 by using protein assay kit (Nippon Bio-Rad Laboratories, Tokyo, Japan).

For phenotypical analysis of lymphocytes, another 15 WKY rats that received NTS at a dose of 0.025 ml/100 g body weight were killed at four hours and on days 1, 3, 6 and 11. Three rats were killed at each time point.

Histological examination. At the times of renal biopsy and sacrifice, each kidney specimen was divided into two parts. The first part was fixed in buffered formalin for light microscopic examination. Sections were stained with hematoxylin and eosin, periodic acid-Schiff and periodic acid silver-methenamine. The number of crescentic glomeruli per 100 glomeruli of each rat was calculated and expressed as a percentage. The second part of the specimen was quick-frozen in n-hexane at $-70°C$ for immunofluorescence study. FITC-conjugated anti-rabbit IgG (Organon Teknika Corporation, West Chester, Pennsylvania, USA), FITC-conjugated anti-rat IgG (Seikagaku Kogyo Co.) and FITC-conjugated anti-rat C_3 (Organon Teknika Corporation) were used for direct immunofluorescence. To analyze cell populations that infiltrated the glomeruli, cryostat sections (2 μM) were stained with mAb MRC-OX8 against the rat CD8 molecule [12, 13], which was prepared as ascites using the corresponding hybridoma (PHLS Centre for Applied Microbiology & Research, Wiltshire, UK), mAb MRC-OX38 [14] (a hybridoma; PHLS Centre for Applied Microbiology and Research), which reacts with rat CD4 molecule, mAb ED1 [15] (Dainippon Seiyaku Co.) for tissue monocytes and macrophages, mAb R73 for T cell receptor $\alpha\beta$ ($\alpha\beta$TCR) [16] (a hybridoma, a gift from Professor T. Hünig, Institut für Virologie und Immunologie, Würzburg, Germany), mAb MRC-OX39 for high affinity receptor of IL-2 (IL-2R) [17] (Dainippon Seiyaku Co.) and mAb 1F4 for CD3 [18] (a gift from Dr. Tanaka, Tohoku University, Sendai, Japan). FITC-conjugated rabbit anti-mouse IgG and IgM, containing 2% normal rat serum, were used as the second antibody. The number of positively stained cells per 100 glomeruli of each rat was counted and expressed per glomerular cross section.

Depletion study of CD8 positive cells in nephrotoxic serum nephritis of WKY rats

Antibodies. MAbs MRC-OX8 (IgG1) and MRC-OX38 (IgG2a) were used to deplete CD8 or CD4 positive cells in the circulation of WKY rats. MAb RC2 (IgG1), which was directed against human renal cell carcinoma antigen and did not react with rat tissues [19], was used as a control mAb (a gift from Professor G. Fleuren, University of Leiden, The Netherlands). The ascitic fluids of these mAbs were centrifuged and adjusted to a concentration of 20.0 mg/ml IgG in phosphate buffered saline (PBS, pH 7.4) as determined by single radial immunodiffusion, using protein A-purified normal mouse IgG as a reference.

In a preliminary immunohistochemical study of cytocentrifuged cell suspensions from normal WKY rats, CD8 positive cells were found to be completely depleted from the circulation, spleen, cervical lymph node and thymus for 10 days after the administration of MRC-OX8. This depletion started at 48 hours after injection. The same treatment induced complete disappearance of CD8 positive cells from the circulation and lymphoid organs of WKY rats that received NTS. However, a single injection of MRC-OX38 was not effective for the depletion of CD4 positive cells in the circulation. After the daily administration for the first three days and following every other day for 14 days, eighty to eighty-five percent of these cells were depleted from the circulation. These data were in accordance with the report by Roser [20]. The optimal amount of MRC-OX8 and MRC-OX38 was determined from the immunohistological study of CD8 and CD4 positive cells in spleen, cervical lymph nodes and thymus of normal rats.

Experimental protocol. To study the depletion of CD8 positive cells, thirty-three WKY rats were divided into five groups: Animals treated with NTS plus MRC-OX8 (group NTS-OX8, N = 6), NTS plus MRC-OX38 (group NTS-OX38, N = 6), NTS plus RC2 (group NTS-RC2, N = 13), normal rabbit sera (NRS) plus MRC-OX8 (group NRS-OX8, N = 4) and NRS plus RC2 (group NRS-RC2, N = 4). NTS and NRS were given intravenously (i.v.) at a dose of 0.025 ml/100 g body weight on day 0. MAbs MRC-OX8 and RC2 were given at a dose of 2.8 mg/100 g body weight intraperitoneally (i.p.) and 0.6 mg/100 g body weight i.v. on day -2. MRC-OX38 was given at a 3.0 mg/100 g body weight i.p., and 0.5 mg/100 g body weight i.v. every day from day -2 to day 0 and every other day until day 14. Renal biopsies were done on day 3 or day 7, and all rats were killed on day 11 under ether anesthesia.

Immunohistochemical examination was performed as described above. For phenotypical analysis of lymphocytes, 15 WKY rats treated with NTS and mAb MRC-OX8 were killed at four hours and on days 1, 3, 6 and 11. Three rats were killed at each time point.

Quantitation of the NTS binding in glomeruli. To quantitate the binding of ^{125}I-anti-GBM IgG, the immunoglobulin fraction of NTS was prepared by 33% ammonium sulfate precipitation. The purified IgG fraction was coupled with ^{125}I by the chloramine T method [21]. Fourteen rats were sacrificed one hour after the injection of ^{125}I-anti-GBM IgG containing NTS.

Circulating antibody and C_3 estimation. Blood samples were taken from the tail vein on days 0, 3, 8 and 10 and stored at $-20°C$. The circulating anti-rabbit IgG level and C_3 level were determined by enzyme-linked immunosorbent assay (ELISA) and the Ouchterlony method [22], respectively. For ELISA, 100 μl of 10 mg/ml normal rabbit IgG was coated on 96-well plates. The wells were incubated with 100 μl of test sera, which was diluted to 1:20. Wells were washed and incubated with horseradish peroxidase-conjugated rabbit anti-rat IgG, diluted 1:50. The color of the reaction product of ortho-phenylene diamine was quantitated by measuring absorbance at 490 nm using an EIA microplate reader (Japan Bio-Rad, Tokyo, Japan). Each sample was assayed with four replicates. Normal rat sera and pooled sera of Wistar rats immunized with rabbit IgG three times served as negative and positive controls, respectively. In order to determine the difference of avidity of antibodies indirectly, sample sera sequentially diluted from 1:4 to 1:4,096

were examined by the dilution-curve and the end-point analysis of the ELISA.

To estimate C_3 level in the circulation, rabbit antiserum against rat C_3 was prepared by immunization with zymosan fixed complement components according to the method of Mardiney and Müller-Eberhard [23]. Anti-rat C_3 serum was found to be monospecific in immunoelectrophoretic analysis. Double diffusion in gel was done using the method of Ouchterlony.

Statistical analysis

Data are expressed as means ± 1 SD. Statistical evaluation was done by Student's *t*-test. All possible pairs were tested.

