

Permanent CD8⁺ T Cell Depletion Prevents Proteinuria in Active Heymann Nephritis

By Mark J. Penny, Rochelle A. Boyd, and Bruce M. Hall

From the Department of Medicine, Liverpool Hospital and the University of New South Wales, Liverpool, New South Wales 2170, Australia

Summary

Active Heymann nephritis (HN) is a rat model of human idiopathic membranous nephropathy in which injury is thought to be mediated by membrane attack complex of complement (MAC) activated by antibody (Ab) to glomerular epithelial cells. Recent work has shown that HN develops in C6-deficient rats which cannot assemble MAC, and that infiltration of activated cytotoxic CD8⁺ T cells and macrophages into glomeruli coincides with proteinuria. This study examined the role of CD8⁺ T cells in mediating glomerular injury in HN by permanent CD8⁺ cytotoxic T cell depletion via adult thymectomy (ATx) and anti-CD8 mAb. Groups of rats were depleted of CD8⁺ T cells either before immunization for HN or 6 wk after immunization when Ab responses and glomerular IgG deposition were well established. These were compared with groups of HN, ATx/HN, and complete Freund's adjuvant (CFA) controls. Neither group of CD8⁺ T cell-depleted rats developed proteinuria, although there was normal development and deposition of Ab. CD8⁺ T cell-depleted rats developed neither T cell or macrophage infiltrates nor their effector cytokines, which are present in glomeruli of rats with HN. Examination of lymph node (LN) draining sites of immunization showed these findings were not explained by altered immune events within these LNs. It was concluded that CD8⁺ cytotoxic T cells are essential to the mediation of glomerular injury in HN and may be relevant to the pathogenesis and treatment of membranous nephropathy.

Key words: Heymann nephritis • glomerulonephritis • rat • CD8⁺ T lymphocyte • lymphocyte depletion

It has been assumed that most forms of glomerulonephritis (GN)¹ are mediated by Ab and complement because they are readily detected in glomeruli. There is increasing evidence that effector T cells mediate injury in proliferative forms of GN (1, 2). In membranous nephropathy, one of the commonest forms of GN in humans, glomerular injury

is thought to be mediated solely by Ab-mediated activation of the membrane attack complex (MAC) of complement (3). Active Heymann nephritis (HN) is a rat model of membranous GN (4) induced in susceptible strains of rats by immunization with a renal tubular antigen preparation (Fx1A) in CFA (5) that results in an anti-Fx1A Ab response. This Ab forms glomerular subepithelial immune deposits that activate MAC (6–8). The recent discovery that C6-deficient PVG rats develop active HN and proteinuria, despite being unable to assemble MAC, casts doubt on the role of MAC as the mediator of injury in this model (9). This study examined whether effector T cells mediate injury to the glomerulus in HN.

Early studies on HN demonstrated classic delayed-type responses to Fx1A (10), lymphocyte cytotoxicity against renal cells (11, 12), and that sensitized lymphocytes can passively transfer disease to tolerant hosts (13). An interstitial mononuclear cell infiltrate of CD4⁺ and CD8⁺ T cells has been described in HN (14, 15), as have glomerular infiltrates of T cells and macrophages (16–18). CD8⁺ T cells infiltrate glomeruli immediately before the onset of proteinuria (18) and temporary depletion of CD8⁺ T cells by

¹Abbreviations used in this paper: ANOVA, analysis of variance; ATx, adult thymectomy; GEC, glomerular epithelial cells; GN, glomerulonephritis; HN, Heymann nephritis; iNOS, inducible nitric oxide synthase; IP, immunoperoxidase; MAC, membrane attack complex (of complement); RT-PCR, reverse transcription PCR.

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anti-CD8 mAb therapy from the time of immunization delays the onset of proteinuria (17). In this study, we examined what role CD8⁺ T cells may play in the pathogenesis of HN by permanent depletion using a combination of anti-CD8 mAb therapy and adult thymectomy (ATx) (19). CD8⁺ T cell-depleted rats did not develop proteinuria, even though they made a normal Ab response to the immunizing antigen and deposited IgG in their glomeruli. This suggests an important effector role for cytotoxic CD8⁺ T cells in HN. This was further supported by the finding of Th1, cytotoxic T cell, and macrophage cytokine mRNA in glomeruli from HN but not in CD8⁺ T cell-depleted glomeruli. CD8⁺ T cell depletion did not alter the production of serum IgG Ab isotypes, or the induction of Th1 (IFN- γ , TNF- β) and Th2 (IL-4) cytokine mRNA in popliteal LN draining sites of immunization when compared with HN rats. This confirmed there was no alteration of either Th1 or Th2 CD4⁺ cell activation in LNs. Taken together, these findings support a role for the CD8⁺ effector T cell as the principal mediator of glomerular injury in HN.

Materials and Methods

Rats. Inbred male Lewis rats (Lew/ssn) were purchased from the Animal Resource Centre, Perth, W.A., Australia. Sprague Dawley rats used to produce Fx1A were acquired from the Animal Breeding & Holding Unit, University of New South Wales, Sydney, Australia. Water and standard chow were available ad libitum. All procedures were performed under general anesthesia, with postoperative buprenorphine analgesia. Animal work was approved by the Animal Care and Ethics Committee, University of New South Wales.

mAbs. MRC OX-8, a mouse anti-rat IgG1, recognizes CD8⁺ T cytotoxic and NK cells (20), and was produced as tissue culture supernatant from the MRC-OX-8 clone (a gift from Dr. Alan Williams, Sir William Dunn School of Pathology, Oxford, UK). Control mAb was A6, a mouse anti-human CD45R0 IgG1 that does not react with rat tissue (21). mAb supernatant had 0.5–1 mg of Ig on assayed immunodiffusion (21) and had <0.6 U/ml of endotoxin as assayed by a Limulus amoebocyte lysate assay (Coatest Gel-LAL, Chromogenix, MoIndal, Sweden).

