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Trafficking of inflammatory macrophages from the kidney to draining lymph nodes during experimental glomerulonephritis

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SUMMARY

Macrophage accumulation within the glomerulus and renal interstitium is a prominent feature of most forms of glomerulonephritis, but the fate of these inflammatory cells is unknown. Macrophage trafficking to the draining kidney lymph nodes (KLN) was assessed in a detailed kinetic analysis of accelerated antiglomerular basement membrane (GBM) disease in the rat. Leucocytes draining to KLN via lymphatic vessels were identified within the marginal sinus by MoAb labelling of tissue sections. In anti-GBM disease, there was a significant increase in the weight of the KLN due to both lymphoproliferation within the nodes and increased lymphatic drainage from the inflamed kidney, as evidenced by prominent dilation of the marginal sinus and increased numbers of cells within the sinus. In non-inflamed lymph nodes, few ED1$^+$ macrophages were present within the marginal sinus ($3{\cdot}0 \pm 0{\cdot}6$/100 nucleated cells). However, in anti-GBM disease, macrophages became the major cell type within the dilated marginal sinus of the KLN, as shown by labelling with ED1, ED2 and ED3 MoAbs, peaking at $74 \pm 2{\cdot}6$ ED1$^+$ cells/100 nucleated cells at day 14. These changes were not simply due to systemic antigen administration, since in the axillary lymph node (ALN) there was no obvious dilation of the marginal sinus and macrophages accounted for a maximum of only $15 \pm 4{\cdot}6$ ED1$^+$ cells/100 nucleated cells. In conclusion, this study provides indirect evidence that there is significant trafficking of the renal macrophage infiltrate to the KLN during experimental glomerulonephritis. This may be a mechanism whereby nephritogenic antigens, released as a consequence of the local inflammatory response, may be presented to T and B lymphocytes within lymph nodes, resulting in the amplification of the immune response in glomerulonephritis.

Keywords macrophage trafficking glomerulonephritis kidney lymph node

INTRODUCTION

Glomerulonephritis is an immune-mediated disease which features prominent leucocyte accumulation within the kidney [1,2]. Glomerular macrophage infiltration is seen in most forms of glomerulonephritis, with T cell infiltration apparent in the more aggressive forms of disease [3–5]. There is also significant interstitial infiltration of both macrophages and T cells in all types of glomerulonephritis except minimal change disease [3,4]. The intensity of interstitial leucocytic infiltration correlates with the degree of renal function impairment [3,6], and predicts long term prognosis of patients [6,7]. Similar patterns of leucocytic infiltration have been described in a wide variety of experimental models of glomerulonephritis [2]. In experimental anti-glomerular basement membrane (GBM) disease, glomerular macrophage infiltration is necessary for the induction of glomerular injury [8], while the accumulation of leucocytes within the interstitium (particularly immune-activated mononuclear cells) correlates strongly with progressive renal dysfunction in this model [9]. However, the fate of inflammatory macrophages within the kidney during glomerulonephritis is unknown.

Lymphocyte recirculation is well characterized [10], and dendritic cells have also been shown to migrate to germinal centres of lymphoid tissues [11], but the fate of macrophages accumulating at sites of tissue inflammation has received little attention. During disease, monocytes enter tissues from the blood, but their fate thereafter is unclear [12]. This question was investigated in a rat model of anti-GBM glomerulonephritis. Leucocytes entering the marginal sinus of the kidney lymph nodes (KLN) during the evolution of disease were phenotyped by MoAb labelling in order to determine whether inflammatory tissue macrophages left the kidney via the lymphatic system.

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MATERIALS AND METHODS

Animals

Inbred male Sprague-Dawley rats, 250–300 g body weight, were obtained from the Monash University Animal Services (Clayton, Australia).

Nephrotoxic serum

Rabbit nephrotoxic serum was raised by repeated immunization of one rabbit with particulate rat GBM emulsified in Freund's incomplete adjuvant (FIA) as previously described [13]. Anti-GBM serum was pooled, decomplemented, and absorbed extensively against normal rat erythrocytes.

Accelerated anti-GBM disease

Passive accelerated anti-GBM disease was induced in rats by subcutaneous immunization with 5 mg normal rabbit IgG (Silenus, Australia) in FIA followed 5 days later by i.v. injection of nephrotoxic serum (10 ml/kg body weight). Groups of four animals were killed at 12 h, days 1, 2, 3, 7, 14, 21 and 28 after injection of nephrotoxic serum. In addition, one group of four normal rats was studied.