Results

Nephrotoxic serum nephritis in WKY rats

Urinary protein excretion. Several NTS doses were intravenously injected to WKY rats. In WKY rats, NTS at a dose of 0.025 ml/100 g body weight was sufficient to induce proteinuria and severe glomerulonephritis. In other strains, Lewis, F344, WKA and SHR rats, the minimal dose of NTS required for overt proteinuria was 0.5 ml/100 g body weight or more (data not shown). In this dose, some became proteinuric on day four and all animals showed significant proteinuria on day 7 and thereafter. All the following data were obtained from the experiments using a NTS dose of 0.025 ml/100 g body weight.

Histology and immunohistochemistry. Glomerular lesions induced with 0.025 ml NTS/100 g body weight on day three were characterized by endocapillary hypercellularity. Mesangial proliferation, severe necrotizing lesion and marked crescent formation were observed on day six and thereafter. In immunofluorescence study, rabbit IgG was intensely stained along the capillary walls in a linear pattern through the experiment. Rat IgG and rat C3 were detected at day 11 and at days three, seven and 11, respectively. But the intensity was similarly faint.

Most characteristic was the infiltration of CD8 positive cells in glomeruli and periglomerular interstitium at four hours, which increased to a peak on day three and decreased thereafter (Fig. 1a, 1c and 1e, Table 1). A considerable number of mAb ED1 positive-macrophages were also observed at four hours and on days 1 and 3; the number peaked on days 6 and 11 (Fig. 1b, 1d and 1f, Table 1). The peak influx of ED1 positive cells in glomeruli occurred later than that of CD8 positive cells (Table 1). However, CD4 positive cells (MRC-OX38 positive) were not observed until day six, when a small number of positive cells began to appear (Table 1).

In serial sections (2 μM) that were alternatively stained with MRC-OX8 and ED1, the cells stained with MRC-OX8 were not in accordance with those stained with ED1 (Fig. 2 a and b). For further characterization of CD8 positive cells, mAbs to $\alpha\beta$TCR, IL-2R and CD3 were used. There were few $\alpha\beta$TCR-positive cells in glomeruli at four hours and day 1. On day 3, two or three positive cells were detected. On day 11, several positive cells were detected only in the interstitium. Thus, the number of $\alpha\beta$TCR positive cells in glomeruli was less than that of CD8 positive cells. These $\alpha\beta$TCR positive cells were not in accordance with CD8 positive cells in serial sections (2 μM) alternatively stained with mAbs R73 and MRC-OX8 (Fig. 2 c and d).

IL-2R positive cells (MRC-OX39 positive cells) were not observed in glomeruli throughout the experiment. Small numbers of IL-2R positive cells were observed in the interstitium. No CD3 positive cells (1F4 positive cells) were detected in glomeruli throughout the experiment. Using serial sections, CD8 positive cells did not express CD3 nor IL-2R in glomeruli.

Depletion study of CD8 positive cells in nephrotoxic serum nephritis of WKY rats

Urinary protein excretion. After the injection of NTS and mAb RC2, all animals showed significant proteinuria on day eight and thereafter (Fig. 3). Urinary protein excretion reached 105.3 ± 11.6 mg/day on day 10. The amount of the urinary protein excretion was the same in rats given NTS alone. MRC-OX38-treatment did not reduce NTS-induced urinary protein excretion on day 10 (113.4 ± 24.0 mg/day; Fig. 3). In contrast, group NTS-OX8 showed complete absence of urinary protein excretion during the experiments (4.6 ± 4.8 mg/day on day 10; $P < 0.001$ compared with groups NTS-RC2 and NTS-OX38). NRS-treated control groups showed no proteinuria (1.8 ± 0.5 mg/day on day 10 in group NRS-RC2 and 2.0 ± 0.1 mg/day in group NRS-OX8).

Histology and immunohistochemistry. No proliferative glomerular lesion and crescent formation were observed in group NTS-OX8 (Fig. 4 a and b). Quantitative study revealed that the frequency of crescentic glomeruli was drastically decreased in group NTS-OX8 (2.7 ± 2.9% of glomerular cross section in group NTS-OX8 vs. 94.3 ± 2.6% in group NTS-RC2; $P < 0.001$; Fig. 5). Glomerular hypercellularity was observed in groups NTS-OX38 and NTS-RC2 on day three and thereafter (Fig. 4c). Severe mononuclear cell proliferation, necrotizing lesion and crescent formation were observed on day 7 (Fig. 4d). No histological changes were induced in groups NRS-RC2 and NRS-OX8.

In immunofluorescence, rabbit IgG was detected along the capillary walls in a linear pattern both in groups NTS-OX8 (Fig. 6a), NTS-RC2 (Fig. 6b) and NTS-OX38. There were no changes in the intensity of rabbit IgG in either group. Rat IgG and rat C_3 were detected in the glomeruli on day 11 and on days 3, 7 and 11, respectively. But their intensity was similarly faint in these three groups. The NRS-RC2 and NRS-OX8 groups showed no deposition of these components in the glomeruli.

Marked intraglomerular infiltration of mononuclear cells was induced in group NTS-RC2. In contrast, a few of infiltrating cells were observed in the glomeruli of group NTS-OX8. No CD8 positive cells were detected in the glomeruli or periglomeruli of group NTS-OX8 throughout the experiment (Fig. 6c). In contrast, the infiltration of CD8 positive cells was observed in glomeruli and periglomeruli on day one and later in group NTS-RC2 (Fig. 6d, Table 2).

A small but significant number of ED1 positive macrophages infiltrated the glomeruli in group NTS-OX8 at four hours and day one (Table 2). Interestingly, few macrophages existed in the glomeruli in group NTS-OX8 at day three and later (Fig. 6e, Table 2), whereas numerous macrophages infiltrated the glomeruli and periglomeruli in group NTS-RC2 (Fig. 6f).

