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Mechanisms of T cell-induced glomerular injury in anti-glomeruler basement membrane (GBM) glomerulonephritis in rats

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SUMMARY

The effector mechanisms of T cell-dependent acute glomerular injury were studied in autologous phase anti-GBM glomerulonephritis (GN) in rats. Acute proliferative GN was induced in sensitized rats by a subnephritogenic dose of sheep anti-rat GBM antibody. Injury was manifested by proteinuria and glomerular leucocyte infiltration composed predominantly of macrophages but also $CD4^+$ and $CD8^+$ T cells. T cell depletion, using an anti-CD5 MoAb, demonstrated that glomerular leucocyte infiltration and proteinuria were T cell-dependent. Inhibition of T helper cell function using an anti-CD4 MoAb prevented proteinuria and glomerular macrophage and $CD4^+$ T cell influx, but not accumulation of $CD8^+$ T cells. Depletion of $CD8^+$ T cells also prevented proteinuria and the influx of macrophages and $CD8^+$ T cells, but not accumulation of $CD4^+$ T cells. Macrophage depletion, using micro-encapsulated clodronate, prevented proteinuria and glomerular macrophage infiltration, but not the accumulation of $CD4^+$ or $CD8^+$ T cells, indicating that macrophages are the common cellular effectors for both CD4 and CD8 T cell-dependent injury. Evidence for cytotoxic mechanisms of injury (increased numbers of apoptotic cells or accumulation of natural killer (NK) cells in glomeruli) could not be demonstrated. These data suggest that acute glomerular injury in anti-GBM GN is the result of macrophage recruitment, which is dependent on both CD4 and CD8 T cells, and that direct T cell-mediated injury (cellular cytotoxicity) is not involved.

Keywords T cell CD4 CD8 macrophage glomerulonephritis

INTRODUCTION

T lymphocytes have been demonstrated in severe proliferative forms of experimental [1,2] and human glomerulonephritis (GN) [3–5], but their role in the pathogenesis of glomerular injury remains to be fully defined. T cells play important roles in facilitating humoral immunity by enhancing antibody production and in cellular immunity by directing antigen-specific recruitment and activation of macrophages and mediating cellular cytotoxicity. In the past, helper functions (antibody production and macrophage recruitment and activation) have been associated with $CD4^+$ subsets of T cells, whereas cytotoxic functions were associated with $CD8^+$ T cells. This distinction may not be as clear cut as previously thought, as evidence is now being provided that $CD8^+$ T cells may perform helper roles [6] and macrophages may induce apoptosis [7].

Evidence suggesting that T cells may act locally within glomeruli to direct glomerular injury (separate from any role in humoral immunity) is accumulating from studies in experimental GN. Cell transfer [8–11] and depletion studies [12,13] have demonstrated that sensitized T cells, which localize in glomeruli in response to a planted antigen, can direct macrophage accumulation and initiate development of proteinuria and crescents. Immunization of rats with type IV collagen has been shown to induce an autoimmune crescentic GN in which T cells play a prominent role [14]. T cells sensitized to haptens can induce acute transient glomerular injury in the absence of significant antibody deposition [10,11]. However, the effector mechanisms by which T cells direct injury have not been defined.

The presence of both $CD4^+$ and $CD8^+$ T cells in glomeruli in both human and experimental GN indicates the potential for T cells to induce glomerular injury via macrophage recruitment and activation akin to DTH and/or via direct T cell cytotoxicity. Experimental models allow the potential to address these questions by specific *in vivo* depletion of the individual cellular elements of the potential effector pathways. The current studies addressed the effector mechanisms by which glomerular T cells induce injury in a T cell-dependent model of GN in rats. The effector mechanisms were examined by determining the requirement for T cell subsets and macrophages to induce glomerular injury and assessing the contribution of local cytotoxicity by quantifying

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the presence of apoptotic cells and natural killer (NK) cells in glomeruli.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley (SD) rats weighing between 100 and 150 g were obtained from Central Animal Services (Monash University, Clayton, Victoria, Australia).

Induction of accelerated anti-GBM GN

Anti-GBM globulin was raised in a sheep by repeated immunization with a membrane fraction of rat kidney in Freund's complete adjuvant (FCA; Sigma Chemical Co., St Louis, MO). The sheep serum was heat-decomplemented and absorbed twice against rat erythrocytes (10% by volume). A globulin fraction was prepared by precipitation with ammonium sulphate at a final concentration of 50% and was extensively dialysed against PBS. Rats were presensitized with 4 mg of normal sheep globulin in 0·75 ml FCA by subcutaneous injection into each flank. Five days later, GN was initiated by i.v. injection of sheep anti-rat GBM globulin at a dose of 100 μg/g body weight. At this dose, the antibody bound 30 μg of globulin per gram wet weight of kidney (determined by binding studies using ^{125}I-labelled antibody) and did not produce proteinuria in non-sensitized rats. The minimum quantity of glomerular bound antibody required to reliably induce proteinuria (the nephritogenic threshold dose) was 70 μg/g.

Assessment of proteinuria

Rats were housed individually in cages to collect urine over the 24 h before and after anti-GBM globulin injection. Urinary protein concentrations were determined by the Bradford method adopted for a microtitre plate assay [15]. All samples and standards were assayed in duplicate.

Histologic assessment of renal disease

Renal tissue sections (3 μm thickness) were cut from paraffin-embedded tissue fixed in Bouin's fixative and stained with periodic acid Schiff (PAS) reagent to assess light microscopic appearances. Glomerular hypercellularity was estimated by counting cell nuclei in a minimum of 30 equatorially sectioned glomeruli per animal.

