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Anti-glomerular basement membrane glomerulonephritis in the mouse: the role of macrophages

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Summary. An immunohistochemical study was undertaken on fixed, paraffin-embedded mouse kidney in order to elucidate the role and significance of infiltrating macrophages in a mouse model of anti-glomerular basement membrane glomerulonephritis (anti-GBM GN). Tissue was available representing the full gamut of histological features seen in this model. The mouse macrophage-specific antigen F4/80 was detected in tissue sections of glomerulonephritic kidney and the pattern and extent of staining was compared with normal mouse kidney.

In glomerulonephritic kidney, an increase in the number of F4/80-positive cells was evident in close proximity to and surrounding Bowman's capsule of those glomeruli which were severely damaged, with extensive fibrin deposition and well developed cellular crescents. F4/80-positive cells did not feature in the glomerular tuft or in the region of the parietal epithelium of Bowman's capsule even when extensive cellular crescents were present. Breaks in Bowman's capsule were not demonstrated.

We conclude that F4/80-positive macrophages are not a major constitutive cell type of developing crescents in this mouse model of anti-GBM GN but, by virtue of their peri-glomerular localization, may be involved in the destructive process, perhaps producing signalling molecules which contribute to the inflammatory reaction.

Keywords: anti-GBM, F4/80, glomerulonephritis, macrophage, mouse

A characteristic histological finding in human, rapidly progressive glomerulonephritis (GN) is the presence of crescents, which result from the process of inflammation and repair and which cause tuft occlusion. The classic hypothesis of cellular crescent formation is proliferation of the parietal epithelial cells of Bowman's capsule. In the last two decades this has been challenged and there is now considerable evidence that accumulation of infiltrating cells, particularly macrophages, plays an important role (Schreiner 1991).

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In experimental glomerulonephritis, macrophages appear to be implicated in the post-acute stage, following deposition of IgG (autologous or heterologous) in the glomerular basement membrane (GBM). In rabbits and rats, macrophages have been identified as a prominent component of glomerular crescents, where it is postulated that they function in their phagocytic role to eliminate the products of coagulation (Lan *et al.* 1992). In humans, there is certainly accumulation of lysozyme-positive cells around Bowman's capsule, though apparently minimal infiltration into the glomerulus or involvement in crescents if Bowman's capsule is intact. If, however, there is rupture of Bowman's cap-

sule, significant infiltration of lysozyme-positive cells occurs and they appear in large numbers in crescents (Boucher *et al.* 1987; Pusey 1990; M.K. Wetherall unpublished observations).

Several antibodies to mouse macrophages have been developed (Springer *et al.* 1978; Malorny *et al.* 1986; Kraal *et al.* 1987) but most of these have limited application in tissue, in that their use is confined to frozen sections. The macrophage-specific rat monoclonal antibody developed by Austyn and Gordon (1981) has the distinct advantage of being suitable for use in fixed tissue, as its antigen is stable to fixation and processing.

The specificity of the Austyn and Gordon rat monoclonal antibody is to F4/80, a mature mouse macrophage plasma membrane glycoprotein of molecular weight approximately 160 kDa. The antigen is not present on polymorphonuclear leucocytes. Relatively large amounts of F4/80 antigen have been found in normal kidney, as well as in the gastrointestinal tract, liver, haemopoietic and lymphoid tissues (Hume *et al.* 1983; Lee *et al.* 1985). However, the level of antigen per cell varies with cell maturity and is probably reduced when macrophages are in an activated state (Austyn & Gordon 1981). By means of immunohistochemical labelling, Hume and Gordon (1983) demonstrated that F4/80 antigen-bearing cells in the kidney are situated interstitially in the renal medulla and cortex (peritubular and pericapsular) and are present in the juxtaglomerular apparatus.

We have reported, previously, the results of a study in which it was concluded that proliferation of the parietal epithelial cells of Bowman's capsule is the major contributory factor to the formation of crescents in a mouse model of anti-GBM GN (Wheeler *et al.* 1993). We report here the results of a study on fixed tissue from this same model, to evaluate macrophage involvement. Accumulation of extrinsic glomerular cells was examined using the rat monoclonal antibody to F4/80.

Materials and methods

Normal mouse tissue

Kidney was obtained from normal, T70 (Beige) male mice, after sacrifice by cervical dislocation, and fixed in formalin (4% phosphate-buffered formaldehyde) overnight or formal sublimate (4% phosphate buffered formaldehyde saturated with mercuric chloride) for 3 hours.