CD4 positive cells (MRC-OX38 positive) were not observed in either the group NTS-OX8 (Fig. 6g, Table 2), NTS-RC2 (Fig. 6h) or NTS-OX38.
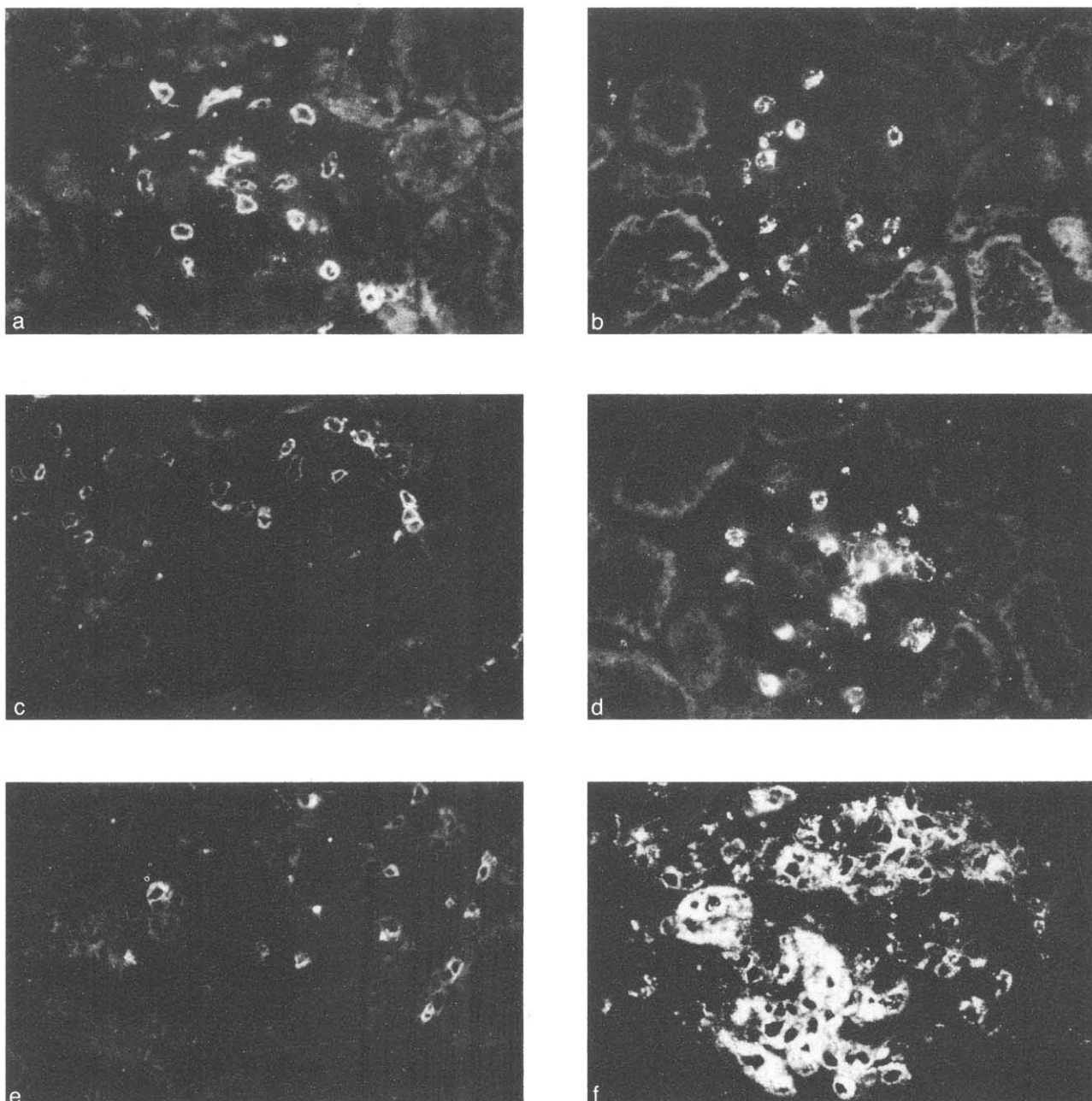
Fig. 1. *Immunofluorescence photomicrograph of glomerular CD8 positive cells* (**a, c and e**) *and ED-1 positive macrophages* (**b, d and f**). A small but significant number of CD8 positive cells is observed on days 1 (**a**), 3 (**c**) and 6 (**e**). The number of macrophages is small on day 1 (**b**), and a considerable number of cells are present on day 3 (**d**). Numerous macrophages are seen in the glomeruli on day 6 (**f**). (Original magnification: ×165)

^{125}I-anti-GBM IgG binding in glomeruli. In group NTS-RC2, the mean percentages of rabbit IgG deposited per one kidney and per 10^4 glomeruli at one hour were 1.20 ± 0.14% and 0.18 ± 0.035%, respectively. Group NTS-OX8 showed respective means of 1.08 ± 0.20% and 0.17 ± 0.045% (Fig. 7). There were no significant differences between the two groups ($P > 0.20$, not significant).

Circulating antibody and C_3 levels. Figure 8 shows the time course of anti-rabbit IgG production in the circulation. Sharp increases in circulating anti-rabbit IgG were observed on day

eight in groups of both NTS-RC2 and NTS-OX8. There were no significant differences between the two groups before 10 days. Group NTS-OX8 showed somewhat higher production of the antibody against rabbit IgG on day 10 ($P < 0.05$). In normal rabbit IgG-injected groups (groups NRS-RC2 and NRS-OX8), antibody production was detected and titers were about half those of NTS-treated groups. In order to evaluate the avidity of the antibody to rabbit IgG indirectly, the amounts of the antibody were examined using serially diluted rat sera. The dilution curves were the same and a 1:512 dilution of sera was

Table 1. Frequency of intraglomerular lymphocytes and macrophages in nephrotoxic serum nephritis of WKY rat

Time	CD4 positive cell	CD8 positive cell	Monocyte/ macrophage
0	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.7
4 hours	0.1 ± 0.4	1.1 ± 1.3	5.0 ± 2.6
Day 1	0.2 ± 0.5	5.6 ± 3.9	10.7 ± 5.4
Day 3	0.2 ± 0.5	10.1 ± 5.2	19.5 ± 7.6
Day 6	0.3 ± 1.1	6.1 ± 3.2	36.8 ± 11.4
Day 11	0.4 ± 1.1	3.2 ± 2.8	35.6 ± 5.2

Data are expressed as the number of stained cells per glomerular cross section, mean ± 1 SD. One hundred glomeruli were examined in each of three or four rats. All rats were injected with 0.025 ml/100 g body weight of anti-GBM sera.

the end-point in both NTS plus RC2- and NTS plus OX8-treated groups, indicating that the avidity of the antibodies was the same in both groups. In addition, no significant differences in the circulating C_3 levels were observed in any group.

Discussion

WKY rats are more susceptible to nephrotoxic serum nephritis than other strains such as Lewis, F344, WKA and SHR rats. This strain developed progressive proteinuria and proliferative, necrotizing, crescentic nephritis at a dose of NTS that was subnephritogenic to other strains. The present study found that pathological changes and urinary protein excretion are closely related to the appearance of CD8 positive cells in the glomeruli of WKY rats given NTS, since the depletion of CD8 positive cells by MRC-OX8 administration completely prevented proteinuria and lessened histological changes such as crescent formation.

There are several reports that lymphocytes play a role in glomerulonephritis. Tipping et al [9] reported that small numbers of helper T lymphocytes can be detected by mAbs in the glomeruli of rats with an accelerated form of autologous nephrotoxic serum nephritis. This early and brief T cell influx is consistent with ultrastructural [4] and kinetic studies [24]. In addition, these T cells are sensitized to the heterologous antibody [2, 3]. These previous reports emphasized a significant increase in helper phenotypic T cells and no attention has been paid to a role of CD8 positive cells in nephrotoxic serum nephritis. In the present study, the most characteristic finding was a significant intraglomerular and periglomerular influx of CD8 positive cells in nephrotoxic serum nephritis of WKY rats. Histological study revealed necrosis and crescent formation.

Most CD8 positive cells have suppressor or cytotoxic functions but some may be subsets of macrophages [25] or NK cells [26, 27]. Since NK cells are known for target-cell lysis in the MHC-unrestricted reaction [28, 29], these cells may be important mediators of renal injury and consequent crescent formation. Since the location of CD8 positive cells and mAb ED1 positive cells did not overlap in serial sections, CD8 positive cells are not macrophages but either classical suppressor/ cytotoxic cells or NK cells. No expression of CD3, high affinity of IL-2R nor αβTCR on the CD8 positive cells in the glomeruli suggested that most of these cells were not classical suppressor/ cytotoxic cells, but rather NK cells. Granados et al [10] briefly mentioned the possibility that the susceptibility of WKY rats

depended on NK activity and ADCC activity. From this point of view, further functional analyses of CD8 positive cells are needed.