The presence of T cells and macrophages in glomeruli was determined by immunohistochemistry on periodate-lysine-paraformaldehyde (PLP)-fixed kidney tissue sections using specific MoAbs to rat leucocyte antigens and a three-layer horseradish peroxidase (HRP)-conjugated antibody to HRP (PAP) technique. Mouse anti-rat MoAb OX-19 (anti-CD5) [16] was used as a pan T cell marker, R73 (anti-T cell $\alpha\beta$ receptor) [17] as a T cell receptor marker, W3/25 (anti-CD4) [18] as a T helper cell marker, OX8 (anti-CD8) [19] as a T cytotoxic/suppressor marker, 3.2.3 (anti-CD56) [20] as an NK cell marker, RP3 [21] as a neutrophil marker and ED1 as a macrophage marker [22]. Other rat macrophage markers ED2 and ED3 are not expressed in normal rat glomeruli [23] and are infrequently expressed on infiltrating cells in anti-GBM GN [23,24]. Leucocyte populations labelled by MoAbs were counted in a minimum of 20 glomeruli cut in equatorial sections from each rat. The mean glomerular cell count from each group was expressed as cells per glomerular cross section (c/gcs).

Assessment of humoral mediators of glomerular injury

Cryostat cut frozen renal tissue sections were stained for rat IgG and rat complement C3 by direct immunofluorescence with sheep anti-rat IgG antibody-FITC (Silenus, Hawthorn, Australia) and sheep anti-rat C3 antibody-FITC (Bethyl, Montgomery, TX) using doubling dilutions of antibodies starting at titre of 1 : 100 to 1 : 3200. The end-point titre for each animal was determined as the highest dilution at which positive staining of glomeruli was detectable.

Assessment of systemic rat anti-sheep globulin antibody

Titres of rat anti-sheep globulin antibody were determined by ELISA on serum collected at the end of each experiment. Flat-bottomed polyvinylchloride microtitre plates (Dynatech, Chantilly, VA) were coated with 10 μg/ml normal sheep globulin in carbonate/bicarbonate buffer pH 9·5 by incubation for 3 h at 37°C and then blocked with 2% bovine serum albumin (BSA). The plates were washed twice in PBS/0·1% Tween 20 before incubation with serial dilutions of rat serum. After a further three washes in PBS–Tween, plates were incubated with HRP-conjugated rabbit anti-rat IgG (Sigma) at a dilution of 1 : 4000. The plates were finally washed six times with Tween 20 and incubated with 0·1 M 2,2′-azino-di-3-ethylbenzthiazoline sulphonate (ABTS; Boehringer Mannheim, Sydney, Australia) in 0·02% H_2O_2. The substrate reaction was stopped by adding an equal volume of 0·1 M citric acid/0·01% sodium azide and absorbency at 405 nm was read on a microtitre plate reader (Dynatech). Serum from each rat was tested using doubling dilutions, starting with a dilution of 1 : 10. Serum from six non-immunized rats was tested to provide normal controls.

T cell depletion protocols

In vivo T cell depletion was induced by a single i.v. injection of 5 mg of protein G purified mouse MoAb given to sensitized rats 24 h before anti-GBM globulin. Three MoAbs were used: OX-19 (anti-CD5) which marks and depletes all circulating T cells, W3/25 (anti-CD4) which inhibits $CD4^+$ T helper cell activation *in vitro* [25] and prevents immunological injury requiring T helper cells *in vivo* [26–29], and OX-8 (anti-CD8) which marks and depletes cytotoxic/suppressor T cells and NK cells. CD4 may also be expressed on rat resident peritoneal macrophages [30] and some tissue macrophages [31]. This antibody does not inhibit glomerular macrophage accumulation in a macrophage-mediated passive anti-GBM GN model [13].

Rats were bled under ether anaesthesia into EDTA anticoagulent before presensitization and 24 h after receiving anti-T cell antibodies to assess the extent of T cell depletion. The peripheral blood leucocyte populations were analysed by flow cytometry (EPICS 752; Coulter Electronics, Hialeah, FL) after staining with anti-rat T cell MoAbs OX-19, R73, W3/25 and OX-8 using a two-layer immunofluorescent technique. Total leucocytes were detected using MoAbs OX-1 (anti-leucocyte common antigen marker), and an irrelevant isotype-matched MoAb (mouse IgG1) was used as a negative control.

Macrophage depletion with micro-encapsulated clodronate

Macrophage depletion was induced by administration of micro-encapsulated clodronate, using a 'macrophage suicide technique', similar to that previously described [32]. Stable microspheres containing clodronate were produced in a manner similar to the one used to micro-encapsulate macrophage colony-stimulating factor using the modified emulsion procedure [33]. Briefly,

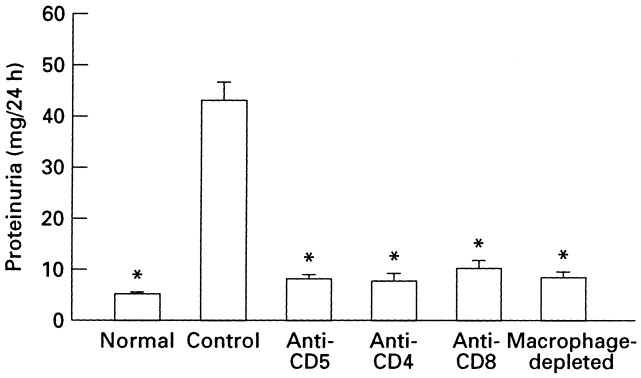

Fig. 1. Effects of T cell and macrophage depletion on proteinuria in anti-GBM glomerulonephritis (GN). * $P < 0{\cdot}001$ compared with isotype control.

clodronate and rabbit albumin (1 : 1 ratio) in aqueous solution were homogenized in olive oil for 10 min. This was followed by sonification for 10 min with a Branson Sonifier set to medium setting (Fisher Scientific, Pittsburgh, PA). Glutaraldehyde was then added to cross-link the albumin. The olive oil was washed off from the microsphere suspension with hexane followed by centrifugation. The microspheres were dried in a vacuum desiccator. Sizing of the microspheres (<1 μm) was achieved by the use of sequential high performance liquid chromatography (HPLC) type nylon filters.

