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Cellular specificity of murine renal C3 expression in two models of inflammation

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SUMMARY

The expression of the complement protein C3 in extrahepatic tissues is highly regulated during the course of inflammation. Hence, systemic acute phase stimuli such as bacterial lipopolysaccharide (LPS) and autoimmune nephritis in aged 'lupus mice' (MRL-lpr/lpr and NZB × NZW F_1) both lead to increased C3 mRNA expression in whole kidney. *In situ* hybridization was used to determine the intrarenal cell type(s) capable of constitutive and regulated C3 mRNA expression. Normal mice injected with *Escherichia coli* LPS show a marked increase in whole kidney C3 mRNA over control (saline-injected) animals. The renal C3 mRNA in LPS-stimulated mice was found in cortical tubular epithelium. By contrast, in aged (18 week) MRL-lpr/lpr mice, which develop lupus nephritis, the increased intrarenal C3 messenger RNA was localized to perivascular inflammatory cells surrounding medium-sized arteries. Similar perivascular infiltrates were seen in the lungs of the MRL-lpr/lpr mice, and focal inflammatory cell infiltrates were also found in the myocardium. Leucocytes in these infiltrates accounted for the increased C3 expression in these tissues. These findings suggest cell as well as tissue specificity of the response to inflammatory stimuli in the local extrahepatic production of the third component of complement.

INTRODUCTION

The complement system is a complex network of soluble and cell-associated proteins which functions not only in host defence against pathogens, but also in the pathophysiology of immune-mediated tissue injury. Alterations in plasma complement levels occur during inflammation. Plasma levels of the third component of complement and the alternative pathway protein factor B rise as a result of new synthesis following acute phase stimuli (reviewed in ref. 1). Immune complex-mediated disorders such as active systemic lupus erythematosus, membranoproliferative glomerulonephritis (MPGN) and acute poststreptococcal glomerulonephritis lead to depressed plasma C3 complement levels as a result of decreased hepatic synthesis, increased catabolism, or both.[2,3]

The third component of complement (C3) is present along the tubular basement membrane in human membranoproliferative glomerulonephritis type I (MPGN type I) and in lupus nephritis.[4] In addition, C3, factor B, and immunoglobulins are found along the tubular basement membrane in experimental interstitial nephritis in guinea-pigs.[5] Linear deposits of C3 and factor B are also present along the tubular basement membrane in allograft rejection in humans.[6] In addition, local complement and immunoglobulin deposition has been observed in non-renal tissues in immune complex-mediated disorders such as systemic lupus erythematosus.[7]

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Experimental and clinical data have provided evidence for the synthesis of complement proteins in liver (the prime source of plasma complement proteins)[8,9] and for the extrahepatic synthesis of complement in all tissues examined thus far (reviewed in ref. 10). In fact, cell types derived from all three embryonic cell layers can synthesize C3; e.g. mononuclear phagocytes,[11] fibroblasts,[12] uterine epithelium,[13] astroglial cells,[14] endothelial cells,[15,16] and alveolar type II epithelium[17] are all sources of C3 protein.

Recent findings from several laboratories have suggested that renal tubular epithelium can synthesize C3. For example, Welch *et al.* found C3 mRNA in tubular epithelial cells in several types of immune complex-mediated renal disease, but not in normal kidneys nor in several types of non-immune renal disease.[18] Brooimans *et al.* documented synthesis of C3 *in vitro* by cultured human proximal tubular epithelial cells.[19]

Constitutive and regulated C3 expression has been detected in murine kidney. That is, C3-specific mRNA is detected at low levels in normal mouse kidney and is markedly up-regulated by intraperitoneal endotoxin (lipopolysaccharide; LPS) injection.[20] Similarly, increased C3 expression has been observed in two models of murine lupus nephritis (MRL-lpr/lpr and NZB × NZW F_1 mice), at a time when plasma C3 levels are actually depressed.[21,22] Because the cell type(s) expressing C3 in these models was (were) unknown, we undertook the investigation of this phenomenon at a cellular level.