FITC- and PE-conjugated mouse anti-rat mAbs (PharMingen, San Diego, CA) used for FACS[®] (Becton Dickinson, San Jose, CA) analysis included: G4.18, which recognizes CD3⁺ T cells (22); MRC OX-35, which recognizes CD4⁺ T cells and some macrophages (23); MRC OX-8, which recognizes CD8⁺ T cytotoxic and NK cells (20); and MRC OX-33 (24), which recognizes a form of CD45 found only on B cells. The mAbs used for immunoperoxidase (IP) staining were: R73 to α/β -TCR receptors on T cells (25); W3/25, which recognizes CD4⁺ T cells and some macrophages (26); MRC OX-8; 3.2.3, which recognizes NK cells (27); ED-1, which recognizes most macrophages and some dendritic cells (28); and MRC OX-12, which recognizes κ light chains and was used to identify glomerular IgG deposition (29). Goat anti-rat C3 Ab was used to identify glomerular C3 deposition on IP.

Antigen and Immunization. Fx1A was prepared from outbred Sprague-Dawley rats as previously described (5, 30). HN (CFA/Fx1A) and control (CFA alone) groups were immunized as previously described (18).

Experimental Protocol. Groups of five male Lewis rats immunized with Fx1A/CFA were depleted of CD8⁺ T cells either before immunization or 6 wk after immunization, and were compared with untreated HN and control groups. All rats were immunized from the same batch of Fx1A/CFA.

CD8⁺ T Lymphocyte Depletion. CD8⁺ T cells were permanently depleted by combined ATx and MRC OX-8 as described (19). ATx was carried out 2 wk before immunization. MRC OX-8 was administered as 1.0 ml of tissue culture supernatant intraperitoneally daily for 10 d, beginning on the day of ATx for the early CD8⁺ T cell depletion group and 6 wk after immunization for the late CD8⁺ T cell depletion group.

Assessment of Disease Activity. 24-h urine protein excretion was measured by colorimetric assay (Bio-Rad, Hercules, CA). Anti-Fx1A Ab titers were determined by ELISA as previously described (18), using alkaline phosphatase-conjugated Abs: sheep anti-rat Ig Fab fragments (Boehringer Mannheim, GmbH, Mannheim, Germany) and mouse anti-rat IgG1, IgG2a, and IgG2b (PharMingen, San Diego, CA). Glomerular Ig deposition was demonstrated by IP of renal cortex using MRC OX-12. Glomerular C3 deposition was demonstrated by IP of renal cortex using anti-C3 Ab.

FACS[®] Analysis. Lymphocyte subset analysis of PBLs and LNs was performed by FACS[®] analysis (FACSscan[®]; Becton Dickinson). Single cell suspensions of lymphocytes were prepared as previously described (31), and directly stained with FITC- and PE-conjugated mouse anti-rat mAb to cell surface antigens (see above). Full blood counts were performed on an automated counter (Coulter Corp., Miami, FL).

Isolation of Intact Glomeruli. Glomeruli for PCR and IP were isolated by graded sieving to >95% purity as previously described (18, 32).

IP Cytochemistry. IP staining of renal cortex cross-sections and isolated glomeruli for all Abs except anti-C3 was performed as previously described (18), using an indirect peroxidase-antiperoxidase technique: step one, mouse anti-rat mAb (described above); step two, rabbit anti-mouse Ig (Dako, Copenhagen, Denmark); step three, peroxidase-monoclonal mouse antiperoxidase (Dako). C3 staining of renal cortex cross-sections used a single-step technique with goat anti-rat C3 Ab peroxidase immunconjugate (Nordic Immunology, Tilberg, The Netherlands).

Semiquantitative Reverse Transcription PCR. Reverse transcription (RT)-PCR for mononuclear cell cytokine mRNA in renal cortex, isolated glomeruli, and popliteal lymph nodes was performed as previously described (18). Semiquantitation of RT-PCR product involved cycle titration at five-cycle intervals (33). Primer and probe oligonucleotide sequences for IL-2, IL-4, IL-5, IL-6, IL-10, granzyme A, IFN- γ , perforin/cytolysin, TNF- α , lymphotoxin/TNF- β , and GAPDH have been previously described (18). Other primers and probes included IL-12 (34), granzyme B (35), and inducible nitric oxide synthase (iNOS; reference 36). GAPDH was used as a positive control gene for intact RNA and efficiency of RT.

Data Analysis. Urine protein estimations, serum anti-Fx1A Ab levels, leukocyte counts, popliteal LN weight, and lymphocyte FACS[®] analysis for each group were expressed as mean \pm SEM. Comparisons between groups were made by analysis of variance (ANOVA), and when significant were examined by Bonferroni-Dunn multiple comparisons post hoc test. Semiquantitative RT-PCR results were treated as parametric data with comparisons between groups made by ANOVA and a Bonferroni-Dunn post hoc test, and expressed graphically as the median PCR cycle number at which PCR product was first identified.