Micro-encapsulated clodronate (5 mg) was reconstituted in 3 ml of saline and administered intravenously 48 h before initiation of anti-GBM GN. Macrophage depletion in tissues and in blood smears was assessed by immunoperoxidase staining with an anti-rat macrophage MoAb ED1 as described above. In a pilot experiment, spleen and liver sections showed >95% elimination of macrophages and Kupffer cells and circulating monocytes were reduced by >90%, 48 h after clodronate administration. This degree of macrophage depletion was maintained for a further 72 h. Circulating lymphocyte numbers and subsets and neutrophil numbers were unaffected by clodronate treatment.

In situ *apoptosis detection*

Kidney tissue was fixed in 4% neutral buffered paraformaldehyde, embedded in paraffin, and 3-μm sections were cut and mounted on Superfrost Plus microscope slides (Biolab Scientific, Melbourne, Australia). Apoptotic cells were identified using an ApopTag In

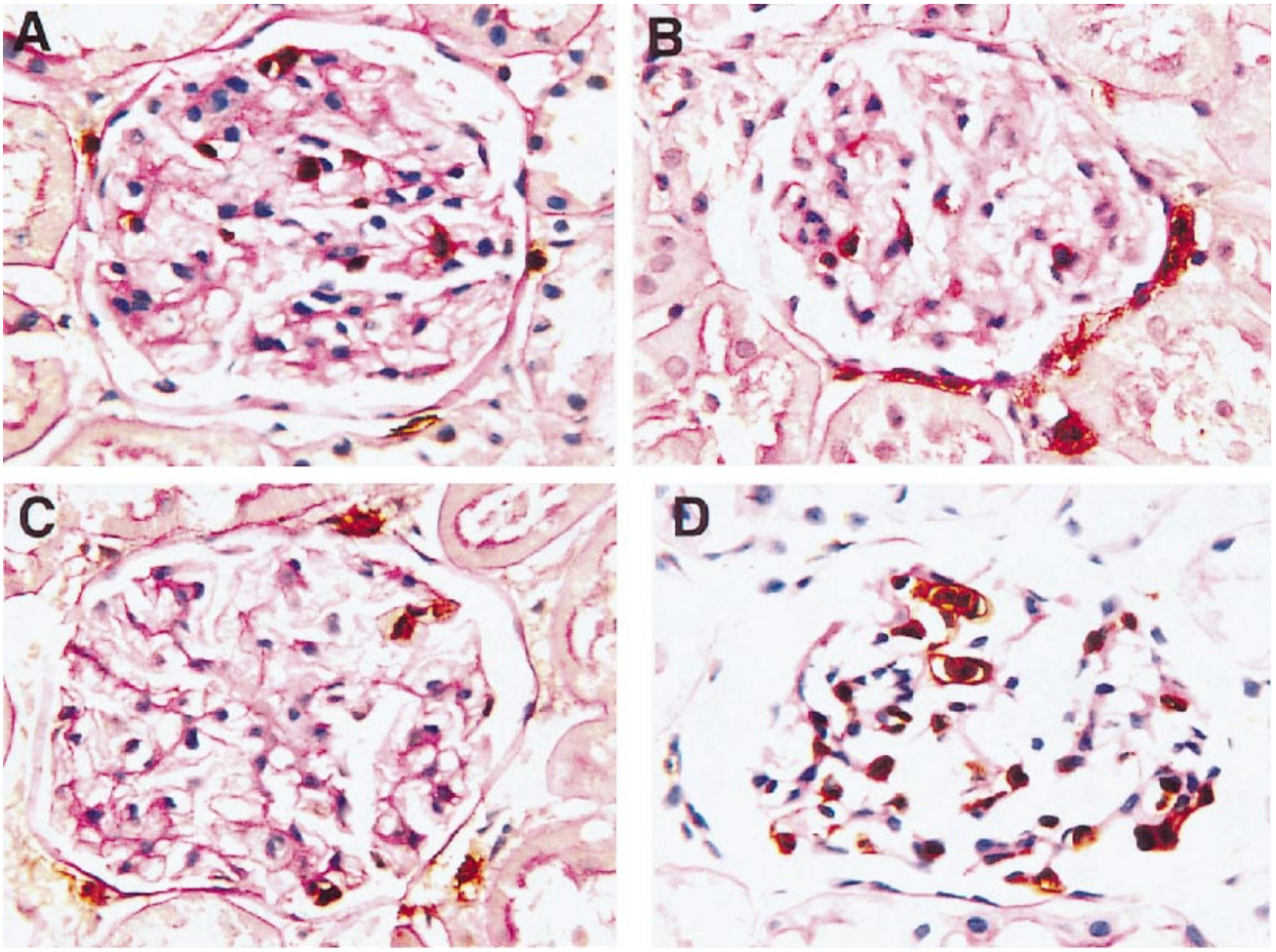

Fig. 2. Photomicrographs of leucocyte accumulation in glomeruli 24 h after initiation of anti-GBM glomerulonephritis (GN) demonstrating the presence of $CD5^+$ cells (A), $CD4^+$ cells (B), $CD8^+$ cells (C) and $ED1^+$ macrophages (D). (Immunoperoxidase with PAS counterstain, mag. ×400.)