The acute inflammatory process following NTS administration is, in general, characterized by the deposition and activation of complement components such as C3a and C5a [30, 31] and by early glomerular infiltration of PMNs, followed by a later phase of host antibody deposition dominated by monocytes and macrophages. The current study clearly demonstrated that a lower dose of NTS induced nephritis and that the deposition of host antibody and complements was negligible and the influx of PMNs no more severe than in other strains (data not shown). Furthermore, antibody production, avidity of the antibody and complement level in the circulation were not affected by administration of MRC-OX8. These findings suggest that the higher susceptibility of WKY rats may not be related to complement- or PMN-related mechanisms of glomerular injury, but rather to CD8 positive cell-related injury.

In this model, numerous ED1 positive macrophages were observed in NTS plus RC2-treated group. Interestingly enough, the depletion of CD8 positive cells was not effective for the prevention of the influx of ED1 positive macrophages at four hours and day one, but effective for the blocking of the later infiltration of these cells. From these data, earlier influx of ED1 positive cells may be associated with planted antibody along the GBM through Fc receptor [32] and they may play a role for antigen presenting cell and would be a source of some cytokines which attract CD8 positive cells, but not directly injurious to the glomerular tuft. On the contrary, the depletion of CD8 positive cells prevented the influx of ED1 positive cells in later phase and development of proteinuria and crescent formation. These data indicate that later infiltration of macrophages relate to the glomerular injury and crescent formation. With regard to this matter, Moura et al [33] recently showed that the administration of bacterial lipopolysaccharide, which induced the depletion of macrophages, ameliorated the urinary protein excretion and crescent formation in the same model.

Although it is still a controversial concept that monocyte migration may be dependent on the initial influx and activation of PMNs [34], Bhan et al [2, 3] suggested that a cell-mediated (delayed-type) mechanism may participate in the production of glomerular lesions in nephrotoxic serum nephritis. As a matter of course, monocytes/macrophages possess receptors for several complement components and respond to the chemoattractant peptides, C3a and C5a [30, 31]. Even so, it is possible that complement activation is not critical for monocyte/macrophage infiltration in vivo. Schreiner et al [35] showed that the glomerular influx of monocytes/macrophages is associated with a complement-independent recruitment mechanism. The mechanisms of monocyte/macrophage infiltration in their models have included immune adherence to heterologous antibody planted along the GBM through Fc receptor [32] and T cell-derived chemotactic factors [2, 3, 8]. These factors are directly released during cellular immune responses to the foreign antibody that elicited the excretion of urinary protein. In this case, however, the factors that may have been produced by the influx of CD8 positive cells were not determined. Some functional studies should be done to clarify the role of CD8 positive cells.

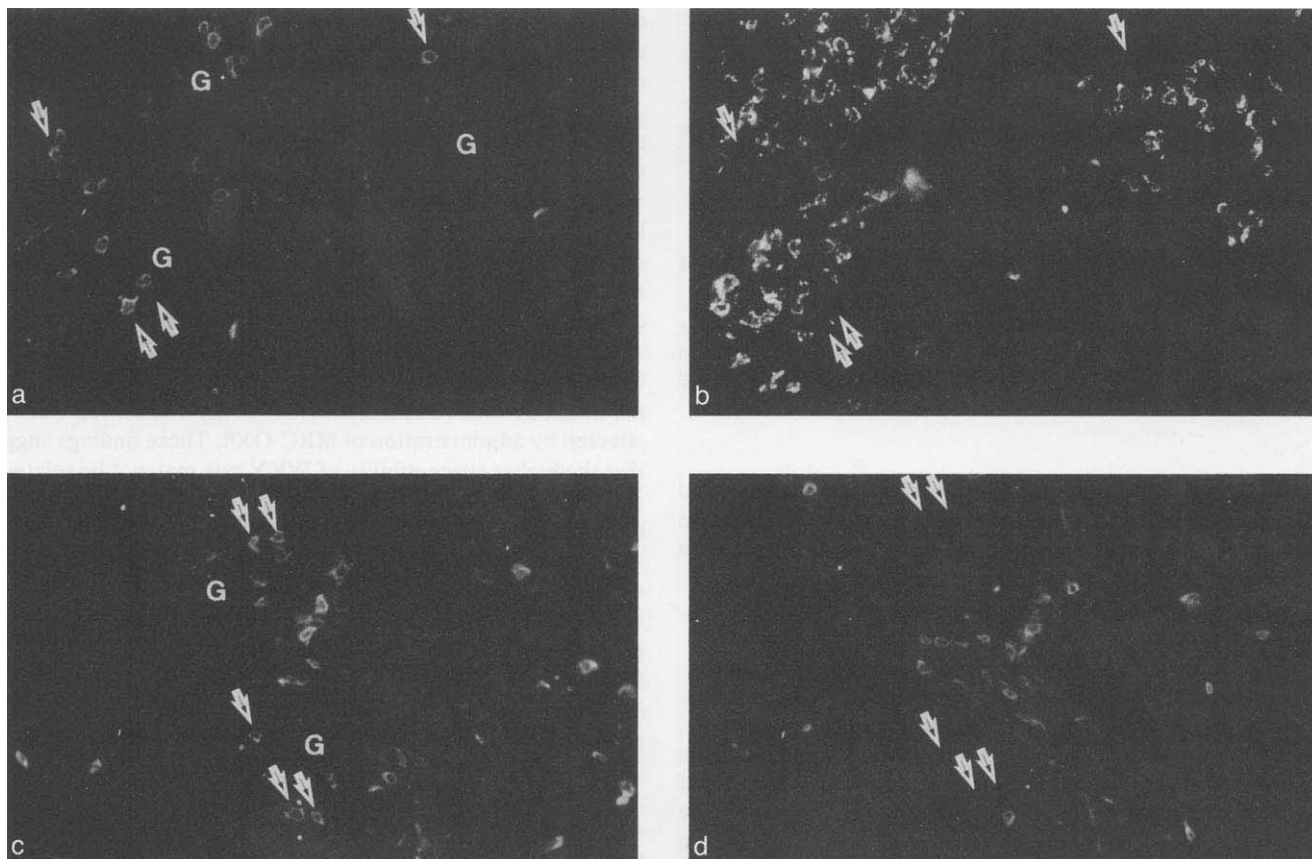There were no differences in the deposition of ^{125}I-NTS-IgG

Fig. 2. *Immunofluorescence findings in serial kidney sections.* CD8 positive cells (a) are not stained with mAb ED1 (arrow) (b). CD8 positive cells (c) do not have αβTCR (arrow) (d). G: glomerulus. (Original magnification: ×125)

between the MRC-OX8-treated group and the control group using the radiolabeling technique. This indicates that urinary protein excretion and glomerular histological changes were not caused by the quantity of bounded NTS, but were directly related to CD8 positive cell infiltration in glomeruli.

In vivo administration of MRC-OX8 induced a profound depletion in CD8 positive cells for more than one week, confirming previous reports [20, 36]. Like et al [37] also mentioned MRC-OX8 induced a functional depletion of the corresponding subset of T lymphocytes. Cells from MRC-OX8-treated animals showed a marked reduction in levels of specific alloreactivity [36] and NK cell activity [37]. According to these reports, the functional decrease in CD8 positive cells may work to lessen pathological changes in this model.