Table 1. FACS[®] Analysis of PBLs Comparing CD8⁺ T Cell-depleted with CD8⁺ T Cell-replete HN Groups

Group	CD8 ⁺ CD3 ⁺ cells/CD3 ⁺ cells (%)			n
	0 wk*	6 wk [†]	12 wk	
CFA control	23.15 ± 2.20	25.68 ± 1.38	24.63 ± 0.98	3
Normal HN	25.53 ± 2.15	24.43 ± 1.91	25.35 ± 2.05	3
ATx/HN	24.97 ± 0.84	22.14 ± 0.76	21.78 ± 0.77	5
Early CD8 depletion/HN	0.47 ± 0.15 [§]	0.40 ± 0.08 [§]	1.01 ± 0.50 [§]	5
Late CD8 depletion/HN	22.57 ± 0.79	20.17 ± 2.07	1.04 ± 0.40 [§]	5

Prolonged CD8⁺ cell depletion was achieved with ATx followed by anti-CD8 mAb therapy (MRC OX-8). Values represent mean ± SEM, percentage total CD3⁺ cells (G4.18⁺) that are CD8⁺CD3⁺ (MRC OX-8+G4.18⁺).

*Time of immunization.

[†]Before CD8⁺ T cell depletion in group 5.

[§]*P* < 0.0001 for comparisons with all CD8⁺ T cell replete groups. ANOVA followed by Bonferroni-Dunn post hoc multiple comparisons test.

This is intentionally conservative analysis of PCR data, as differences in product expression are exponential. *P* ≤ 0.05 was considered significant, unless reduced by Bonferroni-Dunn corrections.

Results

CD8⁺ T Cell Depletion Prevents HN. Lewis rats were depleted of CD8⁺ T cells by combination ATx and anti-CD8 mAb therapy (MRC OX-8), either before (early CD8⁺ T cell depletion group) or 6 wk after (late CD8⁺ T cell depletion group) immunization with Fx1A in CFA. The CD8⁺ T cell-depleted rats were compared with those immunized with Fx1A in CFA (normal HN group), ATx rats immunized with Fx1A in CFA (ATx/HN group), and rats immunized only with CFA (CFA control group). Marked depletion of CD8⁺CD3⁺ PBLs to 0.5–1.0% of CD3⁺ cells was achieved until the completion of the experiment at 12 wk after immunization (Table 1). There was no difference in CD8⁺CD3⁺ PBLs between CFA control, normal HN, and ATx/HN groups (21.8–25.5% of CD3⁺ cells) throughout the course of the experiment. Full blood counts were not different between groups, with white cell counts of 8.4 ± 2.3 × 10⁹/liter in normal HN vs. 6.6 ± 3.1 × 10⁹/liter in CD8⁺ T cell-depleted rats (*P* = NS), and total lymphocyte counts of 1.8 ± 0.3 × 10⁹/liter vs. 1.6 ± 0.4 × 10⁹/liter, respectively (*P* = NS).

In both normal HN and ATx/HN, proteinuria developed by 8 wk and had increased further at 12 wk. In the early and late CD8⁺ T cell-depleted groups, urinary protein remained within the normal range observed in CFA controls (Fig. 1 *a*). There was no evidence of glomerular barrier dysfunction in CD8⁺ T cell-depleted groups. Anti-Fx1A Ab responses were not different between CD8⁺ T cell-depleted, normal HN, and ATx/HN groups (Fig. 1 *b*). CFA controls did not produce an Ab response. In keeping with the Fx1A Ab responses, glomerular Ig deposition was not different between early or late CD8⁺ T cell-depleted and normal HN groups, with none detected in CFA con-

trols (Fig. 1 *c* and see Fig. 4). Glomerular C3 deposition was also not different between early CD8⁺ T cell-depleted and normal HN groups, and was absent in CFA controls (Fig. 1 *c* and see Fig. 4).

These experiments were repeated on four occasions with a minimum of five rats in each treatment group and CD8⁺ T cell-depleted rats never developed proteinuria. At 8–10 wk proteinuria was 7.8 ± 0.8 mg/100 g body wt/day (mean ± SEM) in early CD8⁺ T cell-depleted hosts (*n* = 41) compared with 73.5 ± 14.2 in HN controls (*n* = 18, *P* < 0.0001). The protein measurements in urine were not different from those in CFA immunized controls, which was 3.6 ± 0.3 mg/100 g body wt/day (*n* = 24, *P* = NS). In one of these experiments, an additional control group was treated with A6, an mAb that has the same isotype (IgG1) as MRC OX-8 but no reactivity with rat cells. This mAb had no effect on development of antibodies in sera or glomeruli, nor on proteinuria. At 8 wk, A6-treated rats had 55.5 ± 24.5 mg/100 g body wt/day (mean ± SEM) proteinuria vs. normal HN of 63.5 ± 29.5 mg/100 g/day.

Ab isotype switching is controlled by cytokines produced by T cells. To examine if ATx or CD8⁺ T cell depletion affected such switching, anti-Fx1A IgG Ab isotypes were examined at 4 and 8 wk. Similar levels of IgG1, IgG2a, and IgG2b were found in early and late CD8⁺ T cell-depleted, ATx/HN, and normal HN groups (Fig. 2). In all these groups, at 4 wk only Th2 induced noncomplement-fixing IgG1 was significantly greater than CFA controls. With Th1-induced IgG2a and IgG2b complement-fixing isotypes, the increase was not significant. By 8 wk, the IgG1 response had further increased, and a significant IgG2a but not IgG2b, response had developed. These findings demonstrated that CD8⁺ T cell depletion did not affect IgG isotype switching.