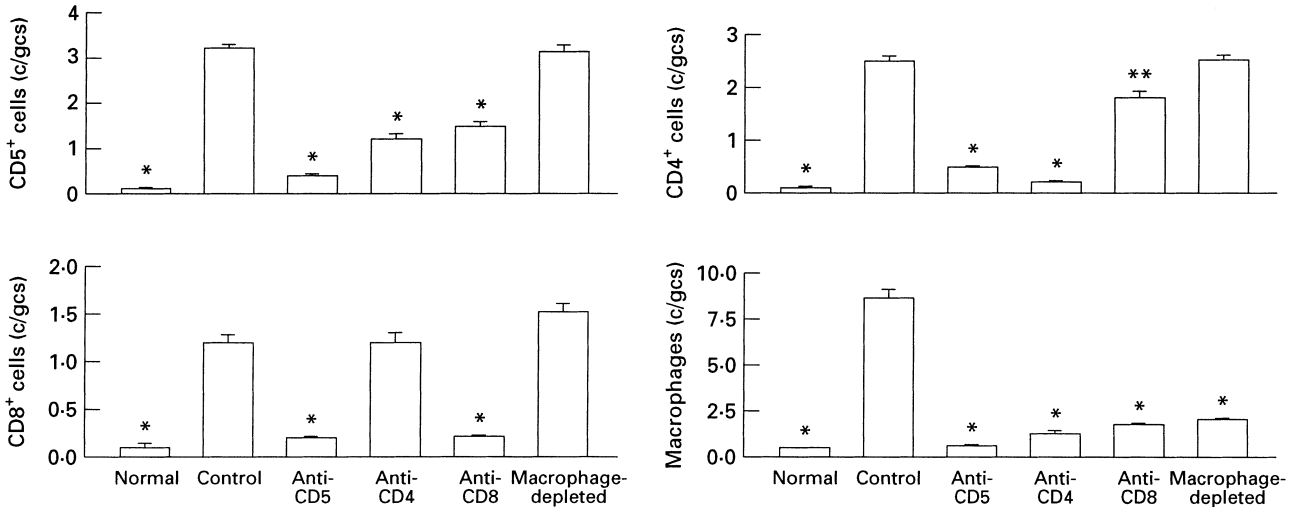

Fig. 3. Effects of T cell, T cell subset and macrophage depletion on glomerular leucocyte accumulation at 24 h after initiation of anti-GBM glomerulonephritis (GN). * $P < 0{\cdot}001$; ** $P < 0{\cdot}05$ compared with isotype-treated control.

Situ Apoptosis Detection Kit (Oncor Inc, Gaithersburg, MD). Sections were deparaffinized and endogenous peroxidase inactivated by incubation with 2% H_2O_2 for 5 min at room temperature. The sections were then incubated with a terminal transferase enzyme/reaction buffer cocktail for 1 h at 37°C. The reaction was terminated by incubation in stop/wash buffer for 30 min at 37°C. The sections were washed in three changes of PBS for 5 min, then incubated with anti-digoxigenin-peroxidase for 30 min at room temperature. Following washes in PBS, sections were incubated with 3,3′-diaminobenzadine (DAB; Sigma) substrate for 3–6 min at room temperature, then counterstained with haematoxylin. In each animal, 30 glomerular cross-sections were examined and TDT-positive (apoptotic) cells were counted (based on positive staining and typical apoptotic morphology). The results were expressed as the number of apoptotic nuclei per equatorial glomerular cross-section (nuc/gcs).

Isolation of apoptotic DNA fragments

Apoptotic DNA fragments were isolated as described by Hermann *et al*. [34] using renal tissue which had been frozen immediately in liquid nitrogen. This tissue was homogenized in lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris–HCl pH 7·5) and centrifuged for 5 min at 1600 ***g***. The supernatant was collected and extraction in lysis buffer was repeated. SDS was then added to a final concentration of 1% and this mixture was incubated with RNase A (5 mg/ml) for 24 h at 37°C followed by digestion with Proteinase K (2·5 mg/ml) for 3 h at 37°C. This was followed by the addition of 1 volume of 10 M ammonium acetate and 5 volumes of absolute ethanol to precipitate DNA. The DNA was dissolved in 10 mM Tris–HCl/0·5 mM EDTA buffer and 10 μg loaded onto a 1·5% agarose gel and subjected to electrophoresis. The gel was finally stained with ethidium bromide to visualize DNA.

Experimental design

T cell depletion was induced by a single i.v. dose of the following anti-T cell antibodies given 24 h before initiation of GN in sensitized rats by anti-GBM globulin: (i) OX19 to deplete $CD5^+$ T cells ($n = 6$); (ii) W3/25 to inhibit CD^+ T cells ($n = 6$; (iii) OX8 to deplete $CD8^+$ T cells ($n = 6$); (iv) an irrelevant isotype-matched (IgG1) mouse MoAb was used as a control ($n = 6$); (v) macrophage depletion was induced by a single i.v. dose of microencapsulated clodronate (5 mg) given 48 h before initiation of GN in sensitized rats by anti-GBM globulin ($n = 6$).

In all groups, GN was induced by administration of anti-GBM globulin to rats sensitized to sheep globulin 5 days earlier. Injury was assessed 24 h after administration of anti-GBM globulin. The results are expressed as the mean ± s.e.m. and the statistical significance of differences between groups was determined by the Mann–Whitney *U*-test.

RESULTS

Anti-GBM GN in control treated rats

Sensitized rats developed an endocapillary proliferative GN with significant proteinuria (43 ± 3·5 mg/24 h, normal 5·3 ± 0·4 mg/24 h, $P < 0{\cdot}001$) (Fig. 1) 24 h after administration of anti-GBM globulin. This injury was associated with significant glomerular accumulation of leucocytes, including T cells, macrophages (Fig. 2a–d) and occasional neutrophils. In non-sensitized rats, the same dose of anti-GBM globulin did not induce a proliferative GN, T cell, macrophage or neutrophil infiltration or proteinuria, indicating that the acute glomerular lesion in sensitized rats is dependent on the active autologous immune response.