MATERIALS AND METHODS

Riboprobe synthesis

A C3 riboprobe was generated by the polymerase chain reaction from a full-length mouse C3 cDNA.[23] Synthetic oligonucleo-

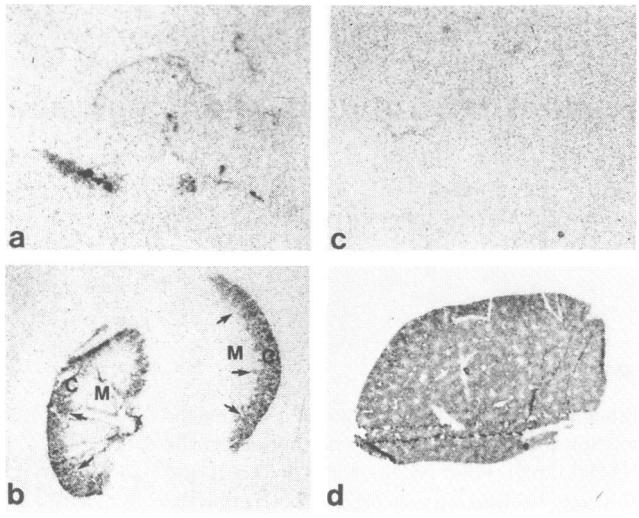

Figure 1. Autoradiographs of B10.PL tissues hybridized with C3 anti-sense and control sense riboprobes. (a) Saline-injected control kidney, C3 anti-sense riboprobe (×3·75). (b) LPS-injected kidney, C3 anti-sense riboprobe (×3·75). (c) LPS-injected kidney, C3 sense riboprobe (×3·75). (d) Saline-injected liver, C3 anti-sense riboprobe (×5).

tides were used as primers for amplification of a fragment spanning exons 4 and 5 (bases 498–715). The resulting 219 base pair (bp) fragment was cloned into pSP72 between *Xho*I and *Bgl*II (using an engineered *Xho*I site at the 5′ end and a naturally occurring *Bgl*II site at the 3′ end). Nucleic acid sequencing confirmed orientation and fidelity of replication of the probe. The resulting construct was cut with *Xho*I to generate the anti-sense probe, and *Bgl*II to generate the control sense probe. Probes were resuspended in RNAse-free water.

Riboprobes were labelled using the Riboprobe Gemini system (Promega Corporation, Madison, WI). One microgram of the sense or anti-sense probe was labelled using 400 μCi ^{35}S-labelled uridine triphosphate (specific activity 1000 Ci/mmol; Amersham, Arlington Heights, IL) and the resulting labelled probes were purified using RNAse-free Sephadex G-25 (Boehringer-Mannheim, Indianapolis, IN). Each labelled probe was adjusted to a concentration of 1×10^7 c.p.m./ml in hybridization solution according to the method of Simmons *et al.*[24] Prior to use the probe preparations were heated to 65° for 10 min to melt annealed strands and centrifuged for 10 min at 14,000 ***g*** in a microcentrifuge to remove aggregates.

Preparation of tissues

B10.PL and MRL-lpr/lpr and MRL-+/+ mice (Jackson Laboratories, Bar Harbor, ME) were maintained in a standard animal facility with *ad libitum* water and standard mouse food. Animals were anaesthetized with a ketamine–acepromazine–xylazine cocktail (Washington University Department of Comparative Medicine, Washington, DC) and perfused through the left ventricle with 10 ml of pyrogen-free saline followed by 30 ml of buffered 4% paraformaldehyde (Sigma Chemical Company, St Louis, MO). When perfusion was complete, organs were removed and stored in 4% paraformaldehyde with 10% sucrose. Tissues were frozen or paraffin-embedded and 5-μm sections were obtained under RNAse-free conditions and mounted on positive-charge (+) slides (Fisher Scientific, Fair Lawn, NJ). Eight-week-old B10.PL mice received a single intraperitoneal injection of 0·1 ml pyrogen-free saline alone or 0·1 ml of pyrogen-free saline containing 10 μg *Escherichia coli* endotoxin (LPS from *E. coli* 0111-B4, phenol extract; Sigma Chemical Company). Perfusion and tissue harvest occurred 24 hr later. MRL-lpr/lpr and MRL-+/+ mice were killed at 18 weeks of age.

