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RAPID COMMUNICATION

Induction of nitric oxide synthase in rat immune complex glomerulonephritis

ALBERTINE JANSEN, TERENCE COOK, G. MICHAEL TAYLOR, PENELOPE LARGEN, VALENTINA RIVEROS-MORENO, SALVADOR MONCADA, and VICTORIA CATTELL

Department of Histopathology, St. Mary's Hospital Medical School, Imperial College of Science and Technology, London, and Wellcome Research Laboratories, Beckenham, Kent, England, United Kingdom

Induction of nitric oxide synthase in rat immune complex glomerulonephritis. Nitric oxide (NO) is a biological mediator which is synthesized from L-arginine by a family of nitric oxide synthases (NOS). Previously we have shown that NO is synthesized *ex vivo* by glomeruli obtained from animals with acute immune complex glomerulonephritis. We have now sought evidence for the *in vivo* induction of NOS in glomeruli by immunohistochemistry using specific antisera raised against a peptide sequence of inducible mouse macrophage NOS and by *in situ* hybridization. The expression of the enzyme was studied in kidneys of rats with acute unilateral immune complex glomerulonephritis, induced by cationized IgG, by immunohistochemistry. Inducible NOS (iNOS) was present in glomeruli in nephritic (left) kidneys at the time of maximum macrophage infiltration, both within intraglomerular mononuclear cells and cells emigrating into Bowman's space. iNOS expressing cells were also present in interstitial infiltrates. There was no expression in normal rat kidneys or in glomeruli in the non-nephritic (right) kidneys of experimental rats. *In situ* hybridization confirmed the immunohistochemical localization. These results provide the first direct evidence for the presence and localization of inducible NOS in glomeruli and support a significant role for NO in the pathogenesis of immune complex glomerulonephritis.

We have previously reported induction of the NO pathway in experimental acute glomerulonephritis, measured as *ex vivo* generation of nitrite (NO_2^-) by isolated nephritic glomeruli. In four different models of immune complex mediated injury the NO_2^- generated correlated with the extent of macrophage infiltration in glomeruli [1–4], and was inhibited by *in vivo* macrophage depletion [2]. The isoform of NOS in macrophages is known to be induced by cytokines such as gamma interferon (γIFN) or by endotoxin. NO is cytostatic/cytotoxic and damages cells by binding to enzymes such as those in the mitochondrial electron transport chain [5] or possibly by formation of the highly reactive peroxynitrite radical [6]. It is therefore a likely mediator of damage in inflammation, and such a role is supported by the protective effects of NOS inhibitors in lung and skin immune complex injury [7].

We sought evidence for *in vivo* induction of NO synthesis in a model of experimental glomerulonephritis using antisera raised against a peptide sequence of inducible NOS. We studied the presence and localization of inducible NOS in kidneys at the time of maximum macrophage infiltration and compared this with *ex vivo* NO generation, measured as NO_2^- accumulation by nephritic glomeruli. Expression of inducible NOS and NO generation by RAW 264.7 cells, the mouse macrophage line from which the inducible NOS peptide sequence used to generate inducible NOS antisera was derived [8], and rat mesangial cells were also studied as controls. In addition, *in situ* hybridization was carried out to confirm the immunohistochemical results.

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Methods

Induction of glomerulonephritis

Unilateral *in situ* immune complex glomerulonephritis was induced in male Lewis rats by preimmunization with human IgG and Freund's complete adjuvant followed by isolated left renal perfusion with 400 μg cationized human IgG (pI > 9.3) as previously described [9]. Rats were killed at four days. Tissue was taken and fixed in 10% formal saline for immunohistochemistry, and *in situ* hybridization. Glomeruli were isolated as previously described [1].

Glomerular isolation

Ex vivo glomerular NO_2^- generation by nephritic glomeruli was studied as previously described with slight modifications [1]. Briefly, glomeruli were isolated by sieving and incubated in triplicate wells at 2000/ml in Dulbecco's modified Eagle's medium without phenol red, with 0.1% low endotoxin bovine serum albumin and antibiotics for 48 hours.