Quantification of the glomerular leucocyte infiltrate

T cells and T cell subsets were present in significantly increased numbers ($CD5^+$ cells 3·20 ± 0·10 c/gcs, normal 0·10 ± 0·03 c/gcs; $CD4^+$ 2·50 ± 0·04 c/gcs, normal 0·10 ± 0·03 c/gcs; $CD8^+$ cells 1·20 ± 0·10 c/gcs, normal 0·10 ± 0·01 c/gcs). However, the predominant infiltrating cells were macrophages (8·20 ± 0·30 c/gcs, normal 0·05 ± 0·01 c/gcs, $P < 0{\cdot}001$) (Fig. 3). Neutrophils comprised only a minor component of the glomerular infiltrate (0·50 ± 0·01 c/gcs, normal 0·01 ± 0·001 c/gcs). A significant influx of NK cells was not observed (0·01 ± 0·01 c/gcs, normal 0·01 ± 0·01 c/gcs).

Glomerular deposition of rat immunoglobulin and complement

Intense linear deposition of rat IgG along the GBM was detected at an endpoint titre of 1 : 1600 by immunofluorescence. Rat C3

Table 1. Circulation T cell subsets (% of total $CD45^+$ leucocytes) in normal rats and T cell-depleted and control treated rats with anti-GBM glomerulonephritis (GN)

Group	$CD5^+$ T cells ($OX19^+$)	α/β TRC^+ T cells ($R73^+$)	$CD4^+$ T cells ($W3/25^+$)	$CD8^+$ T cells ($OX\text{-}8^+$)
Normal rats	52·1 ± 1·7	47·1 ± 1·2	40·1 ± 1·2	19·7 ± 1·2
Anti GBM GN				
Anti-CD5 treated	2·9 ± 0·1**	3·1 ± 0·8**	10·2 ± 0·8**	7·8 ± 1·0*
Anti-CD4 treated	26·1 ± 2·3*	23·0 ± 2·4*	21·7 ± 3·4*	15·9 ± 1·2
Anti-CD8 treated	33·1 ± 3·4*	28·5 ± 2·3*	35·9 ± 2·8	3·5 ± 0·4**
Control treated	52·5 ± 0·9	47·2 ± 0·9	41·4 ± 1·1	21·2 ± 1·8

* $P < 0{\cdot}01$; ** $P < 0{\cdot}001$ compared with normal and isotype-treated control animals.

deposition was also evident along the GBM in a finely granular pattern to an end point titre of 1 : 800.

Circulating rat anti-sheep globulin antibody titres

High titres of rat anti-sheep globulin antibody were detected in the serum by ELISA (Fig. 4).

Effects of T cell and T cell subset depletion

Effects on circulating T cell population (Table 1). Administration of anti-CD5 MoAb (OX-19) induced a profound lymphopenia. In rats before treatment 52·1 ± 1·7% of circulating leucocytes ($CD45^+$ cells) expressed CD5 and 47·1 ± 1·2% expressed $\alpha\beta$ TCR. After treatment, CD5 was expressed on only 2·9 ± 0·1% and $\alpha\beta$ TCR on 3·1 ± 0·8% of $CD45^+$ cells and $CD4^+$ and $CD8^+$ subsets were both significantly reduced. Anti-CD4 MoAb (W3/25) treatment induced only a modest reduction in the percentage of $CD45^+$ cells expressing CD5 (26·1 ± 2·3%) and $\alpha\beta$ TCR (23·0 ± 2·4%). Anti-CD8 MoAb treatment reduced $CD8^+$ cells from 19·7 ± 1·2% to 3·5 ± 0·4% of $OX\text{-}1^+$ cells without significantly affecting $CD4^+$ cells. Circulating T cell populations in isotype control mouse IgG1-treated rats were unaffected.

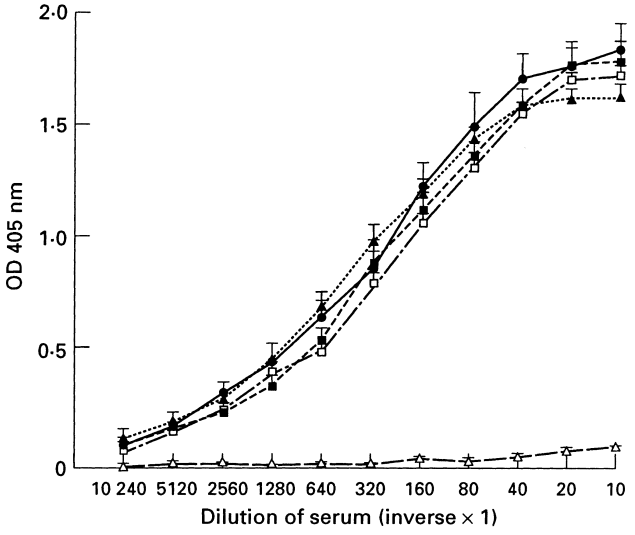

Fig. 4. Serum titres of rat anti-sheep antibody in rats with anti-GBM glomerulonephritis (GN) treated with isotype control (●), anti-CD5 (■), anti-CD4 (▲) and anti-CD8 (□) MoAbs. T cell depletion did not affect the circulating titres of autologous antibody. Titration of normal rat serum was used as a background control (△).

Effects on circulating autologous antibody levels (Fig. 4). T cell depletion using either anti-CD4, anti-CD5 or anti-CD8 had no significant effect on circulating levels of rat anti-sheep globulin antibody, as demonstrated by ELISA.

Effects on glomerular deposition of rat anti-sheep globulin antibody and rat complement C3. T cell and T cell subset depletion did not affect the deposition of autologous rat IgG and C3 deposition in glomeruli, The end point titre for detection of rat IgG (1 : 1600) and C3 (1 : 800) by immunofluorescence in each group of treated rats was the same as in control rats.