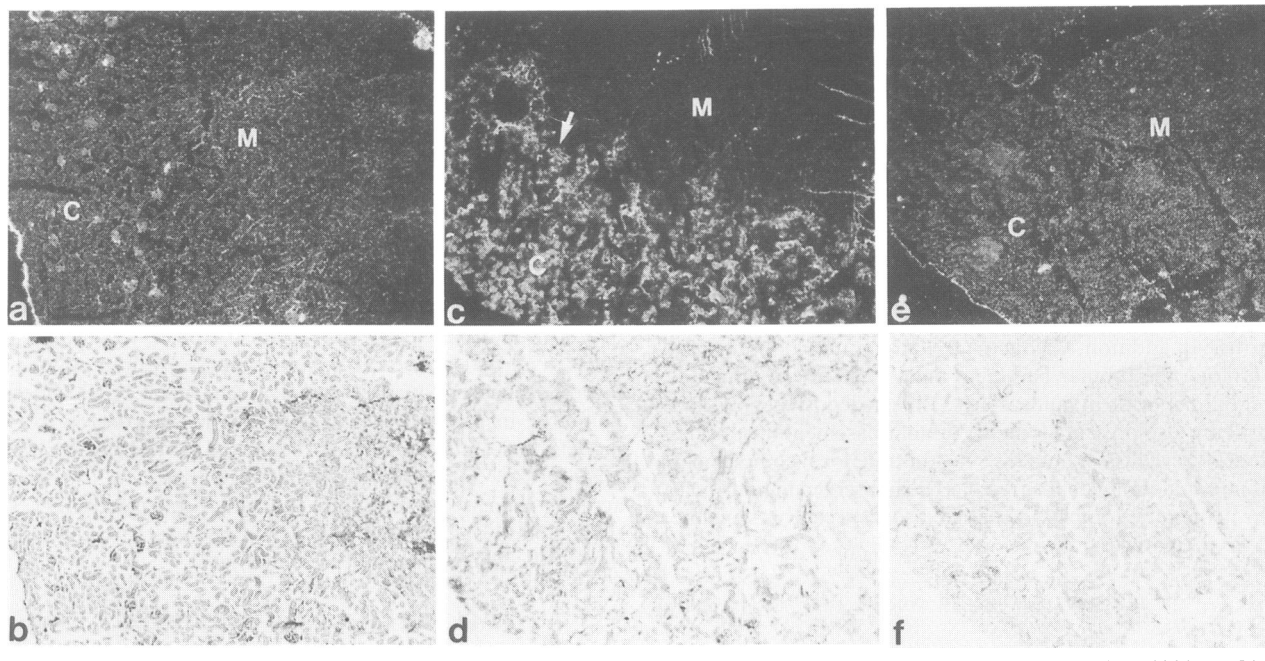

Figure 2. Photomicrographs of kidneys shown in Fig. 1. (a and b) Dark (above) and bright field (below) microscopy of saline-injected kidney, C3 anti-sense riboprobe (×40). (c) and (d) LPS-injected kidney, C3 anti-sense riboprobe (×40). Note striking corticomedullary differentiation of signal (arrow). (e) and (f) LPS-injected kidney, C3 sense riboprobe (×40). C, cortex; M, medulla.

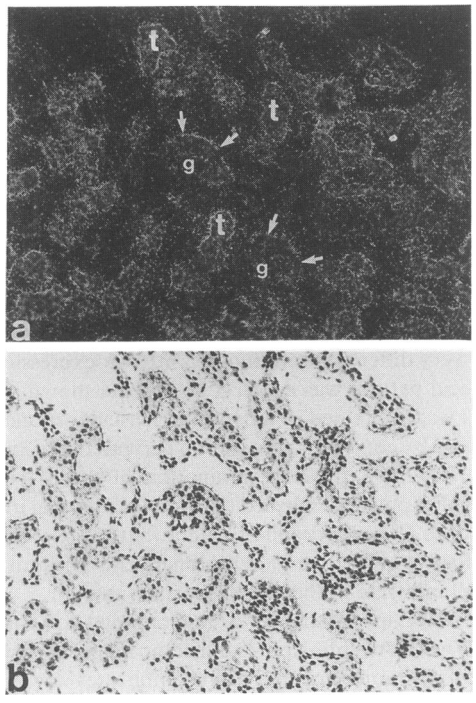

Figure 3. Higher power (× 110) dark (a) and bright field (b) photomicrographs of LPS-injected B10.PL renal cortex, C3 anti-sense riboprobe. Note absence of signal in glomerular tuft (g), although silver grains are present in Bowman's capsule (arrows) and tubules (t).