The relevance of the present observations to human glomerular disease is interesting. Stachura et al [38] showed T cell infiltration in a small number of patients with idiopathic crescentic glomerulonephritis, membranous glomerulonephritis and membranoproliferative glomerulonephritis. In contrast to our results, the helper/inducer phenotype of T cells was dominant in their study. Nolasco et al [39] revealed not only monocytes but also significant numbers of excess T cells in glomeruli from patients with proliferative glomerulonephritis with crescentic or necrotizing lesions. Recently, Li et al [40] reported that active crescent formation involved the recruitment of macrophages to the glomerulus by sensitized T cells, especially IL-2R positive cells, in human crescentic glomerulonephritis. Thus, the close
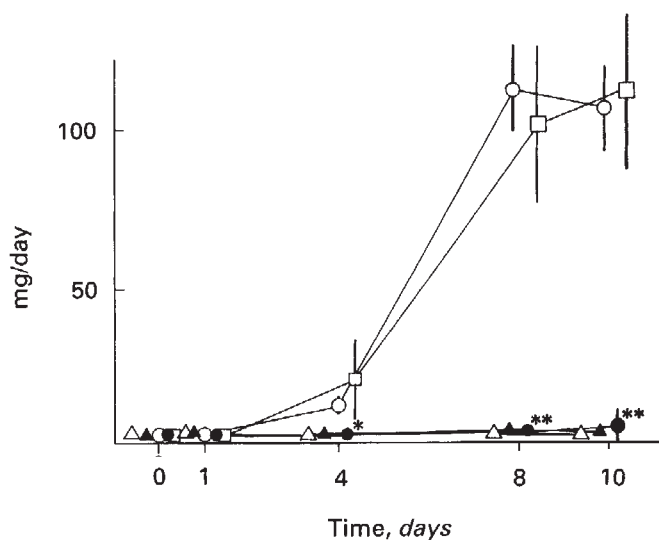

Fig. 3. *Urinary protein excretion in WKY rats treated with NTS plus MRC-OX8* (●), *NTS plus RC2* (○), *NTS plus MRC-OX38* (□), *NRS plus MRC-OX8* (▲) *and NRS plus RC2* (△). Proteinuria is completely absent in group NTS-OX8. *$P < 0.005$, **$P < 0.001$ (group NTS-OX8 vs. groups NTS-RC2 or NTS-OX38).

correlation between T cells and monocytes/macrophages suggests the existence of a local cellular immune reaction in some types of proliferative crescentic glomerulonephritis in humans.
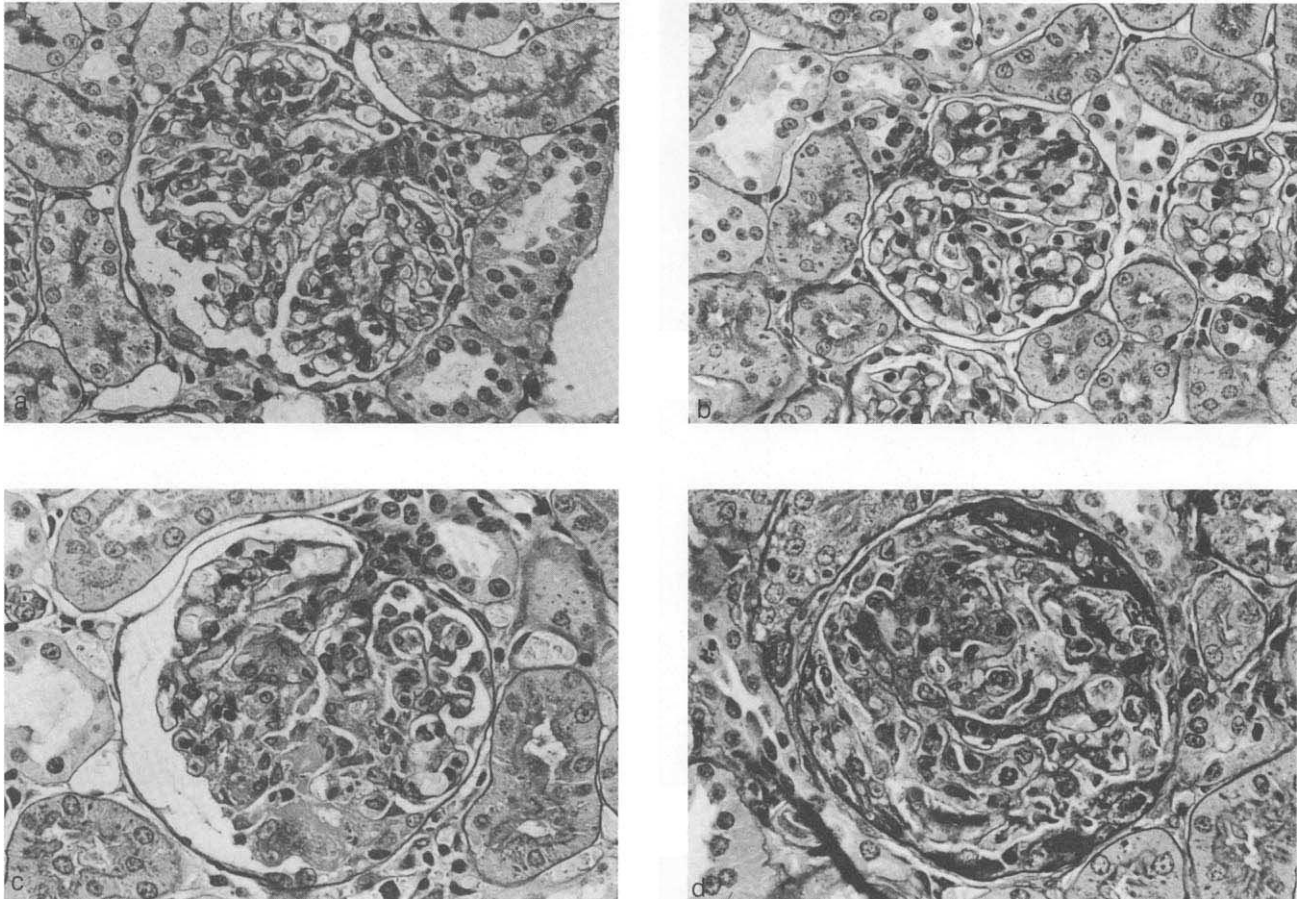
Fig. 4. *Light micrograph showing glomeruli of group NTS-OX8 (***a and b***) and group NTS-RC2 (***c and d***).* Group NTS-OX8 shows a slight increase in mononuclear cells in a glomerulus on day 3 (**a**) and minor change on day 7 (**b**). In group NTS-RC2, PAS positive material is seen in a glomerulus on day 3 (**c**), and mononuclear cell proliferation, exudative change and crescent formation are prominent on day 7 (**d**). Note the increase in glomerular size in **a**, **c** and **d**. (original magnification: ×165) Periodic acid-Schiff stain.
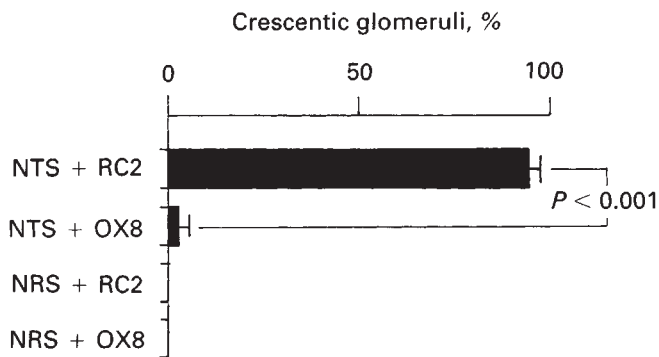
Fig. 5. *Quantitative study of crescent formation in kidney section.* The number of crescentic glomeruli per 100 glomeruli of each rat at day 11 was calculated in Periodic acid-Schiff stained section. The data were expressed as a percentage of crescent glomeruli.