Effects on the development of accelerated anti-GBM GN. The administration of anti-CD4, anti-CD5 and anti-CD8 antibody markedly attenuated the histological appearances of GN. Glomerular hypercellularity was significantly less than observed in control treated animals (Fig. 5a–d). In most animals, the glomeruli appeared normal at a light microscopic level. Accumulation of glomerular T cells and T cell subsets and macrophages was significantly reduced by treatment with anti-CD5 MoAb ($CD5^+$ cells 0·40 ± 0·10 c/gcs, $CD4^+$ cells 0·30 ± 0·04 c/gcs, $CD8^+$ cells 0·20 ± 0·04 c/gcs, macrophages 0·6 ± 0·1 c/gcs). Treatment with anti-CD4 MoAb significantly reduced the accumulation of $CD5^+$ cells (1·2 ± 0·1 c/gcs), $CD4^+$ cells (0·1 ± 0·02 c/gcs) and macrophages (1·2 ± 0·2 c/gcs), but had no effect on the influx of $CD8^+$ cells (1·5 ± 0·1 c/gcs). Treatment with anti-CD8 MoAb significantly reduced the influx of $CD5^+$ cells (1·5 ± 0·1 c/gcs, $CD8^+$ cells (0·2 ± 0·02 c/gcs) and macrophages (1·8 ± 0·5 c/gcs), but did not prevent the influx of $CD4^+$ cells (1·8 ± 0·1 c/gcs) (Fig. 3, Table 2). Treatment with either anti-CD5, anti-CD4 or anti-CD8 antibody prevented the development of abnormal proteinuria (anti-CD5 treated, 8·2 ± 1·5 mg/24 h; anti-CD4 treated, 7·8 ± 1·5 mg/24 h; anti-CD8 treated, 10·4 ± 2·4 mg/24 h; all $P < 0{\cdot}001$ compared with control) (Fig. 1).

Assessment of macrophage depletion with micro-encapsulated clodronate

Macrophage depletion using micro-encapsulated clodronate significantly reduced glomerular macrophage influx (2·2 ± 0·1 c/gcs, $P < 0{\cdot}001$) (Fig. 3, Table 2) and proteinuria (8·4 ± 1·2 mg/24 h, $P < 0{\cdot}001$) compared with control antibody-treated rats with GN (Fig. 1). Macrophage depletion had no effect on the glomerular T cell influx ($CD5^+$ cells 3·1 ± 0·1 c/gcs; $CD4^+$ cells 2·5 ± 0·1 c/gcs, $CD8^+$ cells 1·5 ± 0·1 c/gcs) (Fig. 3).

Assessment of glomerular apoptosis (Fig. 6)