Glomerulonephritic mouse kidney

Formalin/formal sublimate-fixed paraffin embedded kidney from T70 (Beige) male mice was available from previous studies with our mouse model of anti-GBM GN (Wheeler *et al.* 1990; 1993). Thirty-nine cases were selected, demonstrating the full gamut of histological responses observed in this animal model, which is determined by the efficacy of the particular antiserum/bleed employed, i.e. proteinuria, prodocyte vacuolation, fibrin deposition and cellular crescents. Periodic acid Schiff (PAS) stained sections were scored for all four features of histological damage on a 4-point scale of 0 (absent) to 3 (severe) (see Results, Table 1).

Antibodies and reagents

Rat anti-mouse F4/80 monoclonal antibody (IgG2b, supernatant) and biotinylated rabbit anti-rat $F(ab')_2$ fragments were supplied by Serotec, Oxford. A streptavidin/biotin/horse-radish peroxidase complex system was obtained in kit form from Sera-Lab Ltd, Crawley Down, Sussex.

Localization of F4/80-positive cells

Detection of F4/80-positive cells in normal and glomerulonephritic kidney was by an immunoperoxidase method, using an avidin–bitotin complex procedure (ABC) (Gordon *et al.* 1986). This method is suitable for use on tissue fixed in formalin or formal sublimate.

Sections, at 2–4 μm, on lysine coated slides were dewaxed and treated to inactivate endogenous peroxidase (0·3% hydrogen peroxide in methanol for 10 minutes). This was followed by standard trypsin digestion for 5 or 10 minutes, for formal sublimate or formalin fixation, respectively. To minimize non-specific binding of the secondary antibody, sections were flooded with 20% normal rabbit serum (NRS) in Tris-buffered saline pH 7·6 (TBS) for 20 minutes at room temperature. Sections were then incubated overnight at 4°C with a 1 in 10 dilution of the primary antibody, rat anti-mouse F4/80, in NRS. The secondary antibody, biotinylated rabbit anti-rat $F(ab')_2$ fragments, was added at a dilution of 1 in 100 in NRS for 1 hour at room temperature and the sections were subsequently incubated for 30 minutes at room temperature with the streptavidin/biotin/horse-radish peroxidase complex. The chromagen was 3,3-diaminobenzidine (1 mg/ml in TBS) plus imidazole (0·68 mg/ml). Sections were counterstained with Mayer's haematoxylin, precipitates of mercury being removed from formal sublimate fixed tissue by immersion in iodine/sodium thiosulphate solutions, prior to counterstaining. The slides were washed in TBS between stages.