Tissue preparation

Lymphatic ducts and draining kidney lymph nodes were identified following injection of 2% trypan blue dye into the subcapsule and surface of the renal cortex *in situ*. Before removal of the kidneys, the left lateral lumbar nodes around the aortic hiatus (which receive the lymphatic drainage of the left kidney) were removed, weighed, and fixed in 2% paraformaldehyde-lysine-periodate [14]. As control nodes, the left axillary lymph node was removed, weighed, and fixed as above.

Immunohistochemistry

Tissues for histochemistry and immunoperoxidase staining were fixed in 2% paraformaldehyde-lysine-periodate. Cryostat tissue sections (6 μm) were stained for non-specific esterase (NSE) activity using the α-naphthyl acetate method [15]. For immunoperoxidase staining, serial cryostat sections (6 μm) were adhered to gelatin-coated microscope slides and labelled with monoclonal and polyclonal antibodies as previously described [9]. Antibodies used in this study were: OX-19, rat CD5 antigen (pan T cells) [16]; OX-33, B cell-restricted form of the CD45 leucocyte common antigen [17] ED1, macrophages and dendritic cells [18]; ED2, tissue macrophage subsets [18]; ED3, tissue macrophage subsets [18]; horseradish peroxidase-conjugated swine anti-rabbit IgG (Dako Ltd, Denmark).

Quantification of leucocytes within the kidney

Leucocyte subpopulations within the glomerulus and interstitium were analysed on immunoperoxidase-stained cryostat sections. Cells labelled by each MoAb were counted in high-power fields ($\times 400$) of 20 consecutive glomeruli for each animal. To assess tubulointerstitial leucocyte infiltration, cortical areas were selected at random. The number of labelled cells was assessed from 20 consecutive high power fields by means of a 0·02 mm^2 graticule fitted in the eyepiece of the microscope. These fields progressed from the outer to inner cortex, avoiding only large vessels, glomerular and immediate periglomerular areas. For each tissue, the same area was examined in serial sections labelled with different MoAbs. No adjustment of the cell count for tubules and luminal space was made.

Semiquantification of lymphatic drainage in marginal sinus of lymph nodes

Cryostat sections of KLN and axillary lymph node (ALN) were labelled with MoAbs by the immunoperoxidase technique. When dilated, the marginal sinus was easily identified as the space between the capsule of the node and the inner layer of sinus. When there was no dilation, only the single subcapsular layer of cells was used for counting purposes. In view of the anatomical structure of the marginal sinus and the varying degree of dilation apparent in different tissues, quantification of the total number of any one cell population within the sinus was not feasible. Thus, labelled cells within the marginal sinus were counted in high power fields and expressed as the percentage of labelled cells per 100 nucleated cells. In each case, at least 100 nucleated cells were scored.

Statistical analysis

Comparison of lymph node weight and MoAb labelling of cells within the marginal sinus between groups of animals in anti-GBM disease and normal controls was assessed by one way analysis of variance (ANOVA) from the Complete Statistical System (CSS) package.

RESULTS

Macrophage infiltration within the kidney

Figure 1 summarizes the pattern of macrophage and T cell infiltration into the glomerulus and the interstitium during the evolution of anti-GBM glomerulonephritis. Following injection