Occasional apoptotic cells could be detected in normal glomeruli

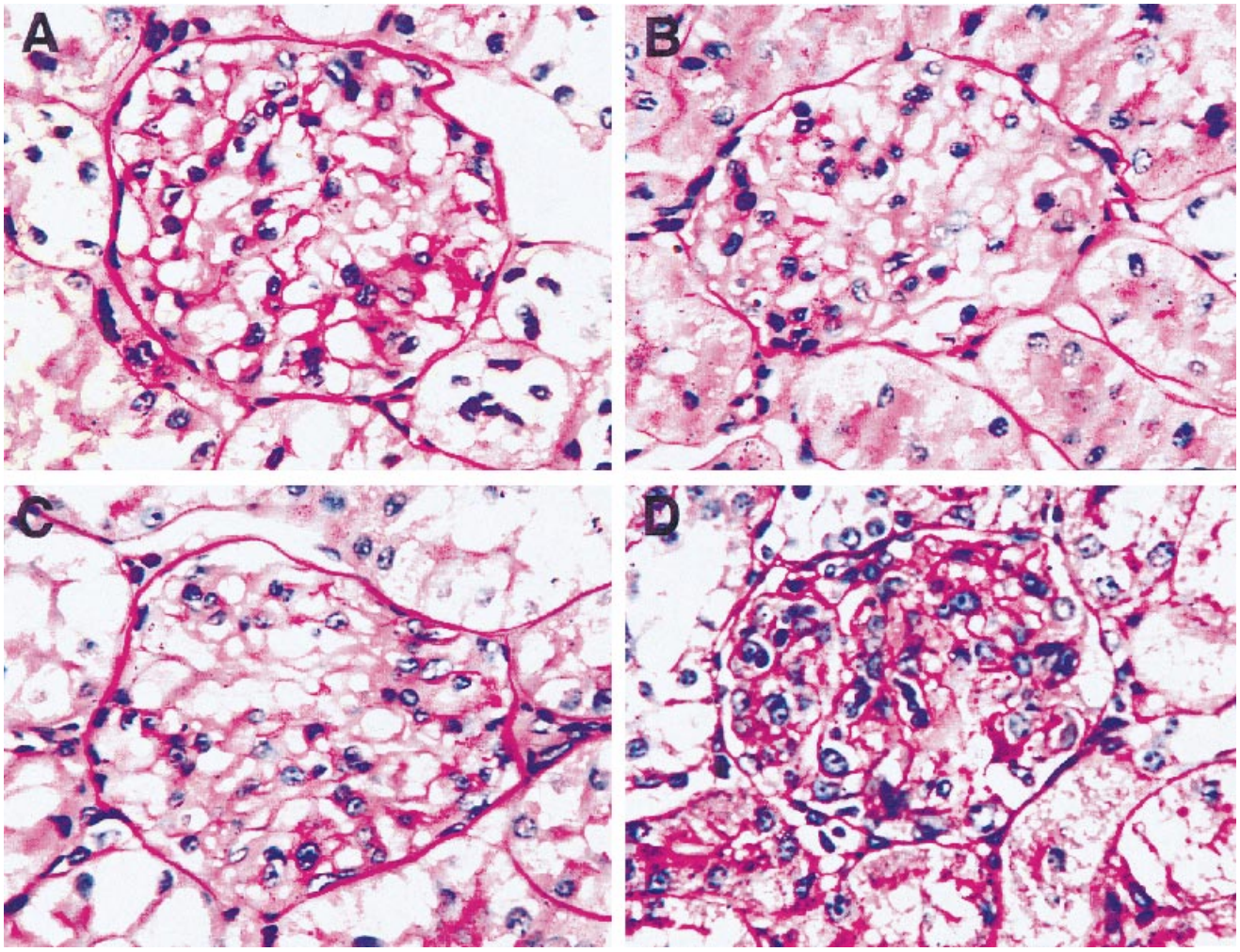

Fig. 5. Photomicrographs of glomeruli from rats with anti-GBM glomerulonephritis (GN) demonstrating the effects of T cell and T cell subset depletion on the histological features of disease following anti-CD5 treatment (A), anti-CD4 treatment (B), anti-CD8 treatment (C) and hypercellularity in isotype control treatment (D). (PAS stain, mag. ×400.)

(0·06 ± 0·01 nuc/gcs). In nephritic glomeruli, no increase in the number of apoptotic nuclei could be detected (0·08 ± 0·01 nuc/gcs). Depletion of $CD8^+$ T cells also did not alter the number of apoptotic cells in glomeruli (0·07 ± 0·01 nuc/gcs). The extent of apoptotic DNA fragmentation assessed by DNA electrophoresis was similar in normal and nephritic glomeruli (Fig. 7).

DISCUSSION

In the current studies, administration of a subnephritogenic dose of anti-GBM globulin to sensitized rats produced acute proteinuria which was associated with a proliferative GN. Macrophages were the predominant inflammatory cell in glomeruli, but infiltration of

Table 2. Effects of T cell, T cell subset and macrophage depletion on glomerular leucocyte accumulation at 24 h after initiation of anti-GBM glomerulonephritis (GN)

Group	$CD5^+$ T cells ($OX19^+$)	$CD4^+$ T cells ($W3/25^+$)	$CD8^+$ T cells ($OX8^+$)	Macrophages ($ED1^+$)
Normal rats	0·10 ± 0·05	0·10 ± 0·05	0·10 ± 0·05	0·50 ± 0·02
Anti-GBM GN				
Anti-CD5 treated	0·40 ± 0·05*	0·50 ± 0·05*	0·20 ± 0·03*	0·60 ± 0·02*
Anti-CD4 treated	1·20 ± 0.10*	0·20 ± 0·05*	1·20 ± 0·10	1·20 ± 0·20*
Anti-CD8 treated	1·50 ± 0·10*	1·80 ± 0·10**	0·20 ± 0·02*	1·80 ± 0·10*
Macrophage-depleted	3·10 ± 0·15	2·50 ± 0·10	1·50 ± 0·10	2·00 ± 0·10*
Control treated	3·20 ± 0·10	2·50 ± 0·10	1·20 ± 0·10	8·60 ± 0·50

Data are expressed as positive cells per glomerular cross-section. * $P < 0·001$; ** $P < 0·05$ compared with isotype-treated control animals.

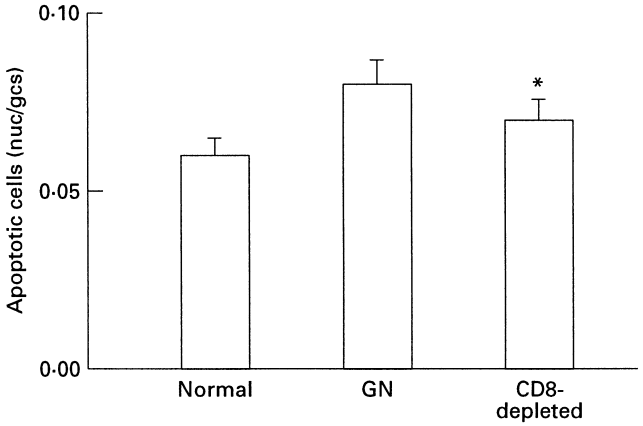

Fig. 6. The number of apoptotic cells (nuc/gcs) in normal, control and CD8-depleted rats with anti-GBM glomerulonephritis (GN) (* $P > 0{\cdot}05$ compared with normal and control rats).

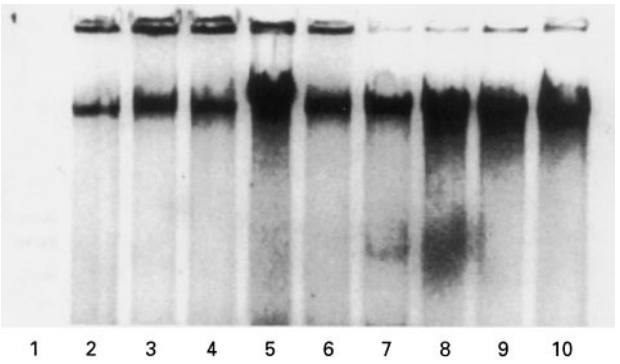

Fig. 7. Agarose gel electropheresis of DNA from normal rats (lanes 2–4) and rats with anti-GBM glomerulonephritis (GN) treated with isotype control MoAb (lanes 5–7) and anti-CD8 (lanes 8–10) demonstrating the absence of 'laddering' seen in apoptotic cells. (Lane 1 contains molecular weight markers.)