The present study provides a good model by which to clarify these mechanisms.

In conclusion, this study provides direct evidence that the active participation of CD8 positive cells is an antecedent to macrophag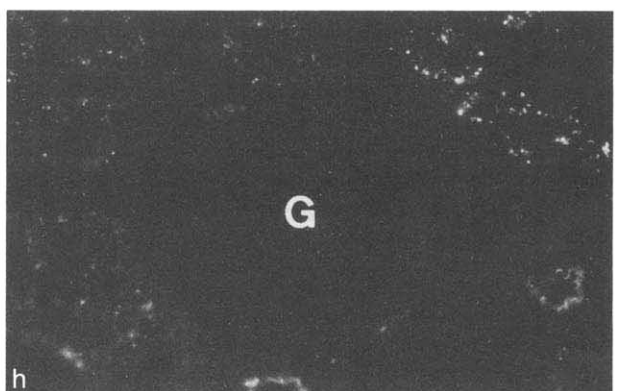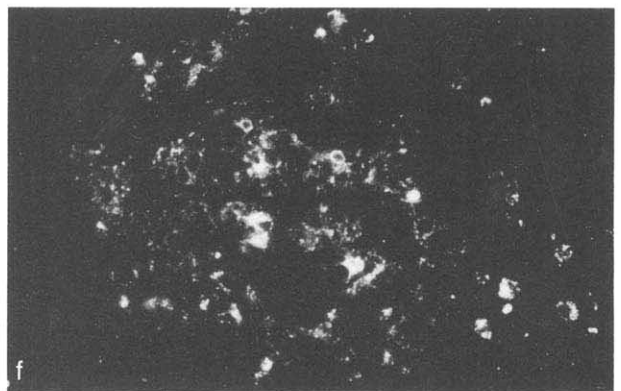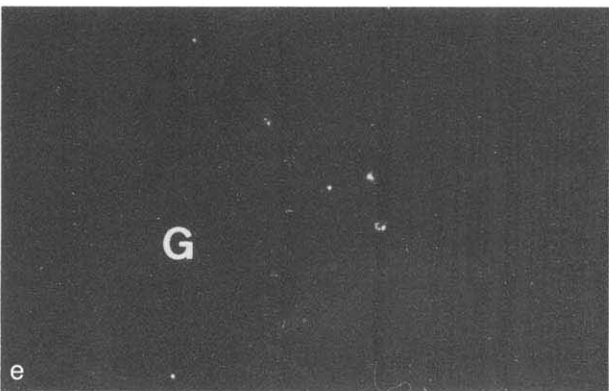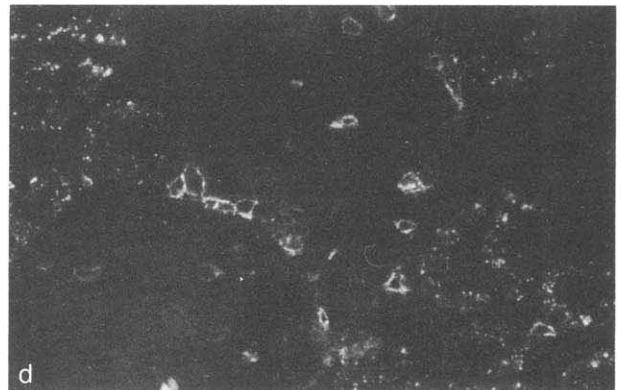e-mediated immune injury in glomeruli. The study on the depletion of CD8 positive cells suggests that a sequence of events was modified. Furthermore, this model may be a useful experimental system for studying the mechanisms that lead to renal injury, especially crescent formation, in humans.

Reprint requests to Dr. K. Kawasaki, Department of Pathology, Institute of Nephrology, Niigata University School of Medicine, Asahimachi 1–757, Niigata 951, Japan.

Appendix. Abbreviations

ADCC; antibody-dependent cell-mediated cytotoxicity, GBM; glomerular basement membrane, IL-2R; receptor of interleukin 2, LAK cell; lymphokine activated killer cell, mAb(s); monoclonal antibody (ies), NRS; normal rabbit serum(sera), NRS-OX8; normal rabbit sera plus mAb MRC OX8-treated (group), NRS-RC2; normal rabbit sera
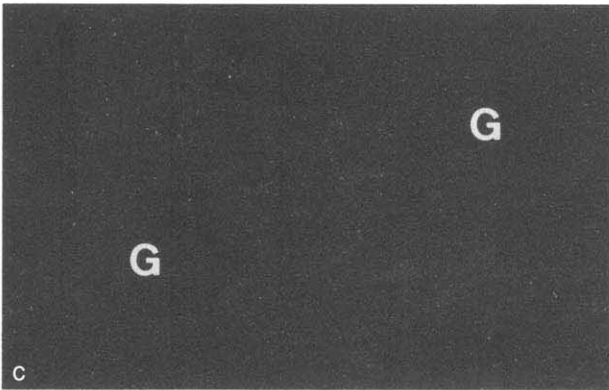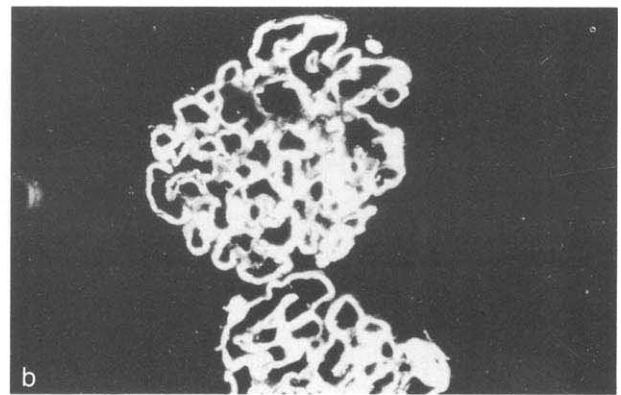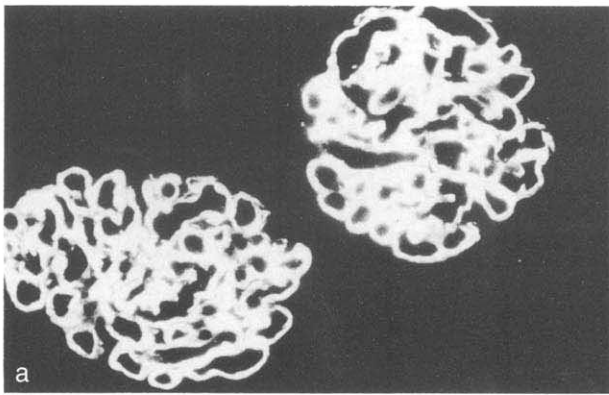
Fig. 6. *Immunofluorescence microscopic findings show the glomeruli of WKY rats treated with NTS plus OX8 (a, c, e and g) and NTS plus RC2 (b, d, f and h).* Rabbit IgG is distributed in a linear pattern along the GBM in both groups (a and b). No CD8 positive cells are seen in a glomerulus of group NTS-OX8 (c). A considerable number of these cells is seen in group NTS-RC2 (d). Few ED1 positive macrophages are seen in a glomerulus of groups NTS-OX8 (e), but many macrophages are infiltrated in a glomerulus of group NTS-RC2 (f). No MRC-OX38 stained cells (CD4 positive cells) are seen in the glomeruli of either group (g and h). All sections were sampled on day 3. G: glomerulus. (original magnification: ×125)