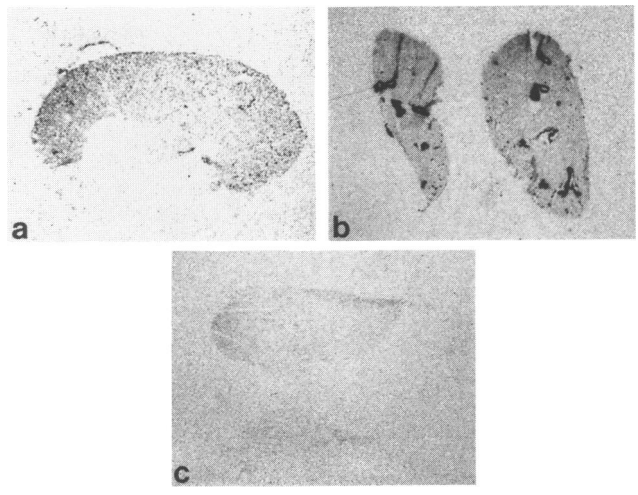

Figure 4. Autoradiographs of 18-week MRL kidney. (a) Control MRL-+/+ kidney, C3 anti-sense riboprobe (× 2·75). (b) MRL-lpr/lpr kidney, C3 anti-sense riboprobe (× 2·75). (c) MRL-lpr/lpr kidney, C3 sense riboprobe (× 2·75).

In situ *hybridization*

In situ hybridization was performed according to the method of Simmons *et al.*[24] Briefly, mounted tissue sections were digested with 10 μg/ml proteinase K (Boehringer-Mannheim Corporation), acetylated with acetic anhydride, dehydrated with ethanol solutions of ascending concentration, and vacuum dried under RNAse-free conditions. Seventy-five microlitres of hybridization solution containing 1×10^7 c.p.m./ml of probe was applied to the dry slides; a cover glass (Richard Allan, Richland, MI) was applied and the edges sealed with DPX mounting medium (BDH Limited, Poole, U.K.). Slides were hybridized overnight on a slide-warming tray (Precision Scientific, Chicago, IL) at 56–59°. The following day the cover glasses were removed and the slides were washed in 4 × saline–sodium citrate (SSC), treated with RNAse A (Sigma Chemical Company) to remove single-stranded RNA, desalted in decreasing concentrations of SSC, and dehydrated in ascending ethanols. Slides were then dried and exposed to X-ray film (XAR; Eastman Kodak Company, Rochester, NY). After exposure slides were delipidated in xylene and ethanol and dipped in NTB-2 autoradiography emulsion (Eastman Kodak Company). Slides were

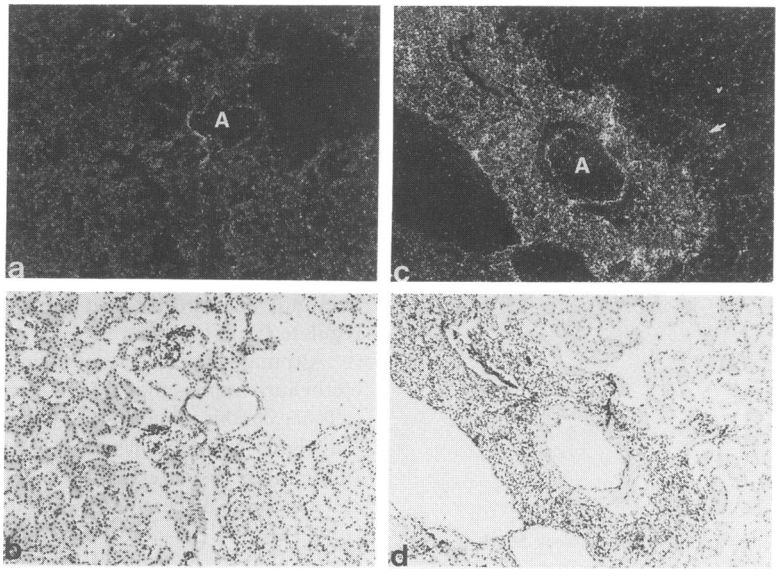

Figure 5. Photomicrographs (× 26) of 18-week MRL kidney. *In situ* hybridization with C3 anti-sense riboprobe. (a) and (b) MRL- +/+. (c) and (d) MRL-lpr/lpr kidney. Note the striking accumulation of C3 signal in perivascular infiltrate around artery (A) and absence of signal in tubules and a glomerulus (arrow) in MRL-lpr/lpr.