Negative method controls were included in which the

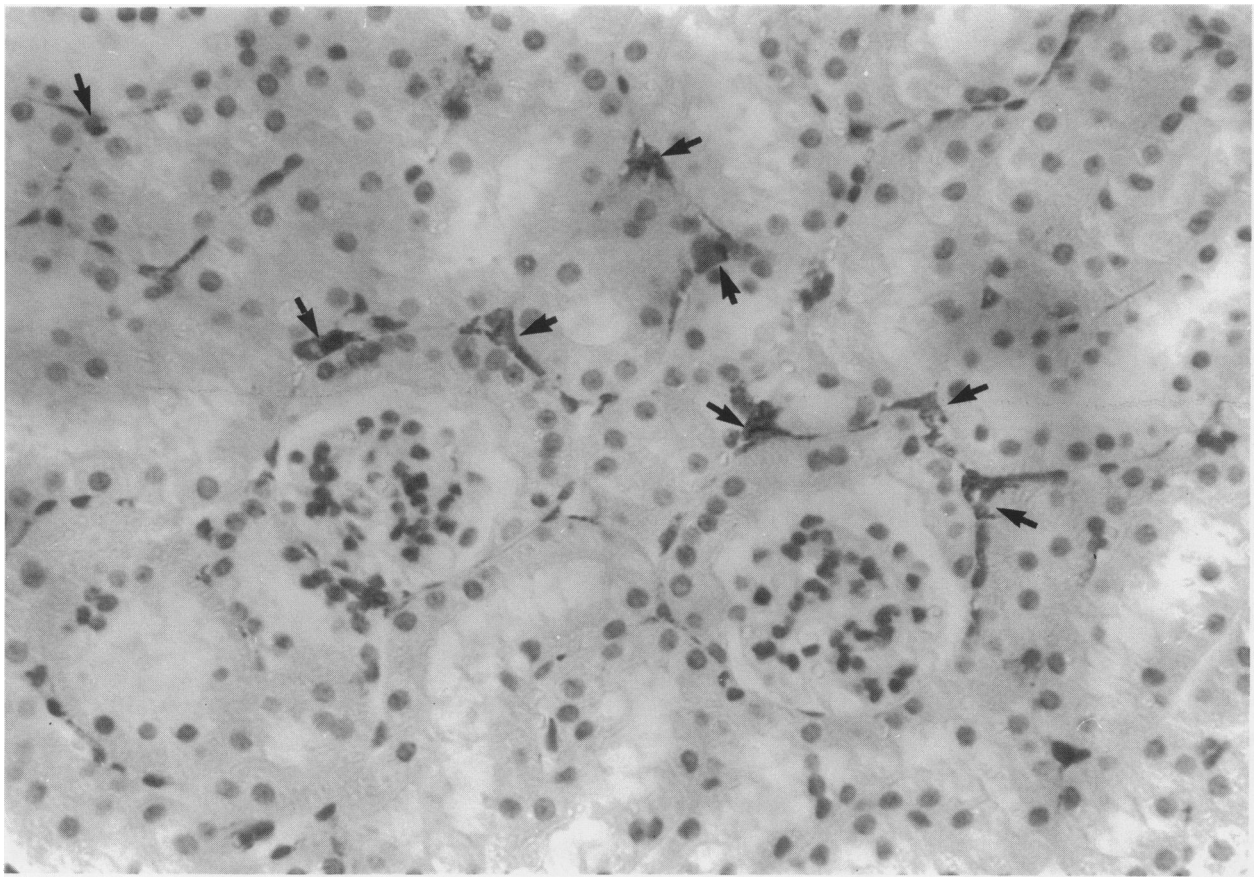

Figure 1. Periglomerular and peritubular F4/80-positive cells (↑) in normal male mouse kidney. ×200.

primary and/or secondary antibodies were omitted at the relevant stages. Positive control sections were of normal mouse kidney.

The presence of macrophages in glomerulonephritic mouse kidney was compared with that in the normal animal and scored on a 4-point scale of 0 (normal) to 3 (substantial increase).

Results

Localization of F4/80-positive cells in normal mouse kidney

A 'non-random' distribution of F4/80-positive cells was found in normal mouse kidney. In longitudinal sections, labelled cells were identified in the medullary interstitial cell population. Such cells were extensive throughout the medulla and surrounded much of the outer surface of the collecting ducts. Similar positive staining of interstitial cells was found in patches throughout the cortex, in direct contact with the outer surfaces of proximal and distal convoluted tubules and with Bowman's capsule (Figure 1). In addition, such stained cells were nearly always found in close proximity to the macula densa and the afferent and efferent arterioles of the glomerular capillary bed, when these features were visible. Glomerular mesangial cells were not F4/80-positive and, indeed, no F4/80-positive cells were ever detected in the glomeruli of normal mouse kidney.

Localization of F4/80-positive cells in glomerulonephritic kidney

Of the 39 kidneys selected, 12 were severely damaged (numbers 28–39 inclusive), with varying degrees of proteinuria, podocyte vacuolation, fibrin deposition and cellular crescents. In 11 of these 12 kidneys, an increased presence of F4/80-positive cells was apparent, predominantly around glomeruli in which fibrin deposition and cellular crescents were evident (Figure 2). An increased presence of F4/80-positive cells was also demonstrated in two other kidneys (numbers 26 and 27) which had