Characterization of Mononuclear Cell Infiltrates in Glomeruli and Interstitium. At 12 wk a glomerular infiltrate of T cells (αβ-TCR⁺, CD4⁺, and CD8⁺) and macrophages, but not NK cells, was identified in normal HN but not in early CD8⁺ T cell-depleted and CFA control groups (Figs. 3 *a*

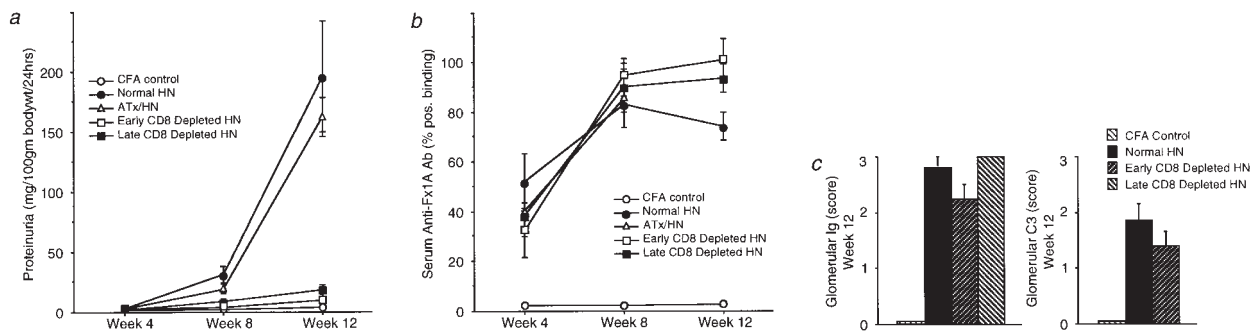


Figure 1. CD8⁺ T cell depletion in HN prevented proteinuria in the presence of elevated serum anti-Fx1A Ab levels and glomerular IgG deposition. (a) Proteinuria in early or late CD8-depleted HN was not significantly greater than that in CFA controls. In normal HN and ATx/HN, proteinuria was detected from 8 wk, and was not different between these groups at 8 and 12 wk. Proteinuria was significantly greater in normal HN and ATx/HN than in early or late CD8-depleted HN, or CFA controls, at 8 ($P < 0.0036$) and 12 wk ($P < 0.0002$), for all comparisons. Values represent the mean \pm SEM ($n = 5$) of urine protein excretion, expressed as mg/100 g body wt/day, measured by colorimetric assay. (b) Serum anti-Fx1A Ab responses were not different between HN, ATx/HN, or early and late CD8-depleted HN at all times. CFA controls did not develop an anti-Fx1A Ab response, with $P < 0.0006$ for comparisons at all times with the other groups. Values represent the mean \pm SEM ($n = 5$) of total anti-Fx1A Ab levels, expressed as percentage binding of known strongly positive serum (Fx1A Ab titer 1:250), measured by ELISA. (c) Glomerular Ig and C3 deposition at 12 wk were not significantly different between normal HN and early or late CD8-depleted HN groups (C3 staining for late CD8-depleted HN not tested). Values represent the mean \pm SEM ($n = 5$) of glomerular IgG (MRC OX-12) or C3 deposition on IP staining, scored from 0 (absent) to 3 (intense). Statistical analysis by ANOVA followed by Bonferroni-Dunn post hoc multiple comparisons test.

and 4). These findings show that depletion of CD8⁺ T cells prevented the accumulation of all mononuclear cell subsets into glomeruli, not just of the CD8⁺ T cell subset. The number of CD8⁺ T cells and NK cells in glomeruli from the CD8⁺ T cell-depleted group was similar to that in CFA controls that do not develop disease. These CD8⁺ T cells were probably CD8⁺CD3⁻ NK cells present in peripheral blood and lymphoid tissue (data not shown). MRC OX-8 mAb therapy initially depletes both CD8⁺ T cells and NK cells, but NK cells rapidly regenerate peripherally, unlike CD8⁺ T cells which require the thymus.

The renal cortical infiltrate identified at 12 wk in normal HN, comprising both T cells (α/β -TCR⁺, CD4⁺, and CD8⁺) and macrophages, but not NK cells, was absent in early and late CD8⁺ T cell-depleted, and CFA control groups (Fig. 3 b). There was a small but significant increase in α/β -TCR⁺ cells and macrophages in the late CD8⁺ T cell-depleted group compared with CFA controls; however, the increase in α/β -TCR⁺ cells was not reflected by an increase in CD4⁺ or CD8⁺ T cells. Only small numbers

of CD8⁺ T cells, less than in CFA controls, were detected in the CD8⁺ T cell-depleted groups.

Analysis of Mononuclear Cell Cytokine mRNA in Glomeruli. The functional potential of the cellular infiltrate in glomeruli, associated with proteinuria in normal HN, was characterized by RT-PCR of mRNA for cytokines (Fig. 5). mRNA for cytokines and effector molecules of Th1 cells (IFN- γ , lymphotoxin, and IL-2), cytotoxic cells (granzyme A and perforin, but not granzyme B), and macrophages (TNF- α , IL-10, IL-12, and iNOS) was increased in normal HN compared with the CD8⁺ T cell-depleted HN groups and CFA controls. Both early and late CD8⁺ T cell-depleted groups had cytokine and effector molecule mRNA levels at or below that observed in CFA controls. This is not surprising as mononuclear cells were not identified within the glomeruli of these groups. There was no difference between groups in the expression of the Th2 cell cytokine IL-4. Other Th2 cytokines such as IL-5 and IL-6 were not studied as their mRNA is not readily detected in isolated glomeruli from normal HN or CFA control rats (18). The persis-

