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A model for the investigation of factors influencing haemorrhagic necrosis mediated by tumour necrosis factor in tissue sites primed with mycobacterial antigen preparations

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SUMMARY

Mycobacterial lesions and skin sites challenged with soluble mycobacterial antigen are very sensitive to the necrotizing effect of tumour necrosis factor (TNF). We have used a model that permits separate quantitative assessment of swelling and haemorrhage to show that when these reactions are elicited in mice that have not been deliberately immunized, pretreatment of the mice with lipopolysaccharide (LPS), or with a MoAb to CR3 which blocks emigration of myeloid cells into the tissues, will block both the swelling and the haemorrhage. On the other hand, treatment with an inhibitor of platelet-activating factor (PAF), or with misoprostol (a synthetic prostaglandin E1 analogue), or with cobra venom factor (CVF) which depletes complement, preferentially blocks the haemorrhagic component, while leaving the swelling relatively unaltered. As swelling occurs before the haemorrhage is seen, it is possible that these factors act at a late stage in the cascade of events leading to the tissue damage. However, LPS and CVF were able to inhibit swelling and haemorrhage in the massive reactions elicited in pre-immunized animals, whereas the PAF inhibitor had no detectable effect.

Keywords Shwartzman reaction tumour necrosis factor platelet-activating factor misoprostol IL-6

INTRODUCTION

Injection of tumour necrosis factor (TNF) into normal tissues causes a minor inflammatory response, but the same dose of TNF will cause haemorrhagic necrosis if injected into sites that are already undergoing certain types of inflammation induced by bacterial components [1,2], some tumours [3], or T cell-mediated responses [4; and Al Attiyah & Rook, manuscript submitted]. This altered state of TNF sensitivity probably corresponds to the 'preparatory' injection of the classical local Shwartzman reaction. Thus Shwartzman observed that injection of Gram-negative organisms into the skin rendered the injected site susceptible to haemorrhagic necrosis which could be provoked by giving an i.v. dose of organisms containing lipopolysaccharide (LPS) 24 h later [5]. It now seems probable that the second dose acts mainly by triggering massive systemic release of cytokines which then cause damage specifically in the 'prepared' site. Support for this interpretation has come from the observation that direct injection of recombinant TNF into skin sites prepared 24 h earlier with LPS or *Corynebacterium parvum* will provoke similar haemorrhagic necrosis in mice [1,2].

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It would be of great clinical relevance to understand the factors which determine the TNF sensitivity of sites of inflammation, since it would be helpful to be able to increase it in tumours before TNF therapy, and decrease it in septicaemia and in any other clinical syndromes where TNF-mediated damage may occur. We suggested previously that much of the tissue damage in tuberculosis, and the haemorrhagic necrosis seen in tuberculin test sites in many tuberculosis patients (Koch phenomenon) may be due to the release of TNF from macrophages which have been activated and triggered in the mycobacterial lesion [6–10].

Mycobacterial lesions are 'prepared sites' in the Shwartzman sense [11–14] and we recently devised a new murine model which allows separate quantification of the swelling and haemorrhage which result from the injection of TNF into sites previously injected with mycobacterial antigen [4; and Al Attiyah & Rook, manuscript submitted]. This model has revealed that the ability of these antigen extracts to prepare sites is subject to complex regulation by both $CD4^+$ and $CD8^+$ T cells, even in animals that have not been deliberately pre-immunized with mycobacteria. In such non-immunized animals the ability of mycobacterial antigen to prepare a site is comparable to that of an injection of 10 μg LPS, whereas in mice pre-immunized with mycobacteria the soluble antigen prepares the site for massive TNF-induced swelling and haemorrhage, approximately 10-fold greater than

can be elicited in LPS-prepared sites [4]. This depends on the presence of CD4+ T cells (Al Attiyah & Rook, manuscript submitted). Here we have identified other factors which regulate the sensitivity of these sites to TNF.