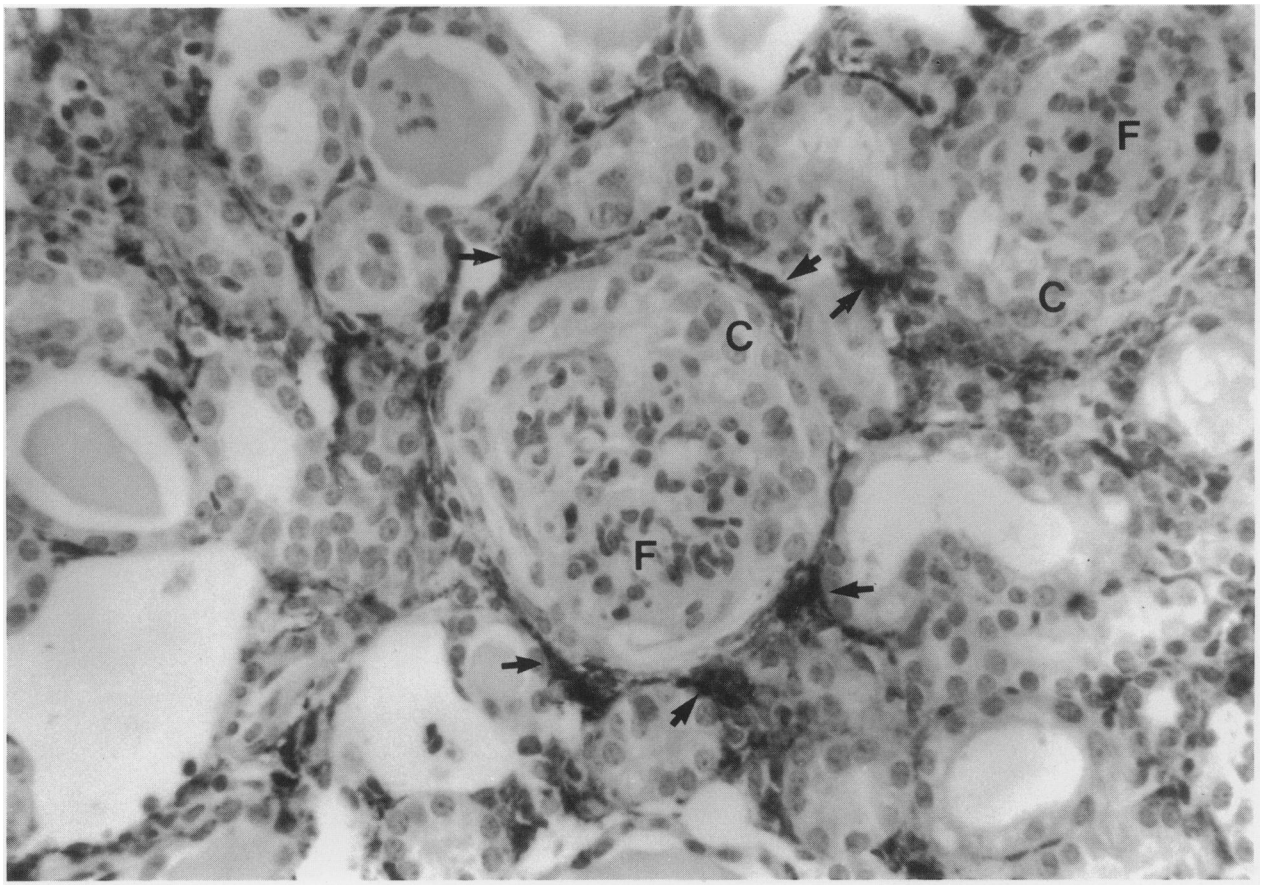

Figure 2. Increased presence of F4/80-positive cells (↑) around severely damaged glomeruli in which tuft fibrin deposition (F) and cellular crescents (C) are also clearly evident. ×200.

evidence of proteinuria, podocyte vacuolation and fibrin deposition, but no cellular crescents. In sharp contrast, a normal distribution of F4/80-positive cells was seen in all 25 remaining kidneys, irrespective of the presence or degree of histological damage. These results are summarized in Table 1.

F4/80-positive cells were never detected within the glomerular tuft or in the region of the parietal epithelium of Bowman's capsule, despite the presence of extensive cellular crescents. Furthermore, there was no evidence of ruptures in Bowman's capsule, even in those kidneys which were severely damaged.

Discussion

It has been demonstrated in recent years that the degree and time-course of macrophage infiltration in glomerulonephritis depend to some extent upon the type of glomerular disease and on the species under study (Wilson 1991). In relation to anti-GBM GN, it is logical to assume that cells of the macrophage lineage will be involved in the inflammatory reaction which follows deposition of IgG in the GBM.

For many years, identification and enumeration of macrophages have presented major challenges to those investigating anti-GBM GN. Many studies, both in human biopsy material and in experimental animals, have concentrated on the intra-glomerular involvement of these cells. In particular, any part played by macrophages in the formation of crescents has been the subject of much speculation (Yoshioka *et al.* 1987; Lan *et al.* 1992).

In this study, localization of the mouse macrophage-specific antigen F4/80 in fixed paraffin embedded kidney was utilized to detect the presence of macrophages in our T70 mouse model of anti-GBM GN. Due to the diffuse nature of the staining and the flattened, periglomerular and peritubular localization of the F4/80-positive cells, enumeration of individual cells was found to be difficult and was not undertaken.