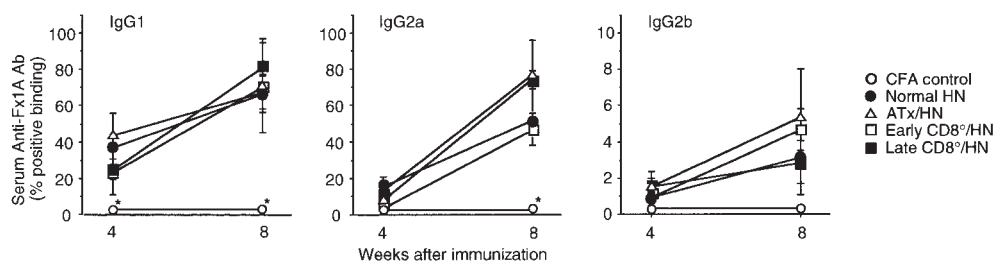


Figure 2. Serum anti-Fx1A Ab IgG isotype switching and subclass production in HN were not altered by CD8⁺ T cell depletion. IgG subclasses were detected in similar proportions at 4 and 8 wk in CD8-depleted HN, ATx/HN, and normal HN groups. CFA controls did not mount an Fx1A Ab response. At 4 wk, only the Th2-mediated IgG1 response was significantly

raised above controls. By 8 wk the IgG1 response had increased further, and a significant Th1 mediated IgG2a response was identified compared with CFA controls. There was no significant-IgG2b response. Values represent mean \pm SEM ($n = 5$) of anti-Fx1A Ab levels, expressed as percentage binding of known strongly positive serum (Fx1A Ab titer 1:250), measured by ELISA. * $P < 0.005$ for comparisons between CFA controls and all other groups. ANOVA followed by Bonferroni-Dunn post hoc multiple comparisons test.

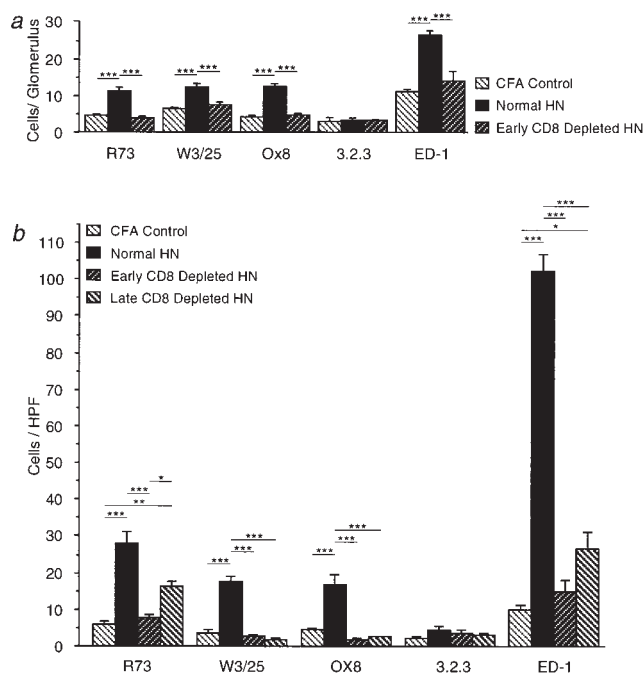


Figure 3. The mononuclear cell infiltrate seen in the glomeruli and renal cortex in normal HN at 12 wk was prevented by early CD8⁺ T cell depletion and significantly attenuated by late CD8⁺ T cell depletion. (a) A glomerular infiltrate of α/β -TCR⁺ (R73), CD4⁺ (W3/25), and CD8⁺ (MRC OX-8) cells, and macrophages (ED-1), but not NK cells (3.2.3), was present in normal HN at 12 wk. This infiltrate was not present in early or late CD8-depleted HN. Values represent the mean \pm SEM ($n = 5$) of cells per glomerulus (10 glomeruli per rat). (b) A renal cortical infiltrate of α/β -TCR⁺, CD4⁺, and CD8⁺ cells, and macrophages, but not NK cells, was present in untreated HN at 12 wk. This infiltrate was absent in early CD8-depleted HN. In late CD8-depleted HN there was a small infiltrate staining only for R73 and ED-1. Values represent the mean \pm SEM ($n = 5$) of cells per HPF (10 HPF per rat). * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$. ANOVA followed by Bonferroni-Dunn post hoc multiple comparisons test.

tent detection of background perforin mRNA despite CD8⁺ T cell depletion is most likely due to CD4⁺ cytotoxic T cells or constitutive expression by NK cells (37, 38).

Lymph Node Responses. To examine whether CD8⁺ T cell depletion influenced early responses in popliteal LN draining the primary sites of immunization, LN from an early CD8⁺ T cell-depleted group were compared with those from normal HN (CFA/Fx1A), CFA control, and normal unimmunized groups at 2 wk.

The weight of LNs from the CD8⁺ T cell-depleted group was not significantly different from normal HN or CFA controls, and all weighed significantly more than LNs from unimmunized rats (Fig. 6 a). CD8⁺ T cell depletion was confirmed in the CD8⁺ T cell-depleted group, with appropriate reciprocal changes in the proportion of other lymphocyte subsets (Fig. 6 b). LNs from CFA controls and normal HN, when compared with unimmunized normals, had a decrease in the proportion of B cells and an increase in the proportion of T cells, especially CD8⁺ T cells.

CD8⁺ T cell depletion did not impair cytokine and effector molecule mRNA expression in LN draining sites of

immunization. Cytokine mRNA for Th1, Th2, and cytotoxic cells was increased in LNs from normal HN and CD8⁺ T cell-depleted HN when compared with unimmunized normals (Fig. 7). The presence in LNs of mRNA for the cytotoxic cell molecules granzyme A, granzyme B, and perforin, despite the confirmed depletion of CD8⁺ T cells, suggests these molecules were from NK or cytotoxic CD4⁺ T cells (37–40).