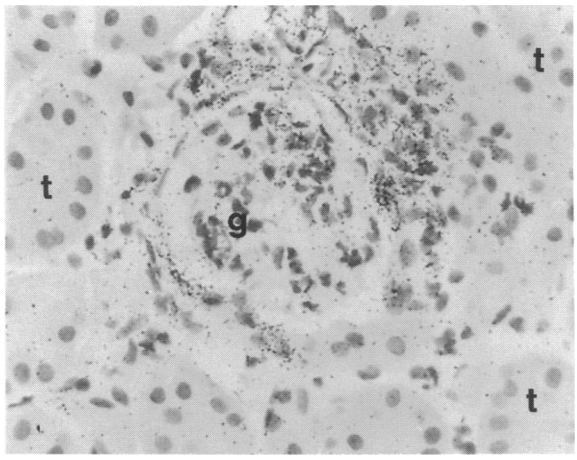

Figure 6. High-power photomicrograph of 18-week MRL-lpr/lpr renal cortex, C3 anti-sense riboprobe, bright field (× 200). Note the absence of signal in the tubules (t) and glomerular tuft (g). C3 signal is present in a periglomerular interstitial infiltrate.

stored at 4° in the dark for 14–42 days; they were then developed with Kodak D-19 developer and fixed with Kodak film strength rapid fixer. Slides were counterstained with haematoxylin and eosin and cover slips applied. Slides were examined by light and dark field microscopy; Kodak Ektachrome and Ektar 100 films were used for photomicroscopy.

RESULTS

Tissue radiographs of control, saline-injected and LPS-injected kidneys are shown in Fig. 1. A faint signal was seen over the entire kidney in the saline-injected mice and a strong signal was localized to the renal cortex in kidneys of mice injected with 10 μg LPS.

Photomicroscopy of kidneys (shown in Fig. 2) revealed a faint signal (apparently non-specific) mostly *between* cells in saline-treated mice (Fig. 2a, b), and a strong signal localized to cortical tubules in LPS-injected kidney (Fig. 2c, d), when tissues were hybridized with the C3 anti-sense riboprobe. The control, sense probe gave no detectable signal (Fig. 2e, f). A higher power view of cortex from the LPS-treated animal hybridized with the C3 anti-sense probe is shown in Fig. 3. Several glomeruli are shown. Although most of the tubules were strongly positive, the glomerular tuft was not. Silver grains can be seen, however, in Bowman's capsule, which contains cells of epithelial origin.

In contrast, the kidneys of 18-week MRL-lpr/lpr mice showed a very different pattern of C3 mRNA expression, i.e. a discrete focal pattern was noted (Fig. 4b). As shown in Fig. 6, this signal was contained in a dense perivascular mononuclear cell infiltrate. By contrast, there was no perivascular signal in the control MRL-+/+ tissue. Examination of the MRL-lpr/lpr tissue at higher power confirmed the absence of signal in tubules and glomeruli (Fig. 6), despite the fact that by 18 weeks there was a diffuse proliferative glomerulonephritis with an inflammatory cell infiltrate in the glomerular mesangium.

Other MRL-lpr/lpr tissues were examined for changes in cellular C3 mRNA expression. For instance, a positive signal for C3 mRNA was noted in perivascular infiltrates and along the visceral pleura of the lung (Fig. 7a,b) and in interstitial mononuclear cells infiltrating the myocardium (Fig. 7c,d) of MRL-lpr/lpr mice at 18 weeks.

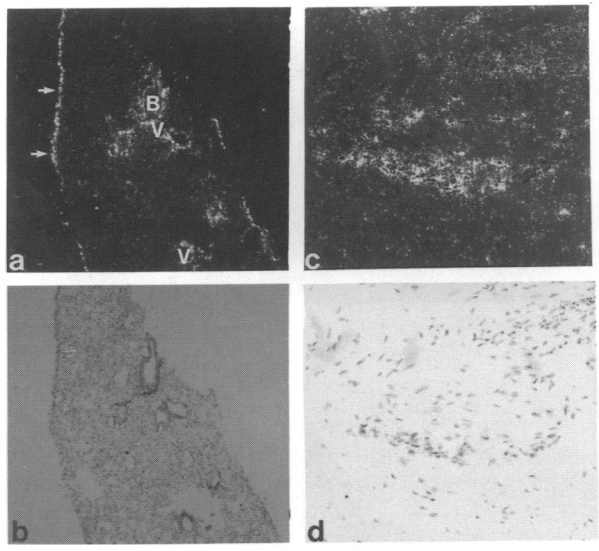

Figure 7. Photomicrographs of lung and heart of 18-week MRL-lpr/lpr mice; C3 anti-sense riboprobe. (a) and (b) lung; note strong signal for C3 in perivascular infiltrates and in the visceral pleura (× 24). B, bronchial epithelium; V, vessel. (c) and (d) myocardium: C3 signal is apparent in leucocytic infiltrate.