The results show an increased macrophage presence

Table 1. Histological damage* and macrophage presence† in experimental anti-GBM GN in T70 mice assessed by PAS staining and immunohistochemical detection of antigen F4/80

Mouse nos.	Proteinuria	Podocyte vacuolation	Fibrin	Crescents	Macrophage presence
1–7	0	0	0	0	0
8–10	1	0	0	0	0
11–14	1	1	0	0	0
15–19	2	1	0	0	0
20	2	2	0	0	0
21–22	2	1	1	0	0
23	1	2	1	0	0
24–25	2	2	1	0	0
26–27	2	2	1	0	1
28	2	2	1	1	1
29	2	2	1	1	2
30	2	1	2	1	1
31	2	2	2	1	0
32	2	2	2	1	2
33	3	2	1	1	2
34	3	2	1	1	1
35	3	2	2	1	2
36	2	1	2	2	1
37–38	3	2	2	2	3
39	3	1	3	3	3

* PAS-stained sections were scored for all four features of histological damage on a 4-point scale of 0 (absent) to 3 (severe).

† The presence of macrophages was compared with that in the normal animal and scored on a 4-point scale of 0 (normal) to 3 (substantial increase).

in close proximity to, and surrounding, Bowman's capsule, only in those glomeruli which were severely damaged, with extensive fibrin deposition and well developed crescents. One may speculate that such periglomerular macrophage accumulation could be the result of chemotaxis due to cytokines released by damaged intrinsic glomerular cells. However, even when glomeruli were extensively damaged, F4/80-positive cells did not feature in the glomerular tuft or within the developing cellular crescents. These results support our earlier studies on this mouse anti-GBM GN model, in which we concluded that proliferation of the parietal epithelial cells of Bowman's capsule is the major contributory factor to the formation of cellular crescents (Wheeler *et al.* 1993). It must be noted however that as F4/80 expression is perhaps reduced upon activation (Austyn & Gordon 1981) there remains the possibility that a macrophage population exists within the glomerulus which does not express the antigen at a level sufficient to permit detection by this method.

The association of an increased macrophage presence in a periglomerular localization with fibrin deposition and cellular crescents in glomeruli is interesting on two counts. First, macrophages are the principal source of pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which have procoagulant activity and cause upregulation and induction of the adhesion molecules ICAM and VCAM, respectively, in endothelial cells (Cotran & Pober 1989). In addition, such inflammatory cytokines have been shown to have various effects on glomerular mesangial cells in culture, such as cytotoxicity and stimulation of production of extracellular matrix components and of TNF-α and IL-6 themselves (Ortiz *et al.* 1994). One may speculate, therefore, that local production of such cytokines around glomeruli could be implicated in fibrin deposition within the glomerular tuft. Secondly, it has been proposed that macrophages secrete growth factors (Boucher *et al.* 1987), which may suggest a role for these cells in the induction of parietal epithelial cell proliferation.

Given the periglomerular accumulation of macrophages and the fact that these cells are known to secrete extracellular matrix-degrading enzymes such as collagenase (Wahl & Mergenhagen 1988), it seems paradoxical that no breaks or ruptures of the basement membrane of Bowman's capsule were seen in this animal model. Such matrix destruction together with pressure from accumulating cells within the renal corpuscle (Morley 1988) could weaken and rupture the basement membrane and such features have been observed in other experimental models of GN (Lan *et al.* 1992). Absence of ruptures in Bowman's capsule may explain the lack of F4/80-positive cells within the glomerular tuft and developing crescents in this animal model. Indeed, studies of human glomerulonephritis by M.K. Wetherall *et al.* (unpublished observa-

tions) support this view, as intra-glomerular, lysozyme-positive cells (macrophages) were found only when there were breaks in the basement membrane of Bowman's capsule.

Recently, other factors produced by infiltrating blood cells and/or intrinsic glomerular cells (mesangial and endothelial), such as platelet-derived growth factor (PDGF) and the transforming growth factors (TGF-β1 and TGF-β2), have been directly implicated in the mediation of glomerular damage, including cell proliferation and modulation of synthesis of extracellular matrix proteins (Abboud 1992).

In conclusion, there is now considerable evidence pointing to an interrelationship between cellular and molecular responses of intrinsic glomerular cells and infiltrating macrophages. This is significant in that it indicates a greater heterogeneity in the processes underlying the progression of crescentic glomerulonephritis than has been widely acknowledged. Ortiz *et al.* (1994) suggest that this interrelationship leads to a 'cascade of events' resulting in development of pathological features such as glomerular fibrosis and crescent formation, so evident in this mouse model of anti-GBM GN. Perhaps future studies should target the systematic identification of cytokines and growth factors (protein and messenger RNA) at various stages in the pathological process *in vivo*. Models of anti-GBM GN could be ideal for such studies.

Acknowledgements

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