Table 2. Frequency of lymphocytes and macrophages in nephrotoxic serum nephritis of MRC-OX8-treated WKY rats

Time	CD4 positive cell	CD8 positive cell	Monocyte/ macrophage
0	0.1 ± 0.2	0 ± 0	0 ± 0
4 hours	0.0 ± 0.1	0 ± 0	2.7 ± 2.2
Day 1	0.1 ± 0.1	0 ± 0	7.6 ± 3.4
Day 3	0.1 ± 0.1	0 ± 0	0.3 ± 0.2
Day 6	0.1 ± 0.1	0 ± 0	0.5 ± 0.3
Day 11	0.1 ± 0.1	0 ± 0	0.5 ± 0.3

Data are expressed as the number of stained cells per glomerular cross section. One hundred glomeruli were examined in each of three or six rats, mean ± 1 SD. NTS (0.025 ml/100 g body weight) and MRC-OX8 (3.4 mg/100 g body weight) were administered in each rat.
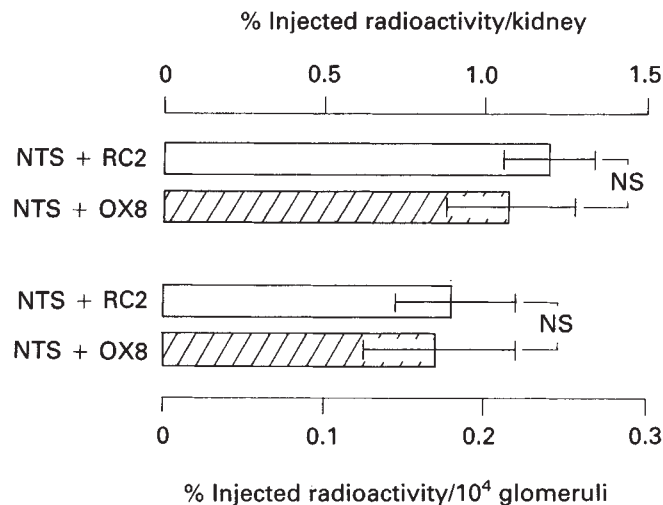

Fig. 7. *Percentage of* ^{125}I-anti-GBM IgG deposition one hour after administration of NTS. Upper and lower columns show the binding of ^{125}I-anti-GBM IgG per kidney and per 10,000 glomeruli, respectively. There are no differences in rabbit IgG deposition between the two groups ($P > 0.05$). Data are means ± 1 SD. NS, not significant. Symbols are: (☐) group NTS-RC2 ($N = 7$); (▨) group NTS-OX8 ($N = 7$).
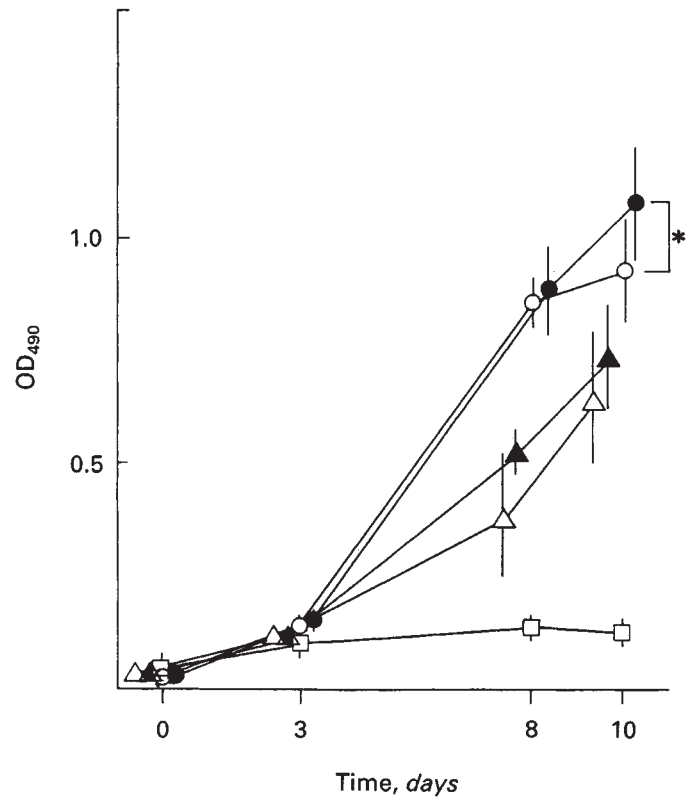

Fig. 8. *ELISA study showing activity against injected rabbit IgG.* ELISA with purified rabbit IgG at a concentration of 1 mg/well showing the reactivity of serum (diluted 1:20) from WKY rats ($N = 4$ or 6) at various time points. Both rats treated with NTS plus RC2 (O) and NTS plus OX8 (●) show good host antibody production. There were no differences of antibody production between groups NTS-RC2 and NTS-OX8 from day 0 to day 8. Antibody production in the rats treated with NTS plus OX8 is higher, not lower, than that in rats treated with NTS plus RC2 at day 10 (*$P < 0.05$). Both groups NRS-OX8 (▲) and NRS-RC2 (△) show a moderate increase in host antibody production. Normal rat sera are also examined (☐). Data shows means ± 1 SD.

plus mAb RC2-treated (group), NTS; nephrotoxic serum(sera), NTS-OX8; nephrotoxic sera plus mAb MRC OX8-treated (group), NTS-OX38; nephrotoxic sera plus mAb MRC OX38-treated (group), NTS-RC2; nephrotoxic sera plus mAb RC2-treated (group), NK cell; natural killer cell.