DISCUSSION

The use of *in situ* hybridization has allowed differentiation, at a cellular level, between the effects of various stimui that up-regulate C3 expression in extrahepatic tissues. Thus, a systemic acute phase stimulus such as endotoxin administration up-regulates C3 in cortical tubule epithelial cells, while in the MRL-lpr/lpr kidney increased C3 expression is mainly due to infiltrating mononuclear cells.

It is not known whether LPS can act directly on renal tubule epithelium, or whether it acts through other mediators to up-regulate C3. Although several cytokines (including interleukin-1, tumour necrosis factor-α and interleukin-6) are capable of up-regulating C3 in cell culture[25–27] and both the cis and trans elements regulating its transcription have been partially defined,[28] the proximate signal(s) for C3 expression is (are) unknown. Brooimans *et al.* showed that interleukin-2 (IL-2) can up-regulate C3 synthesis in cultured human proximal tubular epithelial cells.[19] Mier *et al.*, however, found evidence that IL-2 does not act directly, but through other mediators, to up-regulate C3 in a human hepatocyte cell line (29).

Although the presence of C3 mRNA in renal cortical tubular epithelium implies that C3 protein is also being made by these cells *in vivo*, this remains to be proven. In addition, no studies to date have indicated whether C3 is secreted from the apical (luminal) or basolateral surface of the tubule cell, or from both; i.e. whether C3 secretion by renal tubular epithelium is polar (unidirectional) or non-polar (bidirectional). Although C3 has been found in the urine in glomerulonephritis and in allograft rejection,[30,31] there is as yet no proof that urinary complement proteins are produced locally and secreted into the tubular lumen. C3 expression has been demonstrated in other epithe-

lia,[10] suggesting that local production of complement at epithelial surfaces may be important in first line host defences.

Recent studies have shown that factor B mRNA is present in cortical tubular epithelium in certain strains of mice (B. H. Ault and H. R. Colten, unpublished observations). Volanakis *et al.* have shown that factor D, another alternative pathway protein, is filtered at the glomerulus and can be detected in the urine.[32] Hence, the presence of all three components of the alternative pathway in tubular fluid might allow alternative complement pathway activation by micro-organisms and elimination of bacteria within the urinary tract. In addition, Welch *et al.* have demonstrated constitutive expression of mRNA for C4 in human proximal tubular epithelium, as well as other epithelia throughout the body.[33] The expression of the other classical pathway proteins (C1 and C2) in kidney has not yet been documented, but the finding of C4 mRNA in renal tubular and other epithelia[34] should prompt additional studies.

LPS stimulation up-regulates C3 only in the renal cortex (Figs 1b and 2c, d). This difference between C3 expression in the proximal and distal nephrons may be quantitative or qualitative; proximal and distal tubules have a different embryological origin from the collecting ducts and collecting system.[35] Whether more distal urinary epithelia (ureter and bladder) express C3 remains to be elucidated, but clearly this is an important question in light of the role of mucosal immunity in ascending urinary tract infection.

In contrast to the pattern of C3 expression seen in LPS stimulation, intrarenal C3 mRNA is expressed in MRL-lpr/lpr mice within infiltrating mononuclear cells. The phenotype of the C3-producing cells is unknown. Immunohistochemical staining has shown increased numbers of macrophages in the interstitium and the glomeruli of these mice.[21] Macrophages are capable of synthesizing C3 and are present in the infiltrates; however, there is no evidence of increased C3 signal in the glomeruli, where the macrophages are abundant. A characteristic of MRL-lpr/lpr mice is progressive accumulation of T cells with the phenotype $CD4^-$, $CD8^-$, $B220^+$ in the thymus, lymph nodes, spleen and kidney (and presumably heart and lung).[36,37] T cells infiltrating the kidney have been shown to be autoreactive.[37] T cells are not generally thought to produce complement. However, recent experiments by Melchers *et al.* have provided evidence for a truncated C3 transcript expressed in the spleen, presumably by T cells.[38] Additional experiments will be necessary to identify the cell type(s) responsible for C3 mRNA expression in MRL-lpr/lpr kidney.

The data in this report and others emphasize the potential role of local extrahepatic expression of C3 in the pathophysiological processes leading to end organ damage in the MRL-lpr/lpr model of inflammation and the response of extrahepatic tissues to acute phase stimuli. The recognition of cellular specificity in the stimuli leading to increased extrahepatic complement gene expression suggests new opportunities for modulating the inflammatory response.

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