Cell culture

RAW 264.7 mouse macrophages were studied as a positive control for immunohistochemistry. Macrophages were cultured in Dulbecco's modified Eagle's medium without phenol red, with 10% fetal calf serum (Flow Laboratories) and antibiotics. Macrophages were plated at 4×10^5/250 μl in eight-well Lab-tek chamber slides (Nunc) for immunohistochemistry, or at 1×10^6/ml in 16 mm well plates (Nunc) for NO_2^- assay. Macrophages were stimulated with 50 U/ml rat γIFN (Holland Biotechnology BV, Leiden, The Netherlands) and LPS (1 μg/ml) (*E. coli* 0127:B8, Sigma, Poole, UK) or left unstimulated. After

Fig. 1. *RAW 264.7 mouse macrophages.* **A.** Unstimulated, **B.** stimulated with γ-interferon and LPS for 24 hours *in vitro*. Inducible NO synthase is present in the cytoplasm of stimulated macrophages. Immunoperoxidase/hematoxylin (×390).

Fig. 2. *Glomerulus 4 days after onset of glomerulonephritis.* Inducible NO synthase is localized in the cytoplasm of cells within glomerular capillaries. Immunoperoxidase/hematoxylin (×375).

Fig. 3. *Severely nephritic glomerulus 4 days after onset of glomerulonephritis.* Inducible NO synthase expression is most prominent in cells in Bowman's space. Immunoperoxidase/hematoxylin (×280).

Fig. 4. *Inducible NO synthase expression in interstitial infiltrates in glomerulonephritis.* Only a minority of mononuclear cells are staining. The tubular epithelium is negative. Immunoperoxidase/hematoxylin (×390).

Fig. 5. *Glomerulus double labeled for inducible NO synthase and macrophages.* The majority of inducible NO synthase expressing cells appear positive for the macrophage marker ED_1 (red/brown cells most prominent in the upper part of the glomerulus). There are also cells which express inducible NO synthase alone (long arrow), and macrophages which do not express inducible NO synthase (short arrow). Immunoperoxidase for inducible NO synthase (brown)/immunoalkaline phosphatase for ED_1 (red)/hematoxylin (×390).

Fig. 6. In situ *hybridization showing positive signal for inducible NO synthase in glomerulus with a distribution similar to that of inducible NO synthase immunohistochemistry seen in Figure 3.* Autoradiograph with ^{35}S labeled oligonucleotide counterstained with hematoxylin (×280).

Fig. 7. A. *Glomerulus from normal rat.* There is no localization of iNOS. Immunoperoxidase/hematoxylin (×375). **B.** *In situ* hybridization after ribonuclease treatment carried out on a section adjacent to that in Figure 6. Ribonuclease treatment abolishes the *in situ* hybridization signal. Autoradiograph with ^{35}S labeled oligonucleotide counterstained with hematoxylin (×280).

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24 hours supernatants were removed and RAW cell slides were fixed in acetone for 10 minutes for immunohistochemistry.

Glomerular mesangial cells were isolated from 6- to 10-week-old rats. Glomeruli obtained by sieving were incubated in 750 U/ml collagenase type IV (Sigma) for 20 minutes and then cultured in RPMI 1640 (Sigma) plus 2 mg/ml $NaHCO_3$ supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.25 U/ml amphotericin, ITS (5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite; Sigma) with 20% fetal calf serum (Globepharm, Esher, UK). For measurement of nitrite production passage 6 mesangial cells, grown to confluence in 35 mm wells, were incubated in 1 ml Dulbecco's modified Eagle's medium without phenol red, with 0.1% low endotoxin bovine serum albumin and antibiotics for 48 hours under basal conditions, or with 1 nM recombinant human IL-1β. For immunohistochemistry, mesangial cells were grown on coverslips and stimulated with 1 nM recombinant human IL-1β; after 24 hours the cells were fixed for 10 minutes in acetone.

