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Cell-mediated pathology during murine malaria-associated nephritis

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SUMMARY

We have studied the cellular mechanisms involved in the development of nephritis during acute and chronic murine malaria infections induced by *Plasmodium vinckei petteri* and *P. berghei* respectively. Albuminuria and uraemia were observed during the early stages of both types of infection, and were associated with glomerular and interstitial hypercellularity. There was a gradual increase in numbers of $CD45^+$ cells from the early stages of both infections onwards. These infiltrates contained $CD4^+$ and $CD8^+$ cells and mononuclear phagocytes. The interstitial and glomerular hypercellularity was due to an influx of inflammatory cells rather than an increase in renal cell division. These findings indicate the importance of cell-mediated immune mechanisms in the development of nephritis during murine malaria and illustrate an example of naturally occurring infection-induced nephritis.

Keywords malaria kidney immunopathology nephritis

INTRODUCTION

Human malarial infections are often accompanied by nephritis. Tertiary human falciparum infection causes acute transient nephritis with mild clinical symptoms which resolves in response to antimalarial therapy [1,2]. Conversely, nephritis associated with quartan infections may be more chronic, and does not resolve upon chemotherapy and subsequent parasite clearance [3,4].

Previous reports support the hypothesis that immune complexes initiate nephritis in human and experimental malaria [5], but the role of cell-mediated immune mechanisms remains largely unexplored. In particular, the contribution of inflammatory cells has not been examined despite the fact that these cells make a major contribution to the formation of lesions in other forms of experimental nephritides [6,7]. In various experimental forms of nephritis an increase in the number of cells within the glomerulus correlates with the onset of proteinuria [8,9]. The participation of mononuclear phagocytes has been especially examined owing to their appearance in other forms of inflammation, and because of their ability to secrete complement components, chemotactic factors and degradative enzymes.

We have examined the role of cell-mediated inflammation in the initiation and development of nephritis during murine malarial infections by the histological analysis of cell types present at defined stages of infection.

MATERIALS AND METHODS

Parasite maintenance and serum and urine collection

Two lethal strains of murine malaria were used, acute (*Plasmodium vinckei petteri*), and chronic (*P. berghei* clone RC), both kindly provided by Mr B. Robinson (London School of Hygiene and Tropical Medicine, St Albans, UK). Both infections were initiated by blood passage: 5×10^3 infected erythrocytes were inoculated intraperitoneally into 6-week-old female LACA mice (Tuck & Sons, Battlebridge, UK). The infection was followed by preparing daily tail blood smears [10], and calculating percentage parasitaemia in 2000-cell samples in random fields. Terminal blood samples were collected by cardiac puncture, allowed to clot at 37°C for 1 h, centrifuged at 200 ***g*** for 10 min, then serum was removed and stored at −20°C. Urine samples were collected from individual mice every 24 h (before and after infection) by applying gentle pressure on the bladder.

Experimental protocol

Mice were killed in groups throughout the course of each infection. Time points were predetermined by plotting parasitaemia curves for each infection and determining on which days parasitaemias were low (0–2%), medium (3–55%) and high (56% and above). For *P. v. petteri* the average survival was 13 days and stages of infection were: early, days 0–7; mid, days 8–10; late, day 11 until death. Therefore, groups of five *P. v. petteri*-infected mice were killed by exsanguination under terminal anaesthesia on days 5, 7, 9 and 11. For *P. berghei* the stages of infection were: early, days 0–11; mid, days 12–20; late, day 21 until death, and groups of five *P. berghei*-infected mice were killed on days 10, 18, 21 and 25. Our results are obtained from four different experiments with each infection.

Assessment of kidney dysfunction

Albuminuria. A sandwich capture ELISA was used as previously described [10]. The significance of increases in

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albuminuria was tested by the Kruskal–Wallis test, with between five and 12 mice in each group at each stage of infection.

Blood urea nitrogen. Pre- and post-infection serum was measured by the urease/Berthelot reaction using a kit (Sigma Ltd, No. 640-A), the instructions being adapted to smaller volumes (details available on request). Increases in blood urea nitrogen were tested for significance by the Kruskal–Wallis test with between six and 16 mice in each group.

Morphological changes. Kidneys removed from killed mice were cut in half longitudinally, fixed in formal buffered saline, then wax embedded. Sections (4 μm) were cut and stained with haematoxylin and eosin. Stained sections were assessed microscopically.