$CD4^+$ and $CD8^+$ T cells was also observed. This glomerular lesion was dependent on an active immune response, as the same dose of anti-GBM globulin did not produce histological evidence of injury or proteinuria in non-sensitized rats. Injury in this model is thus initiated by an immune response to sheep globulin which acts as a planted glomerular antigen and results in a mononuclear inflammatory cell influx. Its immunopathogenesis may be considered similar to many forms of human GN which are initiated by exogenous antigens planted in glomeruli.

Treatment with an anti-CD5 MoAb which depletes both helper and cytotoxic/suppressor T cell subsets, demonstrated that this glomerular lesion was T cell-dependent in the effector phase. Because rats were sensitized to the disease-initiating antigen 4 days before administration of anti-T cell antibodies, the depletion protocol did not affect the primary immune response to the antigen, as indicated by the levels of circulating anti-sheep globulin antibodies and the extent of autologous antibody deposition in glomeruli. This treatment prevented accumulation of $CD4^+$ and $CD8^+$ T cells, macrophages and proteinuria, demonstrating that acute injury in this model is due to T cells, not to autologous antibody deposition.

The potential for T cells to induce injury in planted antigen models has been previously demonstrated [10,11]. Previous studies examining more delayed manifestations of injury (including crescent formation) in the same rat model of anti-GBM GN, demonstrated marked attenuation but not complete abrogation of injury after T cell depletion [13]. At later time points in the disease, the levels of circulating autologous antibody were much greater and glomerular levels would also be likely to be higher, making a functional role for antibody-induced injury more likely. This may provide an explanation for the differences in the degree of T cell dependence in early and later phases of this model. Alternatively, the less complete response to T cell depletion in the later phase of disease may be due to the technical difficulty of maintaining complete T cell depletion over that extended period.

Although many studies have demonstrated the potential for T cells to induce both acute and more delayed glomerular injury, the effector mechanisms have not been defined. There are two potential mechanisms of T cell-induced injury: direct cellular cytotoxicity usually induced by $CD8^+$ T cells (or NK cells), and macrophage-mediated injury dependent upon T helper cell functions. To assess the participation of these two mechanisms in this model of GN, the presence of cytotoxicity in glomeruli and the requirement for macrophages were explored. Cytotoxicity was assessed by comparing the presence of apoptotic cells in normal and nephritic glomeruli, and no significant increase in apoptosis was observed. This is consistent with the observation that infiltration of NK cells could not be detected in glomeruli. Although $CD8^+$ T cells were present and their depletion abrogated injury, this effect was not associated with any change in apoptosis in glomeruli, indicating that these cells are not subserving a cytotoxic role in this disease.

Thus early injury is likely to reflect inflammatory cell infiltration and activation rather than cytotoxicity. This function is traditionally attributed to $CD4^+$ T helper cells which recruit and activate macrophages by T helper cells via DTH mechanisms. Interferon-gamma (IFN-γ) is a pivotal mediator of these responses, and recent studies have demonstrated amelioration of glomerular injury by blocking IFN-γ in Th1-dependent anti-GBM model of GN in mice [35]. Functional inhibition of the T helper cell subset using an anti-CD4 antibody confirmed that this mechanism plays a pivotal role in the acute glomerular injury observed in this rat model. The anti-CD4 antibody (W3/25) has previously been demonstrated to effectively inhibit the function of $CD4^+$ T cells without causing profound depletion [13]. Treatment with W3/25 prevented proteinuria and the glomerular accumulation of $CD4^+$ T cells and macrophages, without altering the influx of $CD8^+$ T cells. Although CD4 is expressed in low amounts on a subpopulation of macrophages, it is unlikely that direct depletion of macrophages contributed to its efficacy, as previous studies show that it does not affect a passive model of GN in which injury is macrophage-dependent and T cell-independent [13].

Administration of the anti-CD8 antibody resulted in complete abrogation of $CD8^+$ T cell infiltration in diseased glomeruli, without affecting the number of $CD4^+$ T cells. CD8 depletion also prevented macrophage influx and proteinuria, suggesting a pivotal role for CD8 cells as well as CD4 cells in inducing injury through macrophage recruitment in this model. A similar role for CD8 T cells has also been demonstrated in other disease models of autoimmunity, including experimental allergic encephalomyelitis (EAE) [36], in diabetic models in non-obese diabetic (NOD) mice [37–39] and in experimental models of mercuric chloride inducing nephritis in rats [40,41].

To confirm that macrophages are the ultimate cellular effector of glomerular injury in this model, macrophage depletion was

induced using micro-encapsulated clodronate. The capacity of this diphosphonate drug incorporated in liposomes to deplete resident macrophages and monocytes in rats is well established [42,43]. Depletion of macrophages by this method has been used *in vivo* to determine the role of macrophages in EAE [44] and arthritis [45]. In the current studies, micro-encapsulated clodronate was created using a new technique which allowed efficient incorporation of the drug into more stable microspheres which could be conveniently stored ready for use for several months. Macrophage depletion in tissues was assessed by staining for the marker ED1. Although other markers for tissue macrophages in rats are available (ED2 and ED3), they are not prominently expressed by macrophages in normal or nephritic glomeruli [23,24]. This macrophage depletion technique abrogated glomerular macrophage influx and proteinuria without altering the influx of both CD4- and CD8-bearing T lymphocytes, indicating that macrophages are cellular effectors of injury.

Taken together with the results of CD4 and CD8 T cell depletion, these studies indicate that macrophages are essential for the development of injury in this model of GN. The presence of both CD4 and CD8 cells is necessary for glomerular recruitment of macrophages, suggesting a cooperative T helper cell role of these T cell subsets in macrophage recruitment and acute glomerular injury. These data, together with the absence of any evidence for direct T cell- or macrophage-mediated cytotoxicity, strongly suggest that the development of acute glomerular injury in this planted antigen model of GN is a manifestation of T cell-dependent DTH in the glomerulus.

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