Discussion

This study implicates CD8⁺ cytotoxic T cells as mediators of glomerular injury in active HN, a disease thought to be principally mediated by the MAC of complement (6, 41). Persistent depletion of CD8⁺ T cells by ATx and anti-CD8 mAb therapy, both early and late in the course of disease, prevented the development of proteinuria. Depletion of CD8⁺ T cells did not affect the Ab response, which is not unexpected, as CD4⁺ T cells provide help for anti-Fx1A Ab responses in HN (42). Glomerular deposition of IgG and C3 was also not different between CD8⁺ T cell-depleted and normal HN groups. CD8⁺ T cell-depleted rats also did not develop the glomerular infiltrate of activated mononuclear cells, including CD8⁺ cytotoxic effector cells and macrophages, seen in HN. ATx alone, which did not result in CD8⁺ T cell depletion, did not alter the natural history of HN. The finding that an isotype-matched control mAb that does not react with rat T cells had no effect on development of disease suggests that the effect of the anti-CD8 mAb is specific and not due to a nonspecific inhibitory or inflammatory reaction. Nonspecific effects from endotoxin are also unlikely, as contamination was minimal, there was no leucocytosis in this group, and the cytokine profiles in the popliteal node at the site of immunization were not enhanced in mAb treated hosts. Furthermore, with the early treatment with mAb it would have been eliminated before immunization. In previous studies we have shown that anti-CD8 mAb therapy alone, which led to only temporary depletion of CD8⁺ T cells, delayed the onset of proteinuria (17). These results, especially of the effect of late CD8⁺ T cell depletion, indicated CD8⁺ T cells were required in the final effector phase that leads to proteinuria.

Prevention of HN with CD8⁺ T cell depletion was not due to the modification of initial immune events in LN draining sites of immunization or the isotype of anti-Fx1A Ab. CD8⁺ T cell-depleted rats had a similar increase in LN size, as well as Th1 and Th2 cell cytokine activation, compared with that in normal HN rats. In both CD8⁺ T cell-depleted and normal HN, IgG1 was produced first, then IgG2a and a lesser IgG2b response, which accords with previous reports in HN (43). Thus ATx and anti-CD8 mAb therapy did not alter the Th1/Th2 response. Further evidence against an alteration of initial immunization events is that late depletion of CD8⁺ T cells, well after the initiation of serum Ab responses and glomerular Ig deposition, prevented disease. Thus, in the presence of a normal serum Ab response to Fx1A and deposition of IgG in