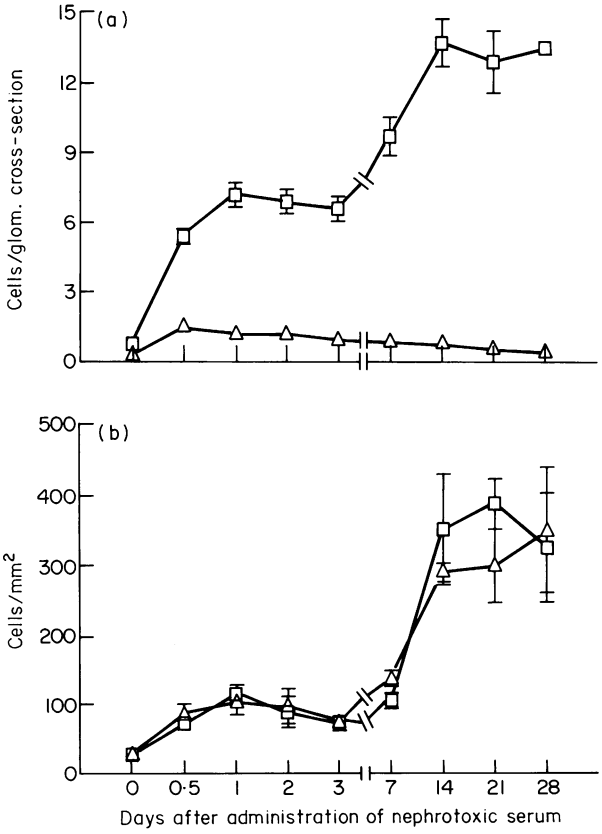

Fig. 1. Leucocyte infiltration into the kidney during the development of anti-glomerular basement membrane (GBM) glomerulonephritis. The number of ED1$^+$ macrophages (□) and OX-19$^+$ T cells (△) in (a) glomeruli, and (b) interstitium, were quantified in MoAb-labelled cryostat tissue sections as described in Materials and Methods. Each point represents the mean count of labelled cells ± s.e.m. for groups of four animals.

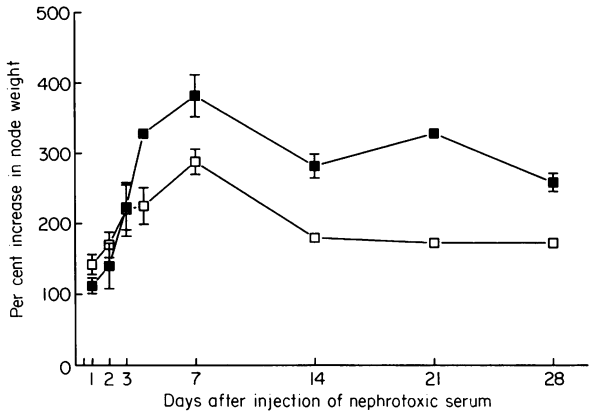

Fig. 2. Increase in lymph node weight during the development of anti-glomerular basement membrane (GBM) glomerulonephritis. The weight of kidney lymph nodes (KLN) and axillary lymph nodes (ALN) was determined at the time of sacrifice and expressed as a per cent increase relative to the weight of non-inflamed nodes from normal animals (normal KLN 4·7 ± 1·1 mg; normal ALN 40·0 ± 2·3 mg). Each point represents the mean ± s.e.m. for groups of four animals. ■, KLN; □, ALN.

of rabbit anti-GBM serum, there was linear deposition of rabbit IgG and then rat IgG along the GBM which led to an accumulation of both ED1$^+$ macrophages and OX-19$^+$ (CD5$^+$) T cells within the glomerulus and the hilar region at 12 h. Although glomerular T cell infiltration was only apparent during the first 3 days, macrophages continued to accumulate within the glomerulus. Following the initial accumulation of ED1$^+$ macrophages and OX-19$^+$ (CD5$^+$) T cells within the periglomerular area, these cells became widespread throughout the tubulointerstitium from day 7 onwards (Fig. 1, [9]). The interstitial macrophage infiltrate was phenotypically heterogeneous with less than 20% of ED1$^+$ cells labelled by ED2 or ED3 MoAbs, while no ED2$^+$ or ED3$^+$ macrophages were seen in the glomerulus.

Histological changes within the KLN

A significant increase in KLN weight was first apparent at day 2 of the experimental course ($P < 0{\cdot}05$ *versus* normal) and this peaked on day 7 (Fig. 2). Microscopically, from day 3 onwards there was extensive dilation of the marginal and cortical sinus of the KLN, with a substantial increase in the number of cells within the sinus, many of which stained positive for NSE, indicating a macrophage or dendritic cell phenotype (Fig. 3a). There was also marked lymphocyte proliferation within the KLN as evidenced by enlargement of germinal centres and a significant increase in the number of cells expressing the IL-2 receptor (IL-2R) within both T and B cell compartments [19].

Antigen (rabbit IgG) was deposited within germinal centres of KLN at 12 h after injection of nephrotoxic serum as assessed by immunoperoxidase staining, and the intensity of antigen deposition increased with time. Similarly, antigen deposited within germinal centres of the ALN at 12 h and increased in intensity with time, although this deposition did not appear as intense as that in the KLN.

To investigate the role of systemic antigen administration in mediating lymph node changes, the ALN was examined. There was an increase in ALN weight which followed the same kinetics

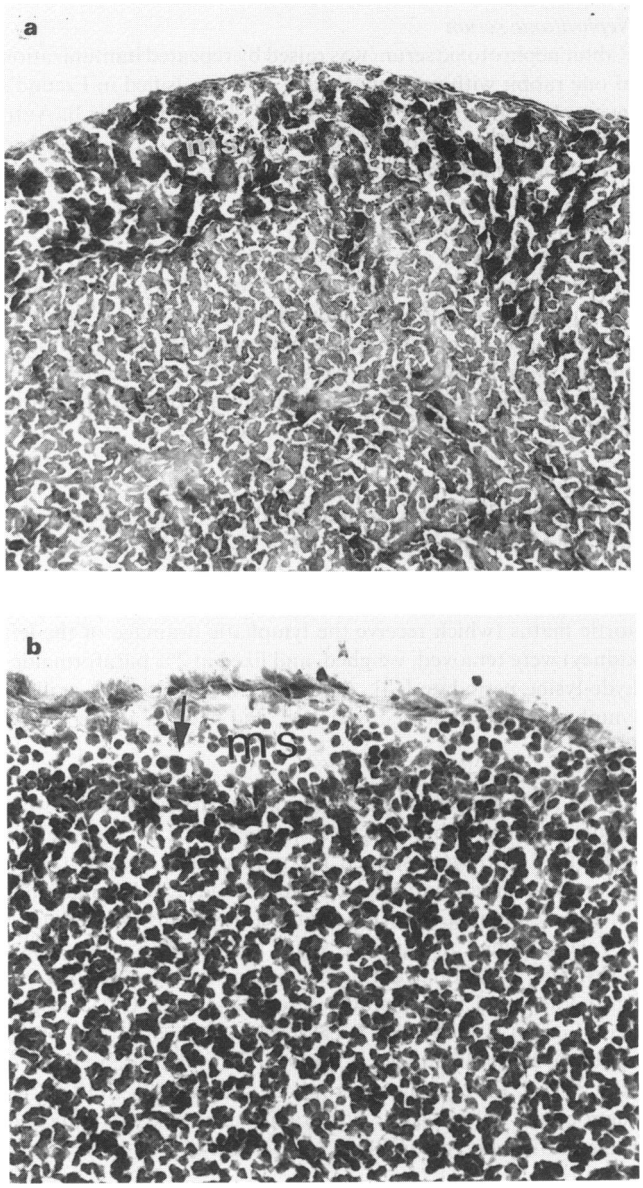

Fig. 3. Dilation of the marginal sinus of lymph nodes in anti-glomerular basement membrane (GBM) glomerulonephritis. Cryostat tissue sections were stained for non-specific esterase (NSE). (a) Day 7 kidney lymph nodes (KLN) showing very marked dilation of the marginal sinus (ms) with many NSE$^+$ cells within the sinus. (b) Day 7 axillary lymph nodes (ALN) showing no dilation of the marginal sinus (ms) with few NSE$^+$ cells present (arrow) (× 250).

as that for the KLN, peaking at approximately three times normal weight on day 7 (Fig. 2). Microscopically, there was no apparent dilation of the marginal sinus, nor an obvious change in the number of both total nucleated cells and NSE$^+$ cells within the sinus, indicating that there was no significant increase in the lymphatic drainage to the ALN (Fig. 3b). There was marked lymphocyte proliferation within the ALN, as shown by enlargement of germinal centres and increased numbers of IL-2R$^+$ cells within both T and B cell compartments, which probably accounts for the increase in ALN weight [19].

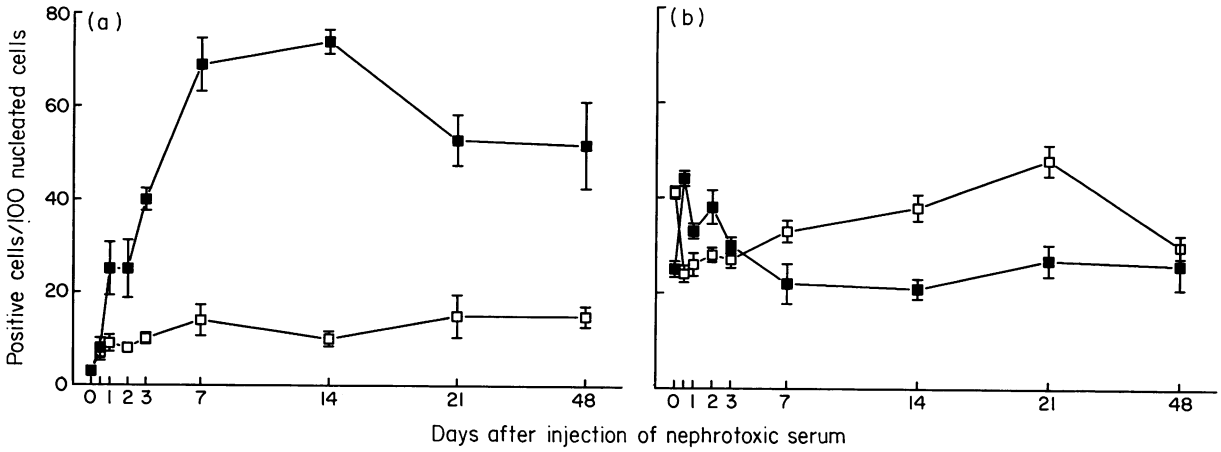

Fig. 4. Semiquantitative analysis of macrophages and T cells within the marginal sinus of lymph nodes during the development of anti-glomerular basement membrane (GBM) glomerulonephritis. (a) ED1$^+$ macrophages, and (b) OX-19$^+$ (CD5$^+$) T cells. Each point represents the mean ± s.e.m. for a group of four animals. ■, Kidney lymph nodes (KLN); □, axillary lymph nodes (ALN).

Phenotype of cells within the marginal sinus

The direct determination of the volume and leucocytic composition of lymphatic drainage from the kidney to the KLN requires cannulation of the lymphatic vessels. Unfortunately, this is not technically feasible in the rat. Thus, in order to assess the phenotype of cells trafficking into lymph nodes via lymphatic vessels, cells within the marginal sinus of lymph nodes were examined by MoAb labelling of cryostat tissue sections. This approach makes the assumption that cells within the marginal sinus reflect the composition of efferent lymphatic drainage.

In non-inflamed lymph nodes the marginal sinus is very small and contains few cells. T cells constituted 25% and 41% of cells within the marginal sinus of non-inflamed KLN and ALN respectively, with very few ED1$^+$ macrophages present (Fig. 4). The remaining cells in the marginal sinus were B cells, as identified by labelling with the OX-33 MoAb (not shown).

In the KLN, there was extensive dilation of the marginal sinus during anti-GBM disease, with a concomitant increase in the number of cells within the sinus. Many of these cells were labelled by the ED1 MoAb, indicating a macrophage phenotype (Fig. 5a), consistent with the results of NSE staining (Fig. 3a). In addition, throughout the experimental course, both ED2 and ED3 MoAbs labelled cells within the marginal sinus in a pattern very similar to that seen with the ED1 MoAb (Fig. 5b). Semiquantitative analysis showed that there was a significant increase in the percentage of ED1$^+$ cells within the marginal sinus at day 1 ($P < 0{\cdot}05$ *versus* normal), which peaked over days 7–14, when ED1$^+$ cells accounted for three-quarters of cells within the marginal sinus (Fig. 4a).

Trafficking of macrophages into the marginal sinus of the KLN was more prominent than that of T cells, as indicated by the relative percentage of the two populations, but significant OX-19$^+$ T cell trafficking was also evident. At 12 h, when there was only mild dilation of the marginal sinus, there was a significant increase in the percentage of T cells within the sinus ($P < 0{\cdot}05$ *versus* normal, Figs 4b and 5d). As disease progressed and the total number of cells within the marginal sinus increased, there was a relatively constant percentage of T cells within the sinus, indicating continued T cell trafficking from the diseased kidney (Fig. 4b).

In the ALN, there was no apparent dilation of the marginal sinus and no obvious change in the total number of cells within the sinus, although there was a minor increase in the percentage of ED1$^+$ macrophages (Figs. 4a and 5b). The percentage of OX-19$^+$ (CD5$^+$) T cells within the sinus showed a mild increase during the experimental course (Fig. 4b).

DISCUSSION

The main finding of this study was that part of the kidney macrophage infiltrate which developed during experimental anti-GBM disease trafficked to the KLN via the lymphatics. This finding is based upon two observations. First, extensive dilation of the marginal and cortical sinus and increased numbers of cells within the sinus indicated a significant increase in the volume of lymph entering the KLN. Second, labelling with ED1, ED2 and ED3 MoAbs demonstrated that macrophages became the major cell population within the dilated marginal sinus of the KLN as disease progressed.

To investigate the influence of antigen administration on lymphatic drainage to lymph nodes, the ALN was examined. In both the ALN and the KLN, there was antigen deposition within germinal centres, enlargement of germinal centres and follicles, and activation of lymphocytes within both T and B cell compartments. However, there was no apparent increase in lymphatic drainage to the ALN, as indicated by the lack of dilation of the marginal sinus. This argues that the changes within the marginal sinus of the KLN were due to cell trafficking from the inflamed kidney, and were not simply a consequence of systemic antigen administration.

How does the appearance of leucocytes within the marginal sinus of the KLN relate to leucocyte infiltration within the kidney during the development of anti-GBM disease? In the kidney, there was progressive accumulation of ED1$^+$ macrophages in both the glomerulus and interstitium [9], and this was reflected in the steady increase of ED1$^+$ macrophages within the marginal sinus of the KLN. T cell infiltration into the glomerulus was a transient event of the first 3 days, which peaked at 12 h, while interstitial T cell infiltration developed from day 7 onwards [9]. Hence, the initial increase in numbers of T cells

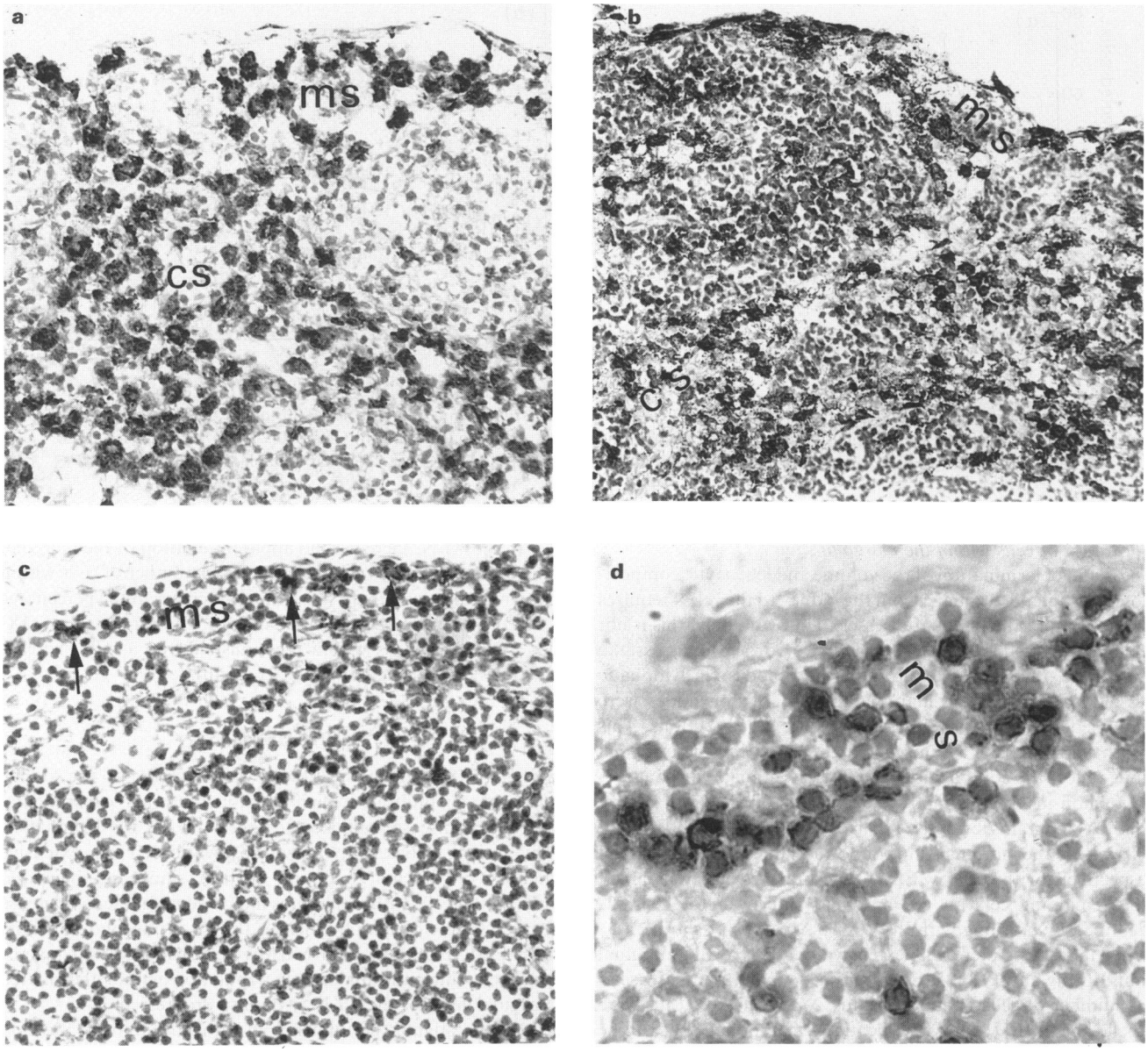

Fig. 5. Phenotypic analysis of leucocytes within the marginal sinus of lymph nodes in anti-glomerular basement membrane (GBM) glomerulonephritis. Cryostat tissue sections were labelled with MoAbs. (a) Day 7 kidney lymph nodes (KLN) showing numerous ED1$^+$ cells within the dilated marginal (ms) and cortical sinus (cs). (b) Day 7 KLN showing the numerous ED2$^+$ cells within the dilated marginal (ms) and cortical sinus (cs). (c) Day 7 axillary lymph nodes (ALN) showing few ED1$^+$ cells within the undilated marginal sinus (ms) (arrows). (d) KLN at 12 h showing numerous OX-19$^+$ (CD5$^+$) T cells within a moderately dilated marginal sinus (ms). (a–c, × 320; d, × 900.)

within the marginal sinus of the KLN may reflect trafficking of the early glomerular T cell infiltrate, while T cell trafficking from day 7 onwards may derive from the interstitial T cell infiltrate.

It is difficult to distinguish between macrophages and dendritic cells in tissue sections due to their similar morphology and variable antigenic phenotype. In the normal rat kidney, resident dendritic cells express the CD4 and CD45 antigens, but lack expression of the ED1, ED2 and ED3 antigens [9,20]. In anti-GBM disease, infiltrating interstitial macrophages expressed ED1 and CD4 antigens, with relatively few cells labelled by ED2 and ED3 MoAbs [9,21]. Thus, expression of the ED1 antigen by many cells within the marginal sinus of the KLN during anti-GBM disease suggests that these cells were macrophages as opposed to dendritic cells. This was confirmed by the labelling of many marginal sinus cells with ED2 and ED3 MoAbs, which recognize subsets of tissue macrophages but do not label dendritic cells isolated from lymphoid tissues [18]. Within the kidney, few macrophages were labelled with the ED2 or ED3 MoAbs, suggesting that macrophages trafficking to the KLN altered their cell surface antigen phenotype during the migration process, or that only a minor subset of renal macrophages is involved in trafficking to the KLN. This is an interesting point warranting further investigation.

Lymphocyte recirculation has been well characterized [10]

and dendritic cells have also been shown to migrate to lymph node germinal centres via the afferent lymphatics following contact sensitization or footpad injection [11,22]. In addition, donor dendritic cells can migrate into the white pulp of spleen via the blood circulation following allograft transplantation [23]. However, the fate of macrophages infiltrating at sites of tissue inflammation is unclear. The results of this study suggest that inflammatory macrophages can traffic to draining lymph nodes. This has important implications, since macrophages are efficient in the processing and presentation of antigens to lymphocytes. Thus, kidney antigens, including nephritogenic antigens released as a consequence of the local inflammatory response, may be transported and presented to T and B lymphocytes within lymph nodes. Therefore, trafficking of inflammatory macrophages could be a mechanism for amplifying both the local cellular-immune response and the systemic humoral response in the progression of glomerulonephritis. Such a mechanism may be relevant to the induction of autoimmune disease following local inflammatory reactions.

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