NO_2^- assay

NO_2^- in glomerular or cell culture supernatants was measured by Griess reaction [10]. Briefly, 100 μl sample and 100 μl Griess reagent were added to a multiplate well and absorbance was read at 550 nm.

Antisera

The amino acid sequence of the RAW 264.7 mouse macrophage cell line inducible NO synthase was subjected to computer analysis similar to that described for the rat brain enzyme [11]. Several regions were predicted to be exposed on the surface of the native protein. The sequence QNGSPQLLTGTAQNVPESLDKLHVT located near the amino terminal end corresponding to amino acids 47-71, was selected to raise antibodies in rabbits. The raising and characterization of the antibodies have recently been described elsewhere [11, 12]. The antiserum reacts with a protein band of 135 kDa in a Western blot of an ADP-eluate of induced macrophages. No reaction was detected in a similar fraction of non-induced macrophages nor with an ADP-eluate of rat brain containing the constitutive NO synthase.

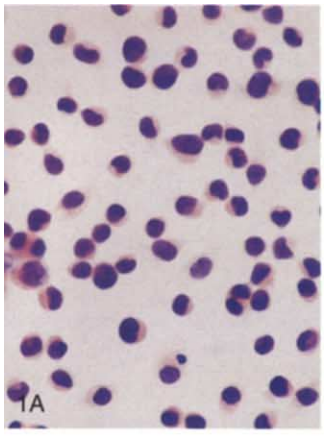
Immunohistochemistry

Renal tissue fixed in formalin was processed for paraffin sectioning. Two-micrometer thick sections were dewaxed in xylene and microwaved in citrate buffer [13]. Tissue sections, RAW cell slides and mesangial cell coverslips were then incubated with inducible NOS-antiserum (1:1500) and normal swine serum at 4°C overnight. For staining a streptavidin-biotin-peroxidase system (DAKO) with diaminobenzidine as chromogen was used. Sections were lightly counterstained with hematoxylin.

To assess macrophages as a possible source of NO, sections were double labeled with inducible NOS antiserum as above and with mouse anti-rat macrophage antibody (ED1; Serotec; 1:500, 60 min at room temperature), stained with alkaline phosphatase.

In situ hybridization

Paraffin sections (4 μm) of renal tissue were mounted on Vectabond-coated slides (Vector Laboratories, Peterborough, UK) and prepared for *in situ* hybridization as previously described [14]. Three 30-base synthetic oligonucleotide probes, with sequences deduced from the cDNA sequence of the inducible mouse macrophage NOS gene [8], were purchased from Oswel DNA Service (Edinburgh, UK). The sequences of these probes were as follows: *NOS-1* (corresponding to amino acids (aa's) 1-10: 5′-TTT-GAA-GAG-AAA-CTT-CCA-GGG-GCA-AGC-CAT-3′; *NOS-2* (aa's 607-616): 5′-AGC-ATA-CCT-GAA-GGT-GTG-GTT-GAG-TTC-TCT-3′; *NOS-3* (aa's 1103-1112): 5′-CTT-GAG-CTG-GAA-GAA-ATA-GTC-TTC-CAC-CTG-3′. The oligonucleotides were 3′ end-labeled to a specific activity of between 1.0 and 2.0 × 10^7 cpm/pmol with ^{35}S dATP. Tissue sections were studied by use of a combined battery of all three antisense probes. Specificity of the hybridization was confirmed by the following control experiments: (1) ribonuclease treatment. Control slides were treated before hybridization with ribonuclease A (20 μg/ml from bovine pancreas; Sigma),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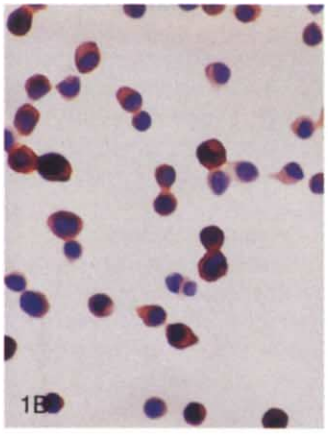
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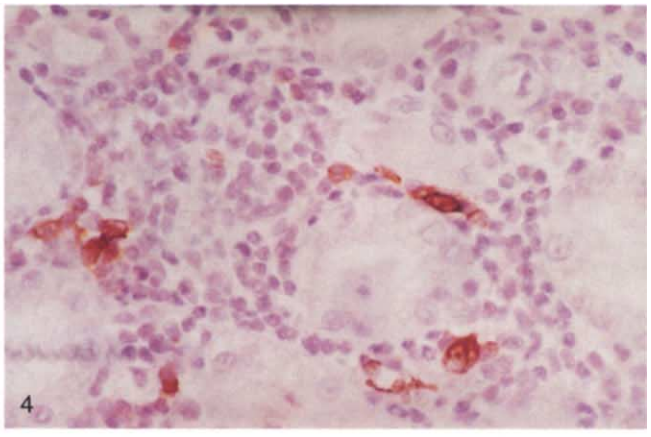
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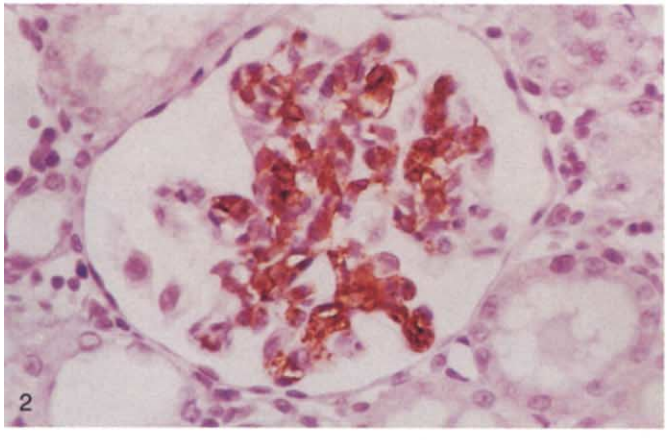
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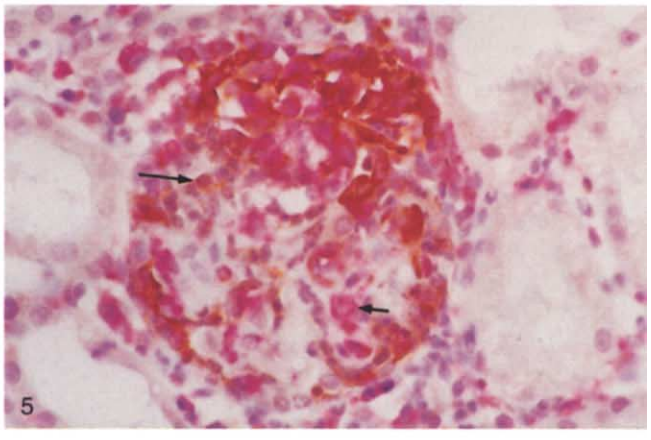
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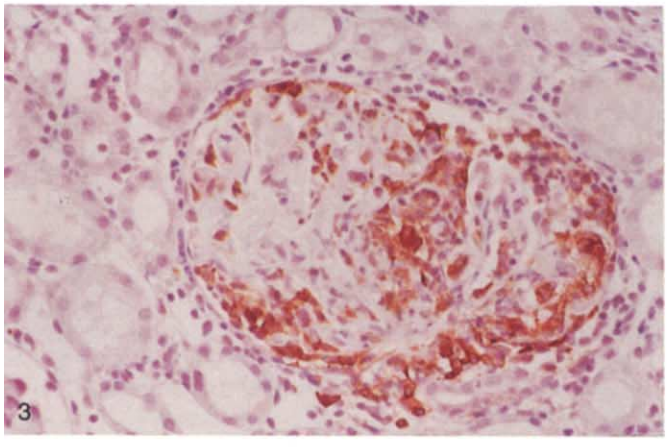
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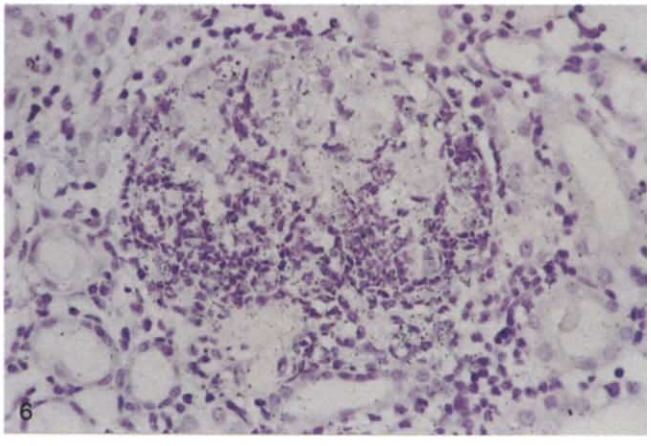
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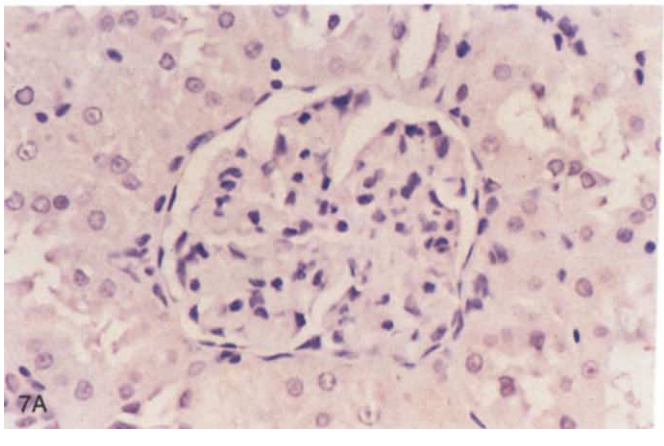
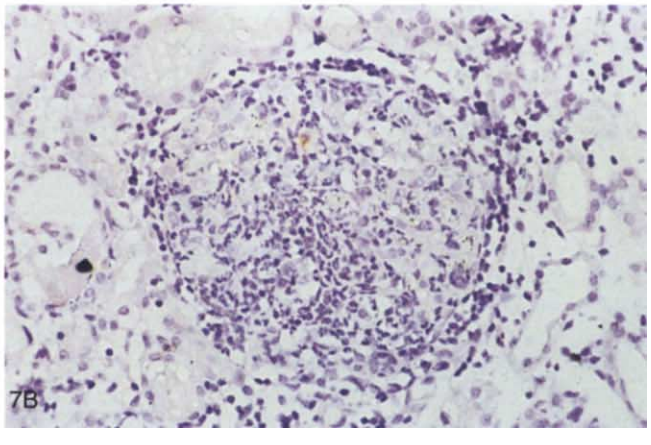

7A


7B

and ribonuclease T_1 (80 U/ml; Sigma) in 10 mM phosphate buffered saline, for 30 minutes at 37°C. (2) Competition with unlabeled probes. To validate the specificity of the method, control slides were hybridized with the radiolabeled oligonucleotides in competition with either the unlabeled NOS probes used at 50-fold excess (350 ng/slide) or with a large excess (10 μg = 1 nmol/slide) of an unlabeled "irrelevant" oligonucleotide of identical G + C composition (47%). (3) Non-homologous hybridization. To further verify hybridization specificity, slides were probed with the irrelevant 30-mer which had been 3′ tailed with ^{35}S dATP. As a further control, RNA from stimulated RAW macrophages was isolated with RNAzol (AMS Ltd, Witney, UK) as described in the manufacturer's instructions, resolved by formaldehyde gel electrophoresis and transferred to a nylon membrane for Northern blot analysis by hybridization with all three probes. Briefly, probes were labeled with ^{32}P dATP (6.7×10^9 dpm/μg; Amersham, UK), purified and added (10 pmol of each probe) to 10 ml hybridization buffer.

Results

Glomerulonephritis

Left kidneys developed a proliferative glomerulonephritis as previously described [9]. By six hours glomeruli were hypercellular with neutrophils and macrophages present within capillary loops. At that time many tubules contained casts. Hypercellularity increased to day 2, by which time most of the infiltrating cells were macrophages. Macrophage infiltration was maximal on day 4, with many glomeruli showing accumulation of macrophages in Bowman's space. Glomerular cellularity remained increased to day 7. From 24 hours onwards there were also focal mononuclear inflammatory infiltrates in the interstitium.

Localization of inducible NOS

Immunohistochemistry. Immunohistochemical staining showed 75% RAW cells stimulated with γIFN and LPS were positive for inducible NOS, and totally negative when unstimulated (Fig. 1). Mesangial cells cultured under basal conditions were negative. After stimulation with IL-1β a small population of stimulated mesangial cells stained strongly for inducible NOS.

Glomeruli from normal kidneys (Fig. 7A) and from right non-nephritic kidneys of glomerulonephritic rats did not show any positive staining. In left nephritic kidneys the strongest inducible NOS staining was localized in the cytoplasm of mononuclear cells within glomerular capillary lumens (Fig. 2), and in cells in Bowman's space in severely affected glomeruli with early crescents (Fig. 3). Weak focal staining was found in some glomeruli over visceral glomerular epithelial cells and occasional capsular cells. Strongly positive mononuclear cells were present in the interstitium (Fig. 4) and in blood vessel lumens in nephritic kidneys. Weak focal staining in occasional cortical proximal tubular epithelium in areas with interstitial inflammation was observed. Large blood vessel endothelium and smooth muscle were consistently negative. Sections preincubated with serum from rabbits immunized with an irrelevant peptide or antiserum preabsorbed with inducible NOS peptides showed no staining. Double labeling for both inducible NOS and the macrophage marker ED1 showed the presence of inducible NOS positive macrophages in the glomerulus (Fig. 5).

In situ hybridization. In Northern analysis the antisense probes used for *in situ* hybridization recognized a single band of 4.6 kb mRNA isolated from induced RAW 264.7 macrophages. In tissue sections the hybridization signal was predominant over nephritic glomeruli and was most intense over severely affected hypercellular segments of glomeruli (Fig. 6), corresponding to the immunohistochemical localization. The distribution of the hybridization signal was cytoplasmic. There was no recognizable labeling of vascular smooth muscle and endothelium nor of tubular epithelium. No signal was seen in normal kidney. Hybridization signal was abolished by ribonuclease treatment (Fig. 7B) and by competition with unlabeled NOS probe, but not by competition with an "irrelevant" oligonucleotide. No signal was detected with radiolabeled non-homologous oligonucleotide.

NO_2^- synthesis

Ex vivo glomerular NO synthesis, measured as accumulation of NO_2^-, was studied to relate NO synthesis by nephritic glomeruli to the presence of inducible NOS within these glomeruli. Consistent with the immunohistochemical localization of inducible NOS, there was *ex vivo* NO_2^- generation by glomeruli (29 ± 6 nmol NO_2^-/2000 glomeruli/48 hrs; $N = 9$) as previously reported in this model [3]. *Ex vivo* NO_2^- accumulation was inhibited in the presence of the L-arginine analogue N^G-monomethyl-L-arginine (L-NMMA; 300 μM) (3.7 ± 1.6 nmol NO_2^-/2000 glomeruli/48 hrs; $N = 9$). There was no NO_2^- generation by normal glomeruli ($N = 6$). RAW cells stimulated with rat γIFN accumulated 66 ± 5.5 nmol $NO_2^-/10^6$ cells/24 hrs ($N = 4$), inhibitable by L-NMMA 22 ± 2.2 nmol $NO_2^-/10^6$ cells/24 hrs ($N = 4$). There was no NO_2^- generation by unstimulated RAW cells. Mesangial cells simulated with IL-1β accumulated 15.6 ± 0.8 nmol NO_2^-/ml/48 hrs ($N = 2$) inhibitable by L-NMMA. There was no NO_2^- generation by unstimulated mesangial cells.