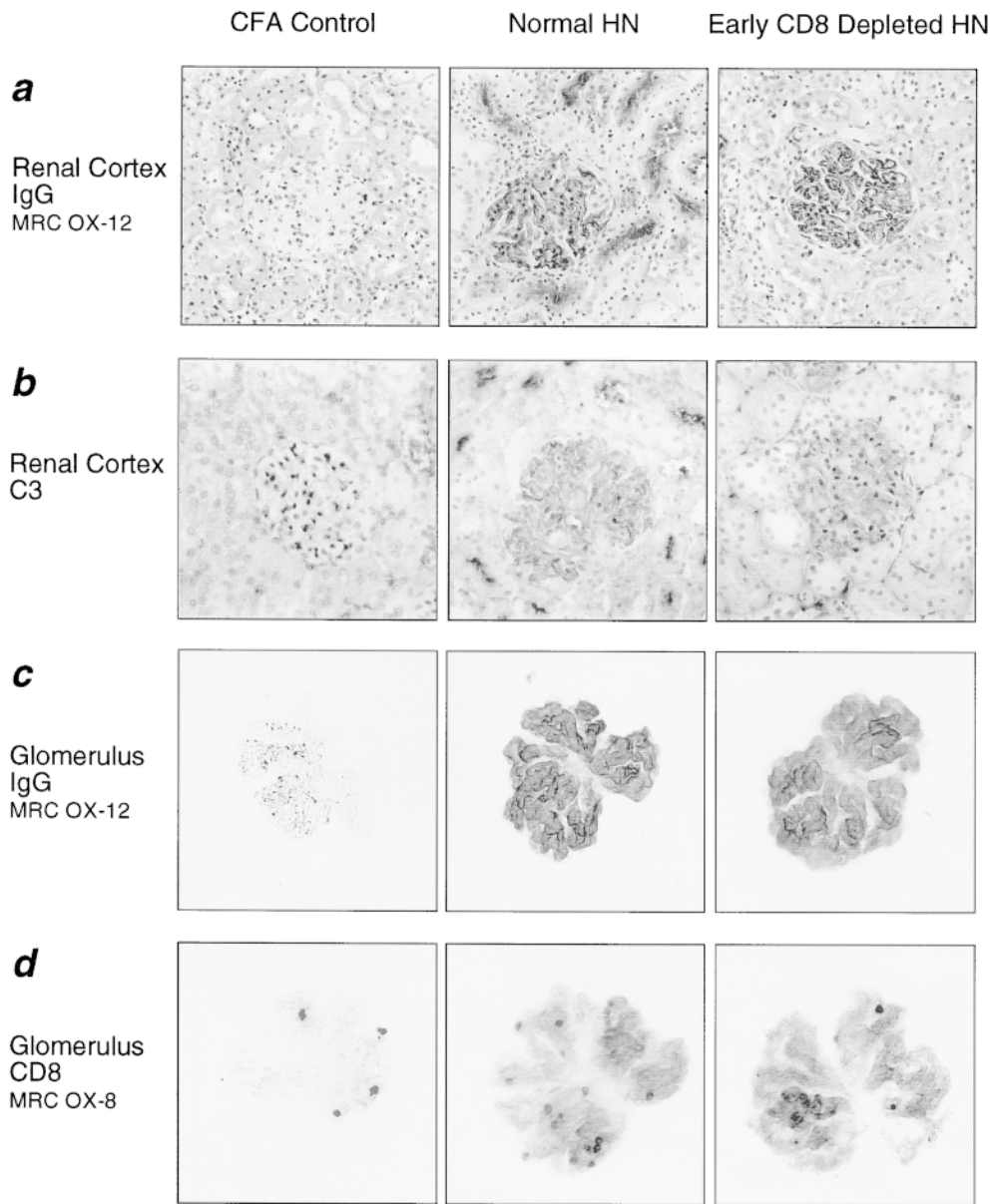


Figure 4. CD8⁺ T cell depletion in HN did not prevent glomerular deposition of IgG or C3, but abrogated the infiltration of mononuclear cells including CD8⁺ cells. (a–d) Glomerular IgG and C3 deposition were not different between normal HN and CD8-depleted HN groups. Note the amorphous staining of IgG and C3 within tubular lumina in the proteinuric normal HN rat. (d) The glomerular infiltrate of CD8⁺ cells in normal HN was absent in CD8-depleted HN hosts.

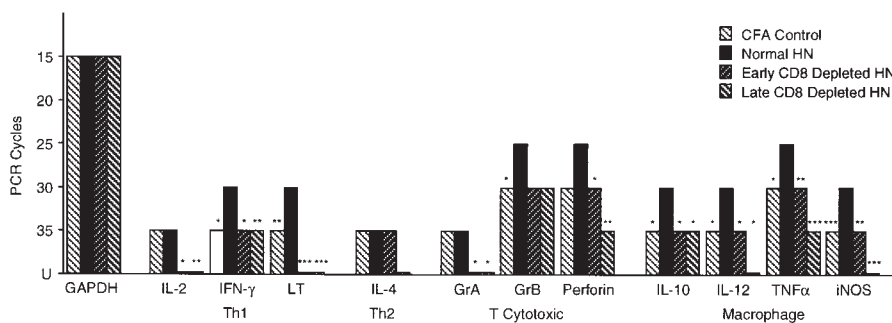


Figure 5. At 12 wk, CD8⁺ T cell depletion prevented the increase in Th1, cytotoxic T cell, and macrophage cytokine expression in glomeruli from rats with HN. Significant increases in cytokine mRNA expression for Th1 cells, cytotoxic cells, and macrophages were seen in normal HN compared with the background expression in CFA controls. These increases were not detected in early and late CD8-depleted glomeruli. Cytotoxic cell molecules in rats depleted of CD8⁺ T cells were probably from NK cells. Results expressed as the median cycle number ($n = 4$) at which PCR product was first detected, measured at five-cycle intervals. * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$. U, PCR product undetectable. ANOVA followed by Bonferroni-Dunn post hoc multiple comparisons test.

product was first detected, measured at five-cycle intervals. * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$. U, PCR product undetectable. ANOVA followed by Bonferroni-Dunn post hoc multiple comparisons test.

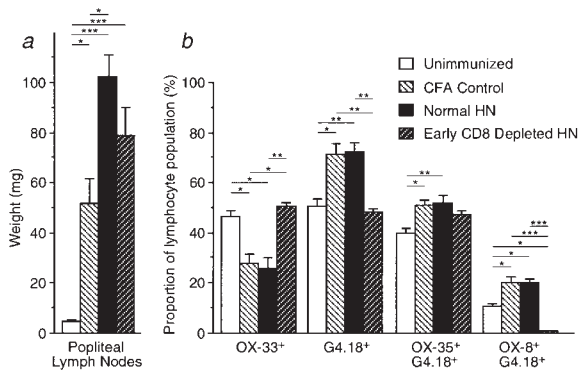


Figure 6. At 2 wk, CD8⁺ T cell depletion did not prevent an increase in the weight of immunized popliteal LNs. (a) There was a significant increase in the weight of all immunized (CFA control, normal HN, early CD8-depleted HN) compared with unimmunized LNs, with LNs from normal HN group being the heaviest. LNs from the early CD8⁺ T cell-depleted group did not differ significantly from CFA controls or normal HN. Values represent the mean \pm SEM ($n = 4$) of popliteal LN weight in grams. (b) There was a significant decrease in the proportion of B cells (OX-33⁺), and an increase in CD3⁺ T cells (G4.18⁺), which included both CD4⁺CD3⁺ (OX-35⁺G4.18⁺) and CD8⁺CD3⁺ (OX-8⁺G4.18⁺) cells. There were virtually no CD8⁺CD3⁺ (OX-8⁺G4.18⁺) cells present in the early CD8⁺ T cell-depleted group. Appropriate reciprocal changes to CD8⁺ T cell-depleted LNs were seen in LNs from the other groups. Values represent the mean \pm SEM ($n = 4$) of labeled lymphocytes as percentage of total gated population identified by FACS[®] analysis. * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$. ANOVA followed by Bonferroni-Dunn post hoc multiple comparisons test.

glomeruli, proteinuria does not develop in CD8⁺ T cell-depleted rats.

HN has been considered the archetypal model for Ab-mediated injury via complement activation, with MAC causing damage to glomerular epithelial cells (GEC). In this study, CD8⁺ T cell-depleted hosts had normal IgG and C3 deposition. This indicates the Ig had bound to GEC and activated complement, but this alone did not result in injury leading to proteinuria. A direct pathogenic role for MAC in active HN was discounted by Leenaerts et al. (9), who demonstrated the development of HN in PVG rats deficient in C6, which are unable to assemble MAC. This

study related increasing size of the subepithelial immune deposits to glomerular barrier dysfunction. However, other studies report no correlation between serum Ab titer or the size of immune deposits and proteinuria (44, 45). Our studies suggest alternative non-Ab-mediated effector mechanisms should be considered.