Assessment of proliferating cells within glomeruli

The renal incorporation of bromodeoxyuridine (BrdU) (Sigma Ltd, Poole, UK) at different stages of infection was assessed to determine whether an increase in glomerular cell proliferation explained renal hypercellularity. Each mouse was injected intraperitoneally with 0·1 ml freshly-made BrdU in PBS (100 mg/kg) 1 h before sacrifice. Kidneys were removed, fixed in formal buffered saline and embedded in wax. Thymus tissue was removed from four uninfected mice for positive controls and was processed similarly. Sections (4 μm) were placed on poly-L-lysine-coated, standard microscope slides, then dewaxed and hydrated.

Endogenous peroxidase was blocked by incubation in a bath of 0·1% hydrogen peroxide (BDH Ltd) in methanol, for 30 min at room temperature, before washing in tap water (twice, 5 min each), then washed in distilled water prewarmed to 37°C (10 min). Sections were transferred to freshly prepared trypsin solution (0·1% trypsin in 0·1% calcium chloride in distilled water, adjusted to pH 7·8 with 1 M sodium hydroxide), at 37°C. After 13 min, slides were washed in cold water for 15 min to inactivate the trypsin, incubated for 15 min in 1 M hydrochloric acid, preheated to 60°C, washed under running tap water for 15 min, then flooded with 0·1 M Tris-buffered saline, pH 7·6, for at least 15 min. Sections were stained for BrdU by an avidin/biotin staining system. All incubations were at room temperature, under humidified conditions. Slides were washed twice in PBS between steps, for 5 min each time. Sections were overlaid with 25 μl of reagent at each stage. Three sequential blocking steps were performed to prevent non-specific antibody binding: sections were overlaid with avidin, then with biotin (20 min each step, Vectastain kit, Vector Labs, Peterborough, UK), and finally with 20% fetal calf serum (FCS) in PBS for 15 min. The blocking stages were followed by adding a 1:30 dilution of mouse anti-BrdU (Dako Ltd, High Wycombe, UK) in PBS supplemented with 10% normal mouse serum (10%NMS/PBS), overnight at 4°C. Controls were prepared from normal kidney, which has very few dividing cells (negative), and thymus (positive). Binding of primary antibody was detected by biotinylated rabbit anti-mouse immunoglobulin (Jackson Immunoresearch Labs, Stratech Scientific Ltd, Luton, UK), and then streptavidin-peroxidase complex (Amersham Ltd, Aylesbury, UK), both at 1:50 in PBS, for 30 min. Sections were flooded with peroxidase substrate solution (400 μg of diaminobenzidine/ml of PBS, containing 0·01% hydrogen peroxidase), counterstained with Harris' haematoxylin for 1 min, then rinsed under running tap water for 1 min. Controls were prepared by selectively omitting MoAb, biotinylated rabbit anti-mouse immunoglobulins, or streptavidin complex. Finally, slides were dehydrated through alcohol and cleared in xylene, mounted, then assessed by microscopy for intracellular brown staining.

Immunohistochemical identification of cell types

Kidneys removed from uninfected and infected mice were cut in half longitudinally, the halves rolled in OCT compound (BDH), mounted on card platforms, snap frozen in hexane, cooled by liquid nitrogen, and stored at −70°C. Cryostat sections (4 μm) on 12-well Multitest slides (Flow Labs, Rickmansworth, UK) were dried for 2 h at room temperature, then fixed at 4°C in acetone for 20 min. Cells were identified by avidin/biotin staining, as above. All incubations were carried out under humidified conditions, and slides were washed between each step (twice for 5 min each). Blocking of non-specific binding was achieved by a three-step phase. Sections were overlaid with avidin, biotin and FCS, as above, and incubated overnight at 4°C with MoAbs specific for murine inflammatory cell surface markers: MOMA-2-anti-macrophages and monocytes (1:50; Serotech Ltd); anti-CD45 (1:20; Boeringher Mannheim Ltd); YTS 191-anti-CD4 (1:10) and YTS 169-anti-CD8 (neat). The last two were supernatants from cultured hybridoma cells obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). Cultures were maintained at $3–9 \times 10^5$ cells/ml in RPMI 1640 + 10% fetal bovine serum (FBS), at 37°C and 5%CO_2, grown to exhaustion and the supernatants collected. After washing off excess antibody, bound antibody was visualized by incubation with biotinylated sheep anti-rat immunoglobulin (1·1 mg/ml, given by Dr S. Hobbs, Chester Beatty, Sutton, UK), then with streptavidin peroxidase complex (Amersham), both diluted at 1:50 in 10% NMS/PBS, for 30 min. Finally, slides were flooded with peroxidase substrate solution (400 μg diaminobenzidine/ml of PBS, containing 0·01% hydrogen peroxide) for 10 min. Control sections were included where MoAb, biotinylated anti-rat immunoglobulin or streptavidin complex were selectively omitted. All preparations were counterstained with Harris' haematoxylin (BDH) and examined for antibody binding. Each section was scored by counting the number of positively stained cells within at least 25 glomeruli per section, at a magnification of ×400. The overall significance was tested by the Kruskal–Wallis test. In addition, differences between preinfection values and each post-infection time period were tested by the Mann–Whitney *U*-test, with between 5 and 13 mice at each stage of infection, for both infections.