MATERIALS AND METHODS

Animals

Female mice, aged 8–10 weeks, of the C57Bl/GrFA [15] strain were bred at the University College and Middlesex School of Medicine. For some experiments these animals were pre-immunized 10 days before use by a single s.c. injection of 10^9 autoclaved *Mycobacterium vaccae*, provided by Dr J. L. Stanford. This organism was used because it retains its immunogenicity when autoclaved, and evokes a reliable and readily standardized response.

Cytokines

Recombinant murine TNF (rMuTNF; $1{\cdot}2 \times 10^7$ U/mg) and human TNF (3×10^7 U/mg) were gifts from Dr G. R. Adolf, Ernst Boehringer Institut für Arzneimittel Forschung, Vienna, Austria. The endotoxin levels in these preparations were less than 0·51 endotoxin units/mg, as measured by the *Limulus* amoebocyte lysate assay.

Bacterial antigens

LPS of *Escherichia coli* 055:B5 (L-2880; Sigma, Poole, UK) was diluted in pyrogen-free saline. *Mycobacterium vaccae* strain R877R (NCTC 11659) was grown on Sauton's medium solidified with 1·5% agar. Soluble antigens were prepared as described [16].

The local Shwartzman reaction (LSR)

Foot-pads were 'prepared' by injecting LPS (10 μg) or sonicate of *M. vaccae* (17 μg) subcutaneously in 40 μl of pyrogen-free saline. At least four mice were used per group (Table 1). Twenty-four hours later, the swelling of the 'prepared' site was measured with a Mitutoyo thickness guage (code no. 7308; Mitutoyo, Japan) and it was then challenged with 1 μg ($1{\cdot}2 \times 10^4$ U) of rMuTNF subcutaneously into the same site. These constitute, respectively, the 'preparatory' and 'challenge' injections and are referred to as such throughout.

Previous studies had shown that in a positive LSR, swelling peaks 16 h after the TNF challenge, and haemorrhage 4 h later. Therefore after 16 h the increase in foot-pad thickness was again determined, and 4 h later the challenged foot was then removed and incubated for 48 in a water bath set at 60°C, in 2 ml of a 1:4 mixture of 10% cetrimide (cetyltrimethylammonium bromide; Sigma, M-7635) and 1 *N* sodium hydroxide, as described. This treatment solubilized the tissues, and converted the haeme moiety of haemoglobin and its breakdown products into haeme derivatives with absorption maxima at 570 nm [17,18]. The alternative peak given by these pigments between 400 and 450 nm was not used because of greater interference from other tissue components. The total quantity of haemoglobin present in each foot was then calculated from a standard curve constructed using a purified human haemoglobin preparation (Sigma, H-7379) dissolved in the same cetrimide/sodium hydroxide mixture.

Effect of pretreatment with LPS on the LSR

In order to find out whether pretreatment with LPS can block subsequent induction of the LSR, C57Bl/GrFa mice received 25 μg of LPS by i.p. injection, 5 h before the preparatory injection of *M. vaccae* sonicate, or 5 h before the challenge injection of TNF, or 5 h before both injections. In some experiments the role of TNF in the effects of pretreatment with LPS was examined by i.v. administration of 1 mg of neutralizing rabbit anti-murine TNF 4 h before the administration of the LPS. This antibody was used as purified IgG isolated from a rabbit antiserum provided Dr G. E. Grau, Geneva, Switzerland.

Effect of a MoAb to CR3 on the development of LSR

The rat IgG2b MoAb (5C6) directed against murine CR3 has been shown to inhibit the emigration of neutrophils and monocytes from blood vessels into inflammatory foci [19,20]. One milligram of the antibody was injected intravenously via the tail vein into C57Bl/GrFa mice, 1 h before either the preparatory or the challenge injection. The control animals were given either pyrogen-free saline or IC5.5H10.A11, an isotype-matched control MoAb.