References

1. DIXON FJ, WILSON CB: Immunological renal injury produced by formation and deposition of immune complexes, in *Immunological Mechanisms of Renal Disease*, edited by WILSON CB, BRENNER BM, STEIN JH, New York, Churchill Livingston, Inc., 1979, pp. 1–34
2. BHAN AK, SCHNEEBERGER EE, COLLINS AB, McCLUSKY RT: Evidence for a pathogenic role of a cell-mediated immune mechanism in experimental glomerulonephritis. *J Exp Med* 148:246–260, 1978
3. BHAN AK, SCHNEEBERGER EE, COLLINS AB, McCLUSKY RT: A cell-mediated reaction against glomerular bound immune complexes. *J Exp Med* 150:1410–1420, 1979
4. KREISBERG JI, WAYNE DB, KARNOVSKY MJ: Rapid and focal loss of negative charge associated with mononuclear cell infiltration early in nephrotoxic serum nephritis. *Kidney Int* 16:290–300, 1979
5. BOLTON WK, TUCKER FL, STURGILL BC: New avian model of experimental glomerulonephritis consistent with mediation by cellular immunity. Non-humorally mediated glomerulonephritis in chickens. *J Clin Invest* 73:1263–1276, 1984
6. BOLTON WK, CHANDRA M, TYSON TM, KIRKPATRICK PR, SADOVNIČ MJ, STURGILL BC: Transfer of experimental glomerulonephritis in chickens by mononuclear cells. *Kidney Int* 34:598–610, 1988
7. FILLIT HM, ZABRISKIE JB: Cellular immunity in glomerulonephritis. *Am J Pathol* 109:227–243, 1982
8. McCLUSKY RT, BHAN AK: Cell-mediated immunity in renal disease. *Hum Pathol* 17:146–153, 1986
9. TIPPING PG, NEALE TJ, HOLDSWORTH SR: T-lymphocyte participation in antibody-induced experimental glomerulonephritis. *Kidney Int* 27:530–537, 1985
10. GRANADOS R, MENDRICK DL, RENNKE HG: Antibody-induced crescent formation in WKY rats: Potential role of antibody-dependent cell cytotoxicity (ADCC) in vivo. (abstract) *Kidney Int* 37:414, 1990
11. KRAKOWER CA, GREENSPON SA: Localization of nephrotoxic antigen within the isolated renal glomerulus. *Arch Pathol* 51:629–639, 1951
12. BRIEDEAU RJ, CARTER PB, McMASTER WR, MASON DW,

WILLIAMS AF: Two subsets of rat T lymphocytes defined with monoclonal antibodies. *Eur J Immunol* 10:609–615, 1980

13. MASON DW, ARTHUR RP, DALLMAN MJ, GREEN JR, SPICKETT GP, THOMAS ML: Functions of rat T-lymphocyte subsets isolated by means of monoclonal antibodies. *Immunol Rev* 74:57–82, 1983

14. ROSER BJ: Cellular mechanisms in neonatal and adult tolerance. *Immunol Rev* 107:179–202, 1989

15. DIJKSTRA CD, DOPP EA, JOLING P, KRALL G: The heterogeneity of mononuclear phagocytes in lymphoid organs: Distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. *Immunology* 54:589–599, 1985

16. HÜNIG T, WALLY H-J, HARTLEY JK, LAWETZKY A, TIEFENTHALER G: A monoclonal antibody to a constant determinant of the rat T cell antigen receptor that induces T cell activation. Differential reactivity with subsets of immature and mature T lymphocytes. *J Exp Med* 169:73–86, 1989

17. PATERSON DJ, JEFFERIES WA, GREEN JR, BRANDON MR, CORTHESY P, PUKLAVEC M, WILLIAMS AF: Antigens of activated rat T lymphocytes including a molecule of 50,000 Mr detected only on CD4 positive T blasts. *Mol Immunol* 24:1281–1290, 1987

18. TANAKA T, MASUKO T, YAGITA H, TAMURA T, HASHIMOTO Y: Characterization of a CD3-like rat T cell surface antigen recognized by a monoclonal antibody. *J Immunol* 142:2791–2795, 1989

19. OOSTERWIJK E, FLEUREN GJ, JONAS U, ZWARTENDIJK J, TE VELDE J, WARNAAR SO: The expression of renal antigens in renal cell carcinoma. *World J Urol* 2:156–158, 1984

20. ROSER BJ: Cellular mechanism in neonatal and adult tolerance. *Immunol Rev* 107:179–202, 1989

21. McCONAHEY PJ, DIXON FJ: A method of trace iodination of proteins for immunological studies. *Int Arch Allergy* 29:185–189, 1966

22. OUCHTERLONY O: Antigen-antibody reactions in gels. *Acta Pathol Microbiol Scand* 26:507–509, 1949

23. MARDINEY MR JR, MÜLLER-EBERHARD HJ: Mouse β1c-globulin: Production of antiserum and characterization in the complement reaction. *J Immunol* 94:879–882, 1965

24. EDDY AA, McCULLOH LM, ADAMS JA: Intraglomerular leukocyte recruitment during nephrotoxic serum nephritis in rats. *Clin Immunol Immunopathol* 57:441–458, 1990

25. KAFFENBERGER W, GRUBER DF, MACVITTIE TJ: Rat monocytes in a model of combined injury express the OX8 antigen. *J Leuk Biol* 42:181–187, 1987

26. WODA BA, McFADDEN ML, WELSH RM, BAIN KM: Separation and isolation of rat natural killer (NK) cells from T cells with monoclonal antibodies. *J Immunol* 132:2183–2184, 1984

27. WODA BA, BIRON CA: Natural killer cell number and function in the spontaneously diabetic BB/W rat. *J Immunol* 137:1860–1866, 1986

28. BOEKSTEGERS A, GRUNDMANN E: What's new in natural killer cells? *Pathol Res Prac* 180:536–552, 1985

29. HERBERMAN RB: Natural killer cells. *Ann Rev Med* 37:347–352, 1986

30. BLANTZ RC, WILSON CB: Acute effects of antiglomerular basement membrane antibody on the process of glomerular filtration in the rat. *J Clin Invest* 58:899–911, 1976

31. BLANTZ RC, TUCKER BJ, WILSON CB: The acute effects of antiglomerular basement membrane antibody upon glomerular filtration in the rat. The influence of dose and complement depletion. *J Clin Invest* 61:910–921, 1978

32. HOLDSWORTH SR: Fc dependence of macrophage accumulation and subsequent injury in experimental glomerulonephritis. *J Immunol* 130:735–739, 1983

33. MOURA LA, MENDRICK DL, RENNKE HG: Modulation of the monocyte/macrophage system affects the expression of antibody-mediated necrotizing and crescentic glomerulonephritis in the rat. (abstract) *J Am Soc Nephrol* 2:55, 1991

34. DOHERTY DE, DOWNEY GP, WORTHEN GS, HASLETT C, HENSON PM: Monocyte retention and migration in pulmonary inflammation. *Lab Invest* 59:200–213, 1988

35. SCHREINER GF, COTRAN RS, PARDO J, UNANUE ER: A mononuclear cell component in experimental immunological glomerulonephritis. *J Exp Med* 147:369–384, 1978

36. HOLMDAHL R, OLSSON T, MORAN T, KLARESKOG L: In vitro treatment of rats with monoclonal anti-T-cell antibodies: Immunohistochemical and functional analysis in normal rats and in experimental allergic neuritis. *Scand J Immunol* 22:157–169, 1985

37. LIKE AA, BIRON CC, WERINGER EJ, BYMAN K, SROCZYNSKI E, GUBERSKI DL: Prevention of diabetes in biobreeding/Woreester rats with monoclonal antibodies that recognize T lymphocytes or natural killer cells. *J Exp Med* 164:1145–1159, 1986

38. STACHURA I, SI L, MADAN E, WHITESIDE T: Mononuclear cell subsets in human renal disease. Enumeration in tissue sections with monoclonal antibodies. *Clin Immunol Immunopathol* 30:362–373, 1984

39. NOLASCO FE, CAMERON JS, HARTLEY B, COELHO A, HILDRETH G, REUBEN R: Intraglomerular T cells and monocytes in nephritis: Study with monoclonal antibodies. *Kidney Int* 31:1160–1166, 1987

40. LI H-L, HANCOCK WW, DOWLING JP, ATKINS RC: Activated (IL-2R+) intraglomerular mononuclear cells in crescentic glomerulonephritis. *Kidney Int* 39:793–798, 1991