RESULTS

Kidney dysfunction

Abnormalities were noted during the early phase of both types of infection. As previously described, mice infected with either *P. v. petteri* or *P. berghei* showed increases in albuminuria from early infection [10]; the results are summarized in Table 1. Significantly increased levels of blood urea nitrogen were observed from the mid or late stages of acute or chronic infection ($P < 0·001$, Kruskal–Wallis test) (Fig. 1 a,b).

Morphological changes

Morphological abnormalities were detected during the early stages of both acute and chronic infections when a patent parasitaemia was observed. At mid-infection stages increased numbers of cells could be seen within glomeruli, and at later

Table 1. Changes in albuminuria during infection with *Plasmodium vinckei petteri* and *P. berghei* RC

	Stage	*n*	Albumin (μg/ml) Median	Range
P. v. petteri	Pre	20	4	0–24
	Early	20	12	2–279
	Mid	20	154	20–782
	Late	6	809	124–1874
P.berghei	Pre	20	4	1–23
	Early	20	6	1–188
	Mid	23	79	13–601
	Late	12	104	46–13 788

stages of infection cells were also seen within the interstitium (Fig. 2 a, b). Interstitial infiltrates were invariably present from around day 20 of *P. berghei* infections, and at late infection in some mice following *P. v. petteri* infection. These infiltrates were made up of parasitized erythrocytes and cells resembling mononuclear phagocytes. Some of these cells contained parasite pigment, which was also seen deposited within glomeruli in mesangial and infiltrating cells.

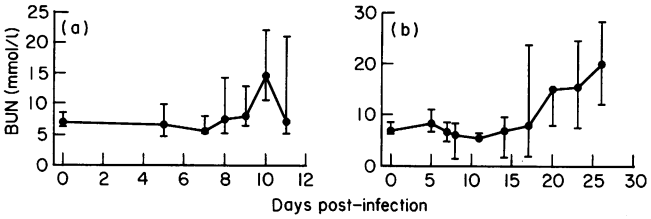

Fig. 1. Median and ranges to show changes in blood urea nitrogen (BUN) during infection with (a) *Plasmodium vinckei petteri*, and (b) *P. berghei*.

Glomerular proliferation

Thymus sections from uninfected mice showed positive staining, indicating that proliferating cells were present, and kidney sections from uninfected mice were negative. Kidneys from mice infected with *P. v. petteri* showed little evidence of cellular proliferation, but some positive cells were observed within tubular epithelium, and at later stages within some glomeruli. Kidneys from early stages of infection with *P. berghei* did not show any BrdU incorporation. However, sections from mice killed at mid infection showed many cells that were positively stained, as shown in Fig. 3a. These cells were predominantly within interstitial infiltrates, but some were also seen within

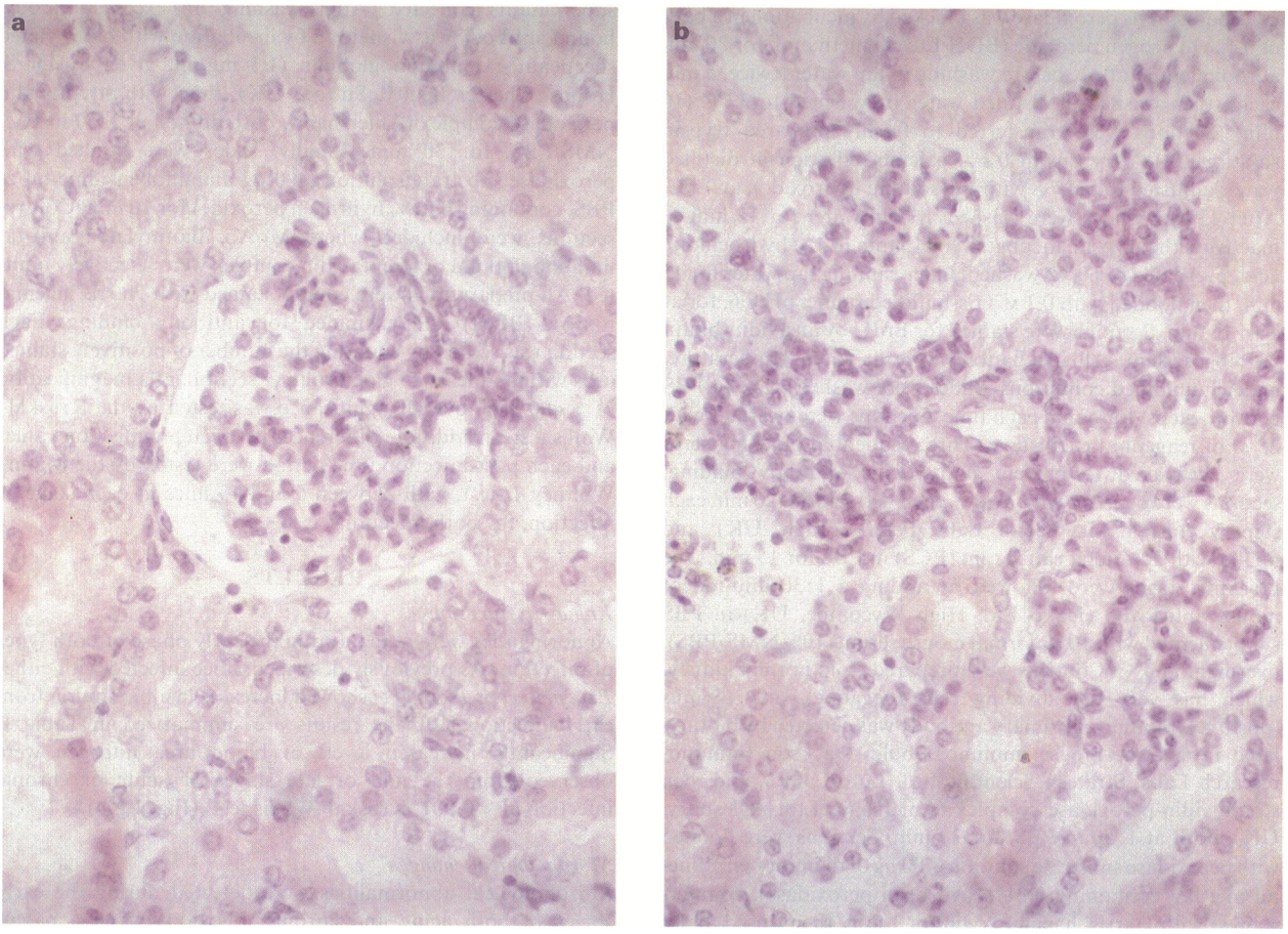

Fig. 2. Morphological changes in the kidney on day 26 of infection with *Plasmodium berghei* RC. Haematoxylin and eosin staining, (a) ×457, (b) ×286.

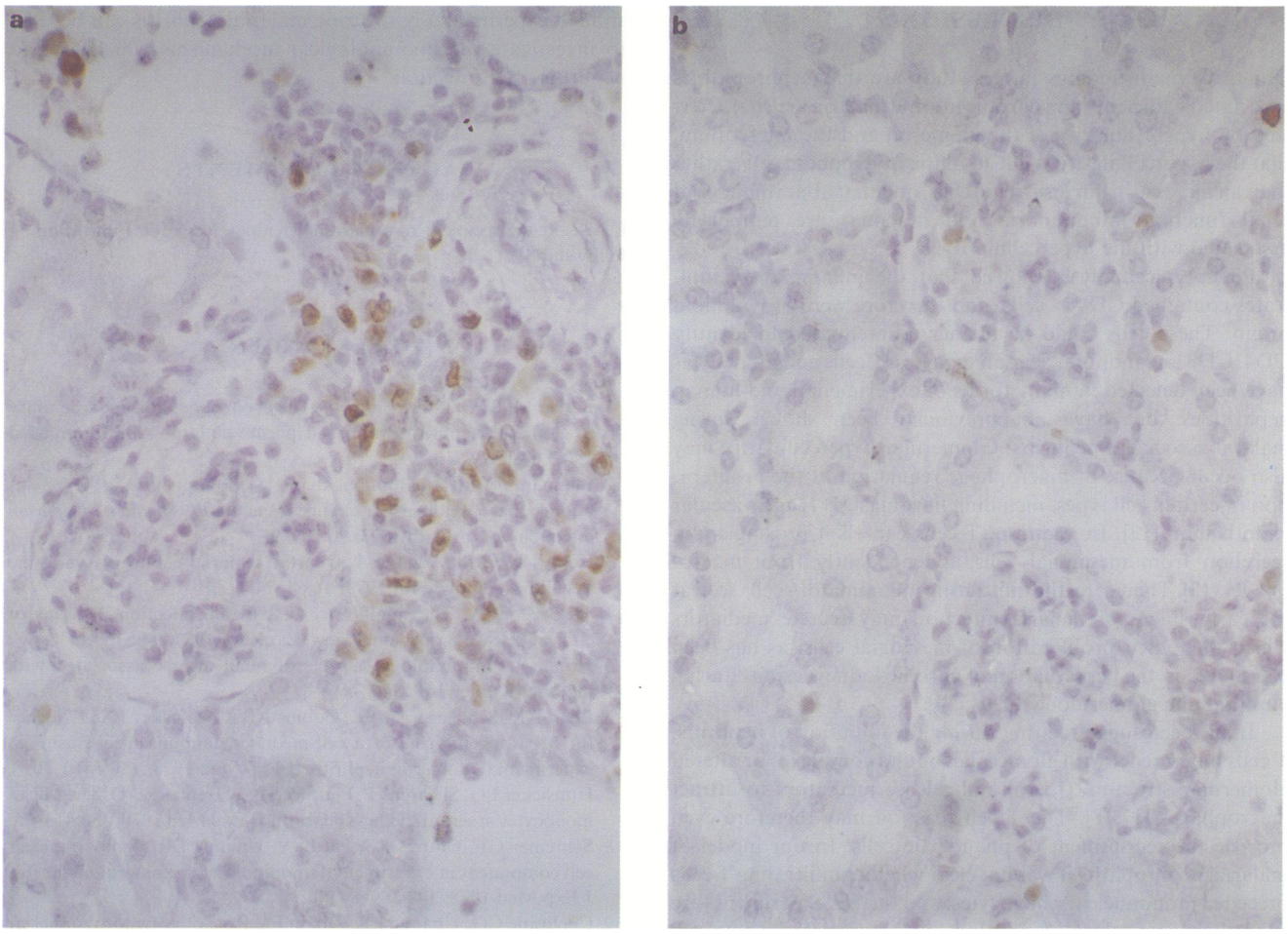

Fig. 3. Detection of proliferating cells within the kidneys during infection with *Plasmodium berghei* RC. Proliferating cells are marked by the incorporation of bromodeoxyuridine (BrdU), shown here as brown peroxidase staining. Generally, proliferating cells were observed within interstitial infiltrates (a, day 26, ×457); with very few positive cells present within glomeruli (b, day 17, ×286).

tubular cells. Several glomeruli also showed one or two dividing cells (Fig. 3b). Parasite pigment within inflammatory cells can be observed as yellow/brown granules. This is the normal colour of such pigment, and is easily distinguishable from BrdU incorporation by its different refractory properties.

Identification of inflammatory cells

Increased numbers of CD45$^+$ cells were found in the glomeruli of animals with acute or chronic infections. The CD45 surface marker is found on all cells of lymphoid origin, and therefore distinguishes infiltrating inflammatory cells from resident kidney cells. Glomerular infiltrates were composed of CD4$^+$ and CD8$^+$ cells, as well as mononuclear phagocytes. These cells were present in low numbers in sections from uninfected mice and numbers rose from early infection. The overall difference between groups was statistically different for both infections ($P < 0{\cdot}001$, Kruskal–Wallis test). In addition, the post-infection increase was significantly higher than preinfection for all markers during both acute and chronic infection ($P < 0{\cdot}005$, Mann–Whitney *U*-test) (Fig. 4a, b). If the numbers of cells found within glomeruli are compared between infections it can be seen that greater numbers of cells are observed within glomeruli during infection with *P. berghei* than with *P. v. petteri*. CD45$^+$ cells were the most predominant cell type throughout the course of both acute and chronic infections; however, it was not possible to determine which particular cell type was the first to infiltrate glomeruli.

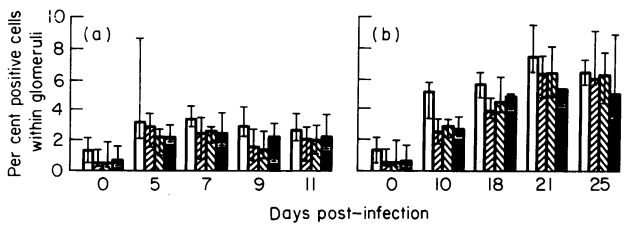

Fig. 4. Median numbers of infiltrating inflammatory cells observed within glomeruli of mice infected with (a) *Plasmodium vinckei petteri* and (b) *P. berghei*. Bars show ranges for each group of mice. □, CD45; ■, CD4; ■, CD8; ■, macrophages.

CD45$^+$ cells were also observed within the interstitial infiltrates of mice killed at the later stages of both infections. This was particularly obvious in mice infected with *P. berghei*, where these infiltrates were present from around day 20. Interstitial infiltrates were found to contain CD4$^+$ and CD8$^+$ cells, as well as mononuclear phagocytes.

DISCUSSION

Our three main findings in this study are that nephropathies associated with acute and chronic murine malaria are characterized by hypercellularity of the glomeruli and the interstitium, that the hypercellularity is due to influx of lymphoid cells rather than an increase in intrinsic renal cells, and that abnormalities of renal function (albuminuria and uraemia) are temporally associated with the hypercellularity.

Phenotypic analysis showed that the inflammatory infiltrates contained $CD45^+$ cells, and therefore were of lymphoid origin. These cells could be further differentiated to identify CD4, CD8 and mononuclear phagocytes. T cells and monocytes have been detected in many forms of experimental and human nephritides, but have not previously been shown during malaria-associated nephritis. Glomerular hypercellularity may also occur because of macrophage regulation of the proliferation of certain cell types, including fibroblasts [11] and vascular endothelium [12]. In addition, T cells can elicit prostaglandin secretion from mesangial cells, independently from macrophages [13]. Therefore, the infiltrating inflammatory cells seen at the early stages of malaria infection may secrete mediators which then elicit proliferation of mesangial cells, as has been postulated in the perpetuation of chronic kidney lesions during autoimmune glomerulonephritis [14].

It has been suggested that during the initiation of nephritis, T cells might react with glomerular-bound complexes or altered glomerular structure [15,16] and release mediators to attract macrophages [17,18]. The T cell reaction may therefore even precede the recruitment of phagocytic cells. In our model of malarial nephropathy it was not clear whether infiltrating T cells preceded mononuclear phagocytes, and further experiments are needed to determine the relationship between these two cell populations.

The occurrence of interstitial nephritis has been reported previously in murine malaria nephropathy [19], but has not yet been characterized immunologically. It was found only in the later stages of infection, in particular during *P. berghei* infection, and these interstitial infiltrates accounted for the majority of proliferating cells. Tubulointerstitial nephritis is presumably a consequence of malaria infection, and is very likely to contribute to renal failure during infection.

The infiltrating inflammatory cells coincided with the onset of renal functional abnormalities and peaked with maximum albuminuria, implying that they caused the functional changes in this model. T cells and macrophages can release cytokines which may cause tissue damage as well as attract further cell populations to the area. Tissue damage may also occur after the release of reactive oxygen intermediates or nitric oxide.

By showing that inflammatory cells are present in the glomerulus and interstitium during murine malarial nephropathy, this study has shown that the pathology of this condition, and very likely the pathogenesis of the disease, has an important cellular component which may be further dissected in experimental models, and even possibly treated in man by various manoeuvres designed to reduce the inflammatory infiltrate.

This study also illustrates the use of plasmodia spp. to initiate nephritis. Whereas most models of experimental nephritis result from immunization with proteins, our model exhibits nephritis caused by a naturally occurring infection. Infection-induced nephritis is a common condition, but has been little studied experimentally. Murine malarial nephritis can be used to investigate the immunological mechanisms which lead to nephritis after infection.

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