The in vivo effect of WEB2170, a platelet-activating factor (PAF) antagonist on LSR

To investigate the involvement of PAF in the LSR we used WEB 2170, a selective PAF antagonist (a gift from Dr H. Heuer, Department of Pharmacology, Boehringer Ingelheim, Ger-

Table 1. Summary of the effects of the agents tested on the induction of haemorrhagic necrosis in foot-pads primed by *M. vaccae* antigen (significant (S) or not significant (NS) inhibition)

	Normal mice		Pre-immunized mice*	
Agent tested	Swelling	Haemorrhage	Swelling	Haemorrhage
LPS	S	S	S	S
5C6 (anti-CR3)	S	S	NT	NT
CVF	NS	S	S	S
Misoprostol	NS	S	NT	NT
WEB2170	NS	S	NS	NS

* 10^9 autoclaved *M. vaccae* subcutaneously 10 days before challenge.
NT, not tested.
LPS, lipopolysaccharide; CVF, cobra venom factor.

many). The *in vivo* duration of action of WEB2170 in mice has been estimated to be 5–6 h after oral administration. The onset of the effect is more rapid after i.v. injection, but the duration is less (Dr H. Heuer, personal communication). Therefore, in order to test the effect of the presence of the PAF inhibitor during the preparatory phase of the LSR, mice received an i.v. dose of 0·024 mg/kg body weight, 40 min before preparation with *M. vaccae*, followed by an oral dose of 10 mg/kg 1 h after preparation. In order to test the effect of the inhibitor on the TNF challenge phase of the LSR, the same doses were given 40 min before and 1 h after the TNF administration. These two groups were compared with saline-treated control animals.

Effect of misoprostol on LSR

Misoprostol, a synthetic prostaglandin E_1 (PGE_1) analogue has been reported to be protective in septic shock. It was provided by G. D. Searle & Co. as a stable dispersion in hydroxypropyl methylcellulose (HPMC). Misoprostol/HPMC was administered orally by mixing it with drinking water at a concentration of 0·5 mg/ml, 3 h before the preparatory injection of *M. vaccae* antigen. The animals were maintained on that treatment during the whole experiment, and their water–drug mixture was replaced regularly. Other groups of animals were treated with the drug in the same way, starting 3 h before TNF challenge. The control groups were given either saline or HPMC alone (0·5 mg/ml).

Effect of complement depletion on the development of LSR

C57Bl/GrFa mice received 10 μg of cobra venom factor (CVF; Sigma, C-8406) or saline intravenously 1 h or 8 h before preparation with *M. vaccae* sonicate (17 μg). They were then challenged 24 h later with TNF (1 μg).

Measurement of serum complement

CVF-treated animals were bled from the tail vein 1 h before the injection of CVF, 1 h later, and then once a day for 2–3 days. Blood was allowed to clot for 30–60 min at 37°C and the serum was then separated and stored at −20°C until assayed for complement level by radial immunodiffusion against sheep anti-mouse C3 (PE280; The binding site, Birmingham, UK).

Statistical analysis

Results are expressed as mean ± s.d. Significance was calculated using the Mann–Whitney *U*-test throughout, and indicated with an asterisk above the appropriate bar.

RESULTS

Inhibition of the LSR by pretreatment with LPS

Pretreatment with sub-lethal doses of LPS can inhibit subsequent attempts to induce TNF-mediated pathology [21–23]. To test this effect in the LSR system, LPS (25 μg) was injected intraperitoneally 5 h before the preparatory injection of *M. vaccae* antigen. This strongly inhibited both the LSR seen in non-pre-immunized mice (data not shown), and the much stronger LSR seen in pre-immunized animals (Fig. 1). It inhibited the swelling ($P < 0.005$) and haemorrhagic ($P < 0.05$) components. In contrast, the same dose of LPS given intraperitoneally 5 h before the challenge dose of TNF was much less inhibitory (Fig. 1), and significantly reduced the swelling ($P < 0.05$) but not the haemorrhage. If LPS was administered 5 h before both injections the LSR was completely abolished. Since TNF release triggered by the LPS could be indirectly mediating the inhibition of the LSR by inducing tachyphylaxis or TNF inhibitor release, rabbit anti-murine TNF was given 4 h before the LPS. This did not diminish the inhibitory effect of LPS treatment. Moreover, the anti-murine TNF polyclonal antibody on its own had no effect on the LSR induced by rMu TNF, suggesting insufficient penetration into the extravascