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A laboratory model of toxin-induced hemolytic uremic syndrome

C. Mark Taylor, Julie M. Williams, Christopher J. Lote, Alexander J. Howie, Andrew Thewles, Jeanne A. Wood, David V. Milford, Faro Raafat, Ian Chant, and Peter E. Rose

Department of Nephrology, The Birmingham Children's Hospital; Renal Research Laboratory, University Hospital, Department of Physiology; Department of Pathology, Birmingham University; Department of Histopathology, The Birmingham Children's Hospital, Birmingham; and Department of Haematology, Warwick Hospital, Warwickshire, United Kingdom

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Background. Verocytotoxin-producing (Shiga-like toxin-producing) *Escherichia coli* infection is the principal cause of hemolytic uremic syndrome (HUS). The pathogenesis is unclear, and there is a need for animal models. These are impeded by the different distribution of verocytotoxin receptors between species. We have circumvented this restriction using ricin, which gains entry into cells via various galactose receptors. Like verocytotoxin, ricin specifically cleaves a single adenine from ribosomal RNA.

Methods. Rats were given ricin at a dose of 6.7 μg/100 g body wt, with or without lipopolysaccharide at 10 μg/100 g body wt. Lipopolysaccharide alone or saline were used as controls. Changes in glomerular filtration rate, hematological parameters, histology, and plasma cytokine concentrations were measured.

Results. Extensive glomerular thrombosis, pyknotic nuclei, and an infiltration of ED1-positive cells into glomeruli were observed eight hours after an injection of ricin. Other vascular beds were unaffected. Histologic changes were preceded by oliguric renal failure, hemolysis, and thrombocytopenia. Ricin produced a rise in plasma concentrations of monocyte chemotactic protein-1, > tumor necrosis factor-α, > interleukin-1β, > interleukin-6. Interferon-γ showed a small increase at the end of the experiment.

Conclusions. Ricin induces glomerular thrombotic microangiopathy, closely resembling that which occurs in verocytotoxin-producing *E. coli*–induced HUS. As in HUS, high concentrations of proinflammatory cytokines are present, which are probably a result of cytokine superinduction by the toxin.

Infection with verocytotoxin (VT; Shiga-like toxin)-producing *Escherichia coli* (VTEC), usually serotype O157, is the major cause of hemolytic uremic syndrome (HUS). Children and the older population appear to be the most vulnerable to this type of infection. VT-1 and VT-2, part of the family of Shiga toxins, are implicated in the pathogenesis of the vascular complications of VTEC infection, including glomerular thrombotic microangiopathy (TMA), which is the hallmark of HUS. However, pathogenic details are incomplete, and research is hampered by the paucity of laboratory models of the condition.

In order to gain cellular uptake, VT requires the receptor Gb3 (CD77 or the blood group Pk), which is expressed in human gut and kidney [1]. The different expression of Gb3 across species is the likely reason why commonly available laboratory animals do not develop HUS after VT exposure. We have overcome this restriction by using the plant toxin ricin. Ricin, like VT, intoxicates cells by inhibition of protein synthesis and has identical enzymatic activity in cleaving adenine from ribosomal RNA at a point in which aminoacyl RNA is assembled [2]. However, unlike VT, ricin gains cell entry through a variety of galactose-bearing surface receptors using clathrin-dependent and -independent pathways [3]. Virtually all mammalian cells have a surface galactose-containing glycolipid or glycoprotein, and therefore, ricin receptors are universally distributed. The clathrin-dependent endocytic pathway is generally regarded as essential for cell viability, whereas the clathrin-independent pathway is thought to be widely distributed but not ubiquitous. We have confirmed that on vero cells, the two toxins have similar effects [4].

In VTEC-induced HUS, there is evidence of an acute inflammatory response with neutrophilia [5], raised concentrations of C-reactive protein, and plasma elastase [6]. The magnitude of these responses predicts adverse outcome. Raised concentrations of the cytokines tumor

Key words: ricin, verocytotoxin, Shiga-like toxin, *Escherichia coli*, thrombotic microangiopathy, cytokines.

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necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and the chemokines IL-8 [7–10] and monocyte chemotactic protein-1 (MCP-1) [11] have been reported. Although the cause for this is unknown, it may be relevant that VT superinduces the production of cytokines from a variety of cells [12, 13]. Ricin also causes the superinduction of proinflammatory cytokines from human [14] and rat monocytes (abstract; Bavari S et al, *Toxicologist* 12:1126, 1992).

We report that ricin administered to rats, with or without simultaneous injection of lipopolysaccharide (LPS), rapidly and reliably produced glomerular TMA, which closely resembled that found in children with diarrhea-associated HUS. The animals exhibited oliguric renal failure, hemolysis, and thrombocytopenia. The model was accompanied by an inflammatory response with a marked increase in the plasma concentrations of inflammatory cytokines and a glomerular infiltrate of macrophages.

METHODS

Experimental protocol

Young adult male rats (Sprague-Dawley strain, weight range 260 to 300 g) were lightly anesthetized with ether and flexible polypropylene cannulae (Portex PP25) implanted in a tail artery and vein. The animals were placed in restraining cages and were allowed to recover from the anesthetic. An initial blood sample was taken from the arterial cannula, and the animals received, via the venous cannula, a bolus of (0.5 ml) 0.7% NaCl solution (control group) or a similar volume of NaCl solution containing LPS (*E. coli* 0111:B4, 10 μg/100 g body wt; Sigma, Poole, Dorset, UK), ricin, a gift from Professor J.M. Lord (University of Warwick, Warwickshire, UK; 6.7 μg/100 g body wt), or a combination of LPS and ricin at these doses.

An infusion of 0.7% NaCl was commenced, via the tail vein, at 6 ml/hr. This was continued for six hours or until the animals were volume expanded by more than 20 ml. Urine was collected by spontaneous voiding, and blood samples (2 ml) were taken at three and five hours. At a minimum of seven hours post-toxin administration, a terminal 2 ml blood sample was obtained, and the animals were anesthetized via the tail vein (sodium pentobarbitone; Sagatal, Rhone Merieux, Dublin, Ireland). After the abdomen was opened, the renal vein was punctured, and the animal was perfusion fixed by flushing the circulation, via the tail artery, with 2×10 ml 0.7% NaCl followed by 10 ml formol saline. The kidneys were excised for histologic examination, together with samples of lung, liver, heart, muscle, gut, spleen, pancreas, and brain.

A separate series of experiments was performed to measure the effects of the toxins on glomerular filtration rate (inulin clearance), uncomplicated by the effects of the large blood sample volumes mentioned earlier in this article. In these experiments, the protocol was similar, except that the initial boluses were given i.p. instead of i.v. and the infusate included inulin (1.5% wt/vol). Blood samples (0.5 ml) were taken from the arterial line for inulin assay at two-hour intervals. When not being used for sampling, the arterial line was used to monitor blood pressure. Urine was collected every two hours by spontaneous voiding. The effects of the toxins were identical (as assessed by blood pressure, body temperature, and histology) whether administration was i.p. or i.v.; therefore, where appropriate, the data from the two series have been pooled. The urine volumes were determined, and the urine osmolality was measured prior to inulin assay.

Hematological parameters were measured in the hematology laboratory at the Birmingham Children's Hospital using a Coulter counter.

Hemolysis

Plasma was obtained from treated rats at eight hours. This was diluted 10-fold with 0.7% NaCl, and the absorbance was measured at 555 nm. The results were compared with standards to determine the degree of hemolysis. To examine the effect of hemolysis alone, 0.5 ml of blood was removed from untreated rats, hemolyzed, and administered to animals. This represented 3% hemolysis. The hemolyzed blood was given as three aliquots at three, four, and five hours into a six-hour infusion of 0.7% saline and inulin.

Histology

After perfusion fixation, the kidneys were removed, bisected longitudinally, and placed in formol saline for 24 hours. These were embedded in paraffin, sectioned, and stained by hematoxylin and eosin and periodic acid-methenamine silver. Sections were also stained for fibrin using an immunoperoxidase method. Endogenous peroxidase was blocked with hydrogen peroxide 0.5% in methanol. Sections were exposed to goat antirat fibrinogen (Organon Teknika Ltd., Cambridge, UK) at 1:100. Donkey antisheep peroxidase-conjugate (1:100) and tetra-amino-biphenyl hydrochloride (diaminobenzidine) were used to visualize binding and sections counterstained with Mayer's hemalum. Glomeruli showing thrombi by fibrinogen staining were counted and expressed as a percentage ($N = 100$). Tissues were stained for the presence of macrophages using an anti-ED1 antibody (Serotec, Ltd., Oxford, UK). The mouse antirat ED1 was used at 1:50 and visualized using rabbit antimouse (1:25), alkaline phosphatase antialkaline phos-

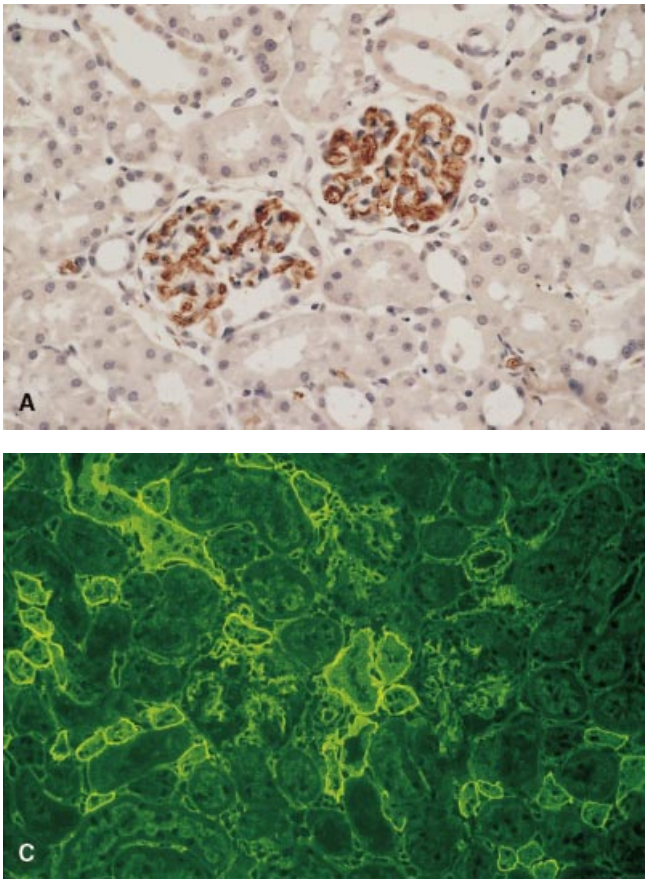

Fig. 1. (*A*) Glomeruli from rat treated with lipopolysaccharide (LPS) and ricin stained with an antifibrinogen antibody that has specifically detected thrombosis in glomerular capillaries (×1600). (*B*) Glomerulus from rat treated with ricin showing pyknotic nuclei in an endocapillary distribution (×2400). (*C*) Frozen section of cortex treated with ricin-fluorescein isothiocyanate (FITC). The strongest binding is to distal tubules, particularly their basement membrane, and there is also binding to glomerular and intertubular capillaries, walls of arterioles, and the brush border of proximal tubules (×1600). Publication of this figure in color was made possible by grants from Fujisawa Ltd. and Roche Pharmaceuticals UK.

phatase (1:50), and the substrate fast red. The number of ED1-positive cells were counted in 100 glomeruli.

Fluorescent staining

Frozen sections of rat kidney were air dried and blocked with 0.1% gelatin in phosphate-buffered saline (PBS). Five μg/ml of ricin-fluorescein isothiocyanate (FITC; Sigma) was applied for one hour at room temperature in a humid environment in the dark. After washing with PBS, sections were fixed with 2% formaldehyde/PBS for 20 minutes and were then mounted in 75% glycerol in 10 mM Tris-buffered saline, pH 8.6.

Electron microscopy

A few small pieces were taken from the cortex after perfusion, placed in glutaraldehyde 2.5% in PBS, and then fixed further in osmium tetroxide. These were embedded in Araldite, sectioned, stained with uranyl acetate and lead citrate, and examined using an electron microscope.

Cytokine/chemokine assays

Levels of plasma cytokines were measured by enzyme-linked immunosorbent assay (Biosource International, Lifescreen, Ltd., Hertfordshire, UK).

RESULTS

The exposure of rats to either saline or LPS alone did not induce glomerular thrombosis. However, when animals were treated with ricin or ricin plus LPS, glomerular thrombosis was marked (Fig. 1A), and pyknotic cell nuclei were seen in endocapillary sites (Fig. 1B). Thrombosis was confined to the glomerular microcirculation with occasional extension into the afferent arteriole. The percentage of glomeruli showing thrombi by fibrinogen staining was control 2.67 ± 1.2, LPS 1.86 ± 0.46, ricin 94.57 ± 3.0, and LPS + ricin 86.33 ± 4.26 (mean ± SEM, $N = 4$). Thrombi were not seen in lung, liver, heart, muscle, gut, spleen, pancreas, or brain of affected animals.

By electron microscopy, glomerular capillaries contained fibrin thrombi interspersed with platelets and areas of endothelial detachment from the basement membrane (Fig. 2). Rats treated with ricin had an increased number of ED1-positive cells in their glomeruli. The number of ED1-positive cells per glomerulus was control 0.59 ± 0.1, LPS 0.74 ± 0.21, ricin 3.53 ± 0.27 ($P < 0.005$), and LPS + ricin 1.37 ± 0.35 (mean ± SEM, $N = 4$). No apparent increase in ED1 staining was observed in any of the other organs examined.

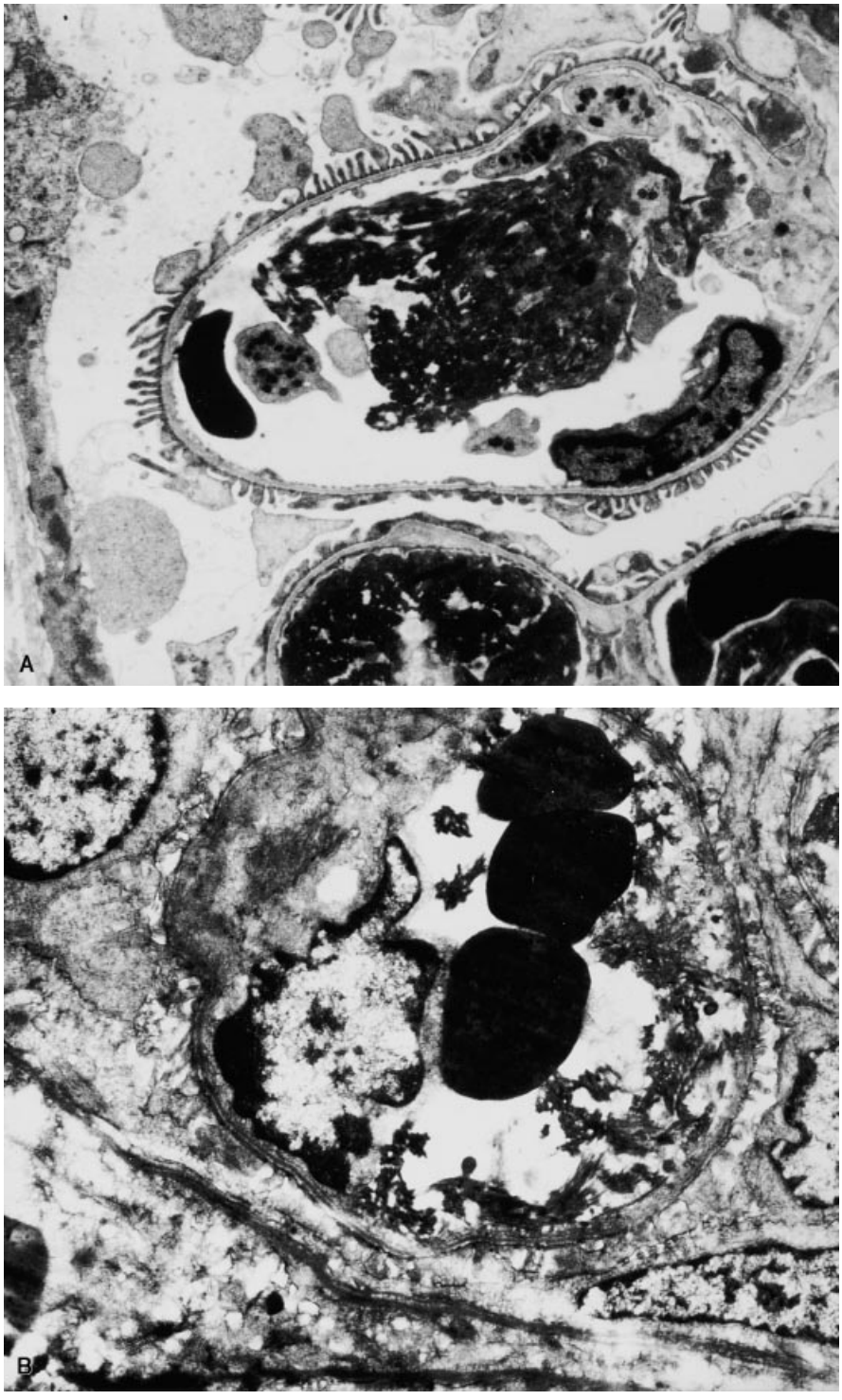

Fig. 2. (*A*) Glomerular capillary loop showing fibrin thrombus and platelets (×4700). (*B*) A capillary showing detachment of endothelial cells from basement membrane and early blebbing of the nucleus (×4700). Publication of this figure in color was made possible by grants from Fujisawa Ltd. and Roche Pharmaceuticals UK.

Ricin-FITC when applied to frozen sections of rat kidney bound most strongly to distal tubules, particularly their basement membrane, and also to glomerular and intertubular capillaries, walls of arterioles, and the brush border of proximal tubules (Fig. 1C).

Decreases in blood pressure were observed only in ricin- and ricin plus LPS-treated rats (Fig. 3A). This commenced at five hours and continued to the end point of the experiment. The glomerular filtration rate (GFR) decreased progressively in these animals from three to seven hours but not in the rats receiving LPS alone (Fig. 3B).

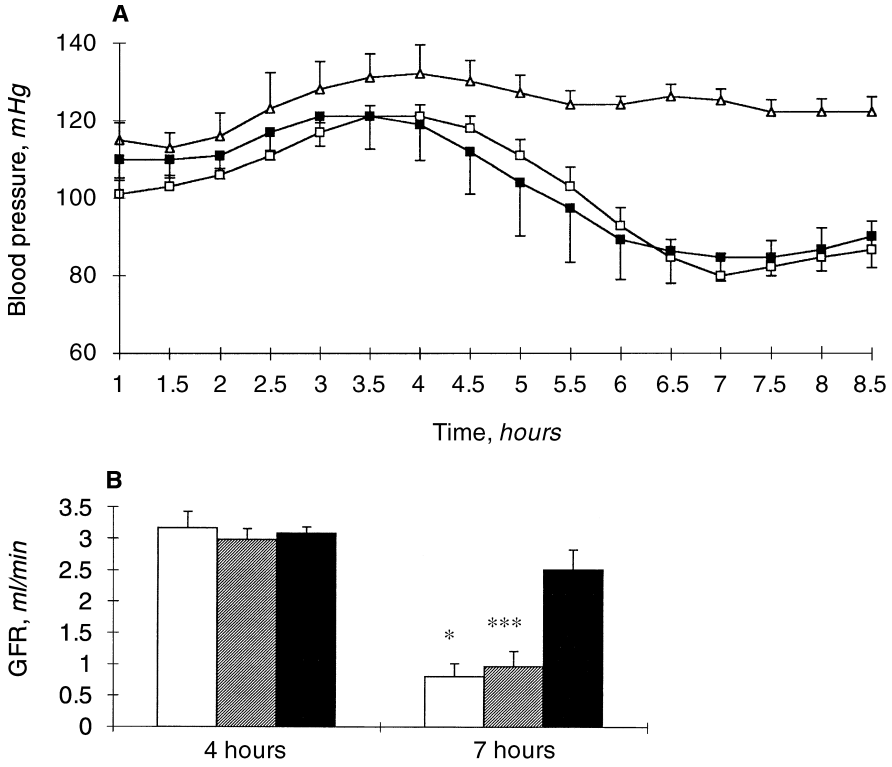

Fig. 3. (*A*) Alterations in blood pressure over time in LPS (△), ricin (□), and LPS plus ricin (■) treated rats. (*B*) The glomerular filtration rate at four and seven hours in LPS (■), ricin plus LPS (▨), and ricin (□) treated rats. Data are mean ± SEM; $N = 3$; $^{*}P < 0.05$, $^{***}P < 0.001$.

Table 1. Hematological values for rats treated with only ricin

	Reference values	Ricin treated	LPS and ricin treated
Erythrocytes $\times 10^{12}$/liter	7.01 ± 0.38	5.9 ± 0.3[b]	4.78 ± 0.19[b]
Haemoglobin g/dl	14.2 ± 0.5	12.3 ± 0.5[b]	10.8 ± 0.84[b]
Leukocytes $\times 10^{9}$/liter	7.84 ± 2.95	11.5 ± 2.0[a]	3.84 ± 0.54[a]
Platelets $\times 10^{9}$/liter	1141 ± 91	603 ± 35.6[b]	239 ± 59.2[b]

Data are mean ± SD; $N = 5$. Reference values are for Sprague-Dawley rats [15]. LPS is lipopolysaccharide.
[a] $P < 0.005$; [b] $P < 0.005$, unpaired t-test.

The administration of ricin with or without LPS induced thrombocytopenia and anemia (Table 1). Schistocytes were not evident on a blood film (data not shown); however, there was visible hemolysis in plasma, calculated to be 3% at eight hours and visible hemoglobinuria from three hours. LPS alone did not have this effect. In separate experiments on normal animals, intravenous administration of hemolyzed blood elicited transient hemoglobinuria; however, glomerular filtration rate and urine flow remained normal, and histologic examination showed no evidence of glomerular thrombosis. Thus, the hemolysis induced by ricin did not itself promote the lesions of this model. Ricin alone caused an increase in peripheral blood total leukocyte count, whereas ricin with LPS together caused a significant decrease.

Ricin alone gave an early and sustained increase in MCP-1 and TNF-α, whereas IL-1β, IL-6, and interferon-γ (IFN-γ) increased more slowly but were significant by seven hours (Fig. 4). LPS alone gave an early and transient rise in TNF-α, MCP-1, and IFN-γ with a decrease from three hours. Synergism was observed between LPS and ricin in the responses of IL-1β, IL-6, and IFN-γ, whereas the effect on TNF-α and MCP-1 appeared to be additive. The terminal concentrations were similar when LPS was added, while with saline there was no increase in any of the cytokines measured.

DISCUSSION

The need for a model with which to explore the pathogenesis of VTEC-induced HUS is widely recognized (report of World Health Organization Working Group Meeting on Shiga-like Toxin-producing *E. coli*, with an emphasis on zoonotic aspects, 1994). However, the models described so far are varied, perhaps because the distribution of VT receptors is different between species. For example, an injection of VT in the rabbit causes focal endothelial lesions in the central nervous system, a site where Gb3 has been identified [16], whereas VTEC infection in mice has been shown to give rise to an interstitial renal lesion without TMA [17]. Given that the distribution of Gb3 in primates may more closely resemble humans, Siegler et al administered Shiga toxin to baboons (abstract; Siegler et al, *J Am Soc Nephrol* 7:1834, 1996). This gave rise to gut and renal lesions, but glomer-

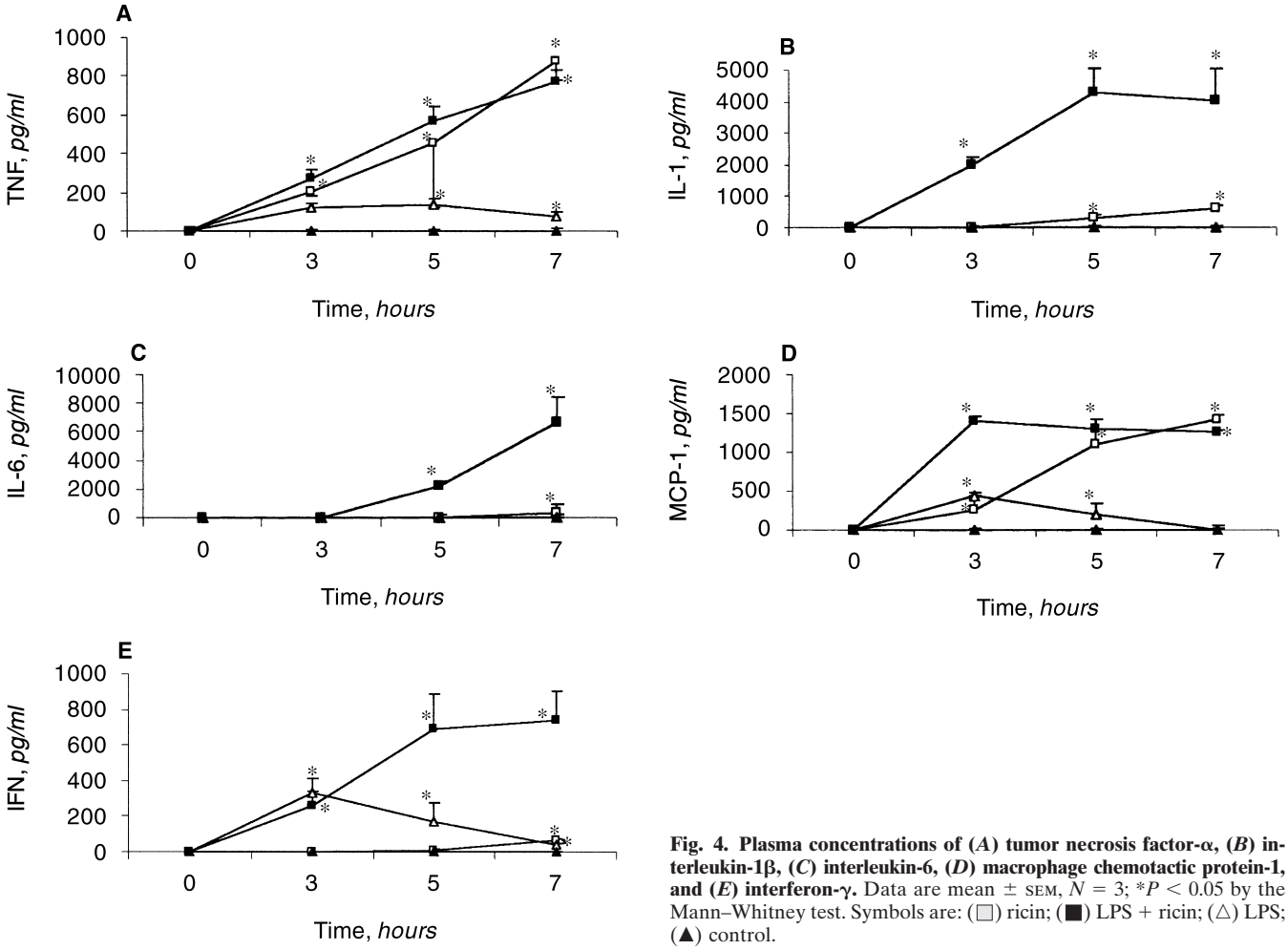

Fig. 4. Plasma concentrations of (*A*) tumor necrosis factor-α, (*B*) interleukin-1β, (*C*) interleukin-6, (*D*) macrophage chemotactic protein-1, and (*E*) interferon-γ. Data are mean ± SEM, $N = 3$; $^*P < 0.05$ by the Mann–Whitney test. Symbols are: (□) ricin; (■) LPS + ricin; (△) LPS; (▲) control.

ular thrombosis was scanty. Another possible model is "Alabama Rot," a naturally occurring VTEC-related disease of greyhounds. In this, dogs develop bloody diarrhea, edema, ulcerating skin lesions, and renal failure. Histologically, the kidney shows extensive glomerular TMA [18]. To bypasses the species restriction of VT receptors, we have used the ribotoxin ricin, which has identical protein synthesis inhibitory effects to VT [2].

The pathogenesis of glomerular TMA is unknown. It has been suggested that the predilection for glomerular thrombosis in humans relates to the glomerular expression of Gb3 [1] and direct vascular toxicity. The ricin model throws doubt on this concept. Ricin-FITC binds to a number of different structures in the renal cortex (Fig. 1C), illustrating that receptor distribution does not account for the specificity of the glomerular TMA; for example, there is obvious binding of ricin to arteriolar endothelium, but thrombi were rarely found in this site. It must be remembered, however, that applying ricin to sections is an artificial condition, as *in vivo* exposure would be via the blood stream. Ricin is delivered to liver or lung during first pass after intraperitoneal or intravenous administration, respectively. Previous studies have shown the toxin to be widely distributed, the majority being found in the liver (46%), spleen (9.9%), and muscle (13%), with less than 2% recoverable in the kidney [19]. It is therefore of interest that within the time frame of the experiment, ricin primarily gives rise to glomerular thrombosis. As in human HUS, extensive fibrin thrombi fill the capillaries that appear engorged and occluded. The obstruction to the microcirculation in the model is sufficient to explain renal failure. Occasionally, we saw thrombus extending into the afferent artery, as recently described in a postmortem study in children [20]. Although our model has the characteristic clinical triad of HUS, oliguric renal failure, thrombocytopenia, and hemolysis, fragmented erythrocytes were not seen. Very little is known about the mechanism that produces schistocytes in HUS, making it difficult to comment on this difference.

Children with VTEC infection and diarrhea-associated HUS have been shown to have an increase in the

cytokines TNF-α, IL-1β and IL-6, and the chemokines IL-8 and MCP-1 in plasma and/or urine. Similarly, in our model, there was a marked increase in these inflammatory mediators, the concentrations far exceeding those induced by LPS. The synergistic response observed between ricin and LPS for IL-1β, IL-6, and IFN-γ indicates that different signaling pathways are involved. In that the shallow rise in IFN-γ occurred late in the course of the experiment after glomerular thrombosis had begun, it is unlikely that this cytokine participates in the model. The pattern of cytokines MCP-1, > TNF-α, > IL-1β indicates the importance of monocytes. Verocytotoxin is known to cause superinduction of human monocytes to secrete TNF-α, IL-1, IL-6, and IL-8 over a prolonged period *in vitro* [13], and ricin has been shown to behave similarly on human peripheral blood mononuclear cells [14]. We have also demonstrated an infiltration of monocytes/macrophages into the glomeruli of the model.

There are similarities between our model and others that give rise to glomerular thrombosis such as the generalized Shwartzman reaction and accelerated nephrotoxic serum nephritis. These reactions are leukocyte and complement dependent [21] and involve proinflammatory cytokines [22, 23], up-regulation of adhesion molecules [24], and tissue factor [25]. The thrombotic lesion can be prevented by combined the administration of TNF-α receptor antagonist and soluble IL-1 receptor in the nephrotoxic serum nephritis model [26]. In the Shwartzman reaction, the challenge dose of LPS can be by replaced by TNF-α and IL-1β [22], whereas anti-TNF-α antibody administered prior to the priming injection gives partial protection against fibrin deposition [27]. The similarity of the proinflammatory cytokine response in all three models of glomerular capillary thrombosis suggests a common pathogenic pathway. The ricin model has the potential to elucidate these mechanisms, as well as providing a framework for investigating therapeutic strategies for HUS.

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Reprint requests to Dr. C. Mark Taylor, Department of Nephrology, The Birmingham Children's Hospital, Steelhouse Lane, Birmingham B4 6NH, England, United Kingdom.

APPENDIX

Abbreviations used in this article are: FITC, fluorescein isothiocyanate; HUS, hemolytic uremic syndrome; IFN-γ, interferon-γ; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; PBS, phosphate-buffered saline; TMA, thrombotic microangiopathy; TNF-α, tumor necrosis factor-α; VT, verocytotoxin; VTEC, verocytotoxin-producing *E. coli.*

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