A possible explanation for the pathogenesis of HN is that after immunization with Fx1A the antigen is presented by host class II MHC that activates Th1 and Th2 CD4⁺ T cells that provide help for B cells in draining LN to produce an Ab response (30, 42). These Ab bind to antigen on GEC and form subepithelial immune deposits. The response of GEC to this causes antigen presentation by class I MHC on these cells to CD8⁺ T cells. This response may also require help from class II-restricted CD4⁺ T cells (17). Alternatively, there may be direct presentation of exogenous antigen to MHC class I-restricted CD8⁺ T cells as this pathway of antigen presentation has recently been described (46). As GEC are capable of expressing MHC class I and II and costimulatory molecules (47–49) they may themselves act as antigen-presenting cells. The activation of CD8⁺ T cells appears to be much slower than the activation of the CD4⁺ T cell-dependent B cell response to Fx1A.

The final effector mechanism, resulting in GEC injury and proteinuria, is most likely direct CD8⁺ T cell-mediated cytotoxicity via the granule exocytosis perforin pathway. This is supported by increased mRNA for granule exocytosis pathway molecules in CD8⁺ T cell-depleted glomeruli compared with HN. Glomerular injury may be compounded by the cytotoxic products of glomerular macrophages (16, 18, 50), whose recruitment into glomeruli is dependent upon activated CD4⁺ and CD8⁺ T cells (51).

Criteria for determining the functional significance of cell-mediated immunity in GN (1) have now been fulfilled for HN. First, an infiltrate comprising the elements of effector cell-mediated immunity, including Th1 and cytotoxic T lymphocytes, and macrophages, has been detected within glomeruli. Second, the influx of effector cells is temporally related to the development of proteinuria. Third, functional activation of the potential effector cells,

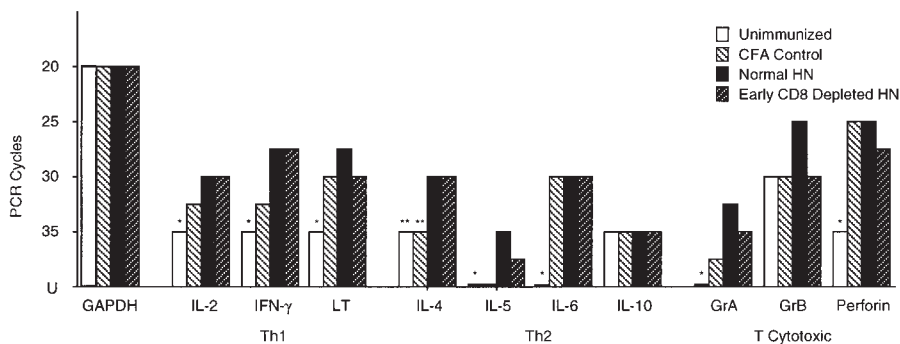


Figure 7. At 2 wk, CD8⁺ T cell depletion did not alter the balance of Th1 and Th2 cytokine expression in LNs from rats with HN. Immunization with CFA and CFA/Fx1A resulted in significantly increased expression of Th1, Th2, and cytotoxic cell cytokines when compared with unimmunized LNs. This was not significantly decreased by CD8⁺ T cell depletion. Cytotoxic cell molecules in rats depleted of CD8⁺ T cells were probably from NK cells. Results are expressed as the median cycle number ($n = 4$) at which PCR product was first detected, measured at five-cycle intervals. * $P < 0.01$, ** $P < 0.001$. U, PCR product undetectable. ANOVA followed by Bonferroni-Dunn post hoc multiple comparisons test.

CD8⁺ T cells and macrophages, has been demonstrated by the detection of the mRNA for their cytokines (TNF- α , TNF- β , IFN- γ , etc.), cytolytic molecules (perforin, granzymes), and other mediators, such as iNOS, reactive oxygen species (16). Production of these molecules could directly or indirectly cause tissue injury. Fourth, lymphocytes have been specifically sensitized to relevant glomerular antigens (10), and transfer of sensitized lymphoid cells induces GEC injury in vitro (11) and in vivo (13, 52). Fifth, depletion of CD8⁺ T effector cells abrogates injury. We have attempted reconstitution of CD8⁺ T cell-depleted rats immunized for HN with sensitized CD8⁺ T cells, but these cells have been short lived and did not reconstitute the CD8⁺ T cell population (Penny, M.J., unpublished observation).

Our study suggests that glomerular injury in HN is mediated by cytotoxic CD8⁺ effector T cells. This is an unusual effector mechanism for autoimmunity, and is best described in experimental immune-mediated diabetes mellitus (53, 54). CD8⁺ lymphocytes are thought to have a role as effector cells in nephrotoxic serum nephritis (55). In the majority of autoimmune models, especially those induced by immunization with CFA, and including those of nephritis (1, 2), injury is mediated by CD4⁺ T cells and macrophages. This work may be of relevance to the pathogenesis of human membranous nephropathy and other forms of GN, and may result in specific therapies that improve treatment.

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Address correspondence to Mark Penny, Department of Medicine, Liverpool Hospital, Liverpool, New South Wales 2170, Australia. Phone: 61-2-9828-3000; Fax: 61-2-9828-4970; E-mail: pennymark@bigpond.com

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