Discussion

These studies provide the first direct evidence of *in vivo* induction of the NO pathway in glomerulonephritis. Until now demonstration of NO synthesis by nephritic glomeruli has entirely depended on our previous studies showing *ex vivo* nitrite synthesis [1–4]. The use of both immunohistochemistry and *in situ* hybridization provides strong evidence for the specific localization of inducible NOS in nephritic glomeruli. The isoform of NOS we have detected is known to be induced by cytokines involved in the mediation of immune complex injury. These results suggest a role for NO in the pathogenesis of immune complex glomerulonephritis, and are consistent with the reported benefits of NO inhibition in immune complex injury [7].

The source of NO is of some interest. Although all the cells expressing inducible NOS in nephritic glomeruli could not be identified by preliminary double labeling procedures, we found that the majority of positive cells were mononuclear, mostly within capillary lumens, and identified as macrophages by ED1. In this model of glomerulonephritis there is a substantial infiltration by both neutrophils and macrophages [3]. There is already evidence implicating infiltrating macrophages as a major source of NO in glomerulonephritis [2]. However, at this stage we cannot exclude that in inflammatory reactions involving neutrophils they may also be a source of NO, as suggested by studies with rat neutrophils [15]. In our study, although the majority of neutrophils (identified by nuclear hypersegmentation) were negative, occasional cells were positive. There was

weak focal staining of glomerular epithelial cells, which suggests they may have the capacity to express inducible NOS.

Further possible sources in glomeruli are mesangial cells and endothelial cells, which have previously been reported to express inducible NOS *in vitro* [16, 17]. We did not find convincing staining of these cells with double labeling. However, we cannot exclude a mesangial cell contribution as staining was present within the mesangium (Fig. 2). We believe, from our double labeling studies, that most of this iNOS reactivity can be accounted for by macrophage infiltration at that site. As we show here, the antibody used in our study does react with the enzyme expressed by rat mesangial cells. In addition, although rat mesangial cell inducible NO synthase has not yet been sequenced, it is relevant that the vascular smooth muscle inducible NO synthase sequence [18] differs by only three amino acids from the peptide sequence used in our studies. The lack of definite staining in mesangial cells suggests either that they do not synthesize NO *in vivo* in this model, or that the level of enzyme expression is very low. It is also possible that NO synthase induction in different cells may peak at different time points. We are now studying the time course of NOS induction in this model with markers to definitively identify the different cell types.

It is clearly of considerable significance that the NO pathway is activated in nephritic glomeruli, and our results, together with preliminary reports that inhibition of NO affects the course of experimental glomerulonephritis [19], provide a stimulus to further work defining the role of NO in the inflamed glomerulus. There are a number of possible effects which NO may mediate within the glomerulus [reviewed in 20], and these could be either injurious or protective. In the quantities generated by the inducible isoform of the enzyme, NO is cytostatic/cytotoxic. The cellular damage may be produced by binding of NO to iron sulphur-containing enzymes such as aconitase or those of the mitochondrial electron transport chain, or through a reaction with superoxide to form the peroxynitrite radical [6]. Other possible effects of NO in glomerular injury are inhibition of mesangial cell contraction [21] and inhibition of mesangial cell proliferation [22]. It may also reduce glomerular thrombosis [23], and possibly have inhibitory effects on leukocyte infiltration [24].

In summary, we have now demonstrated the *in vivo* presence of inducible NOS in glomeruli with acute immune complex glomerulonephritis. This is strong evidence for a role for nitric oxide in the pathogenesis of immune complex-induced inflammation.

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Reprint requests to Dr. Victoria Cattell, Department of Histopathology, St. Mary's Hospital Medical School, 1 Norfolk Place, London W2 1PG, England, United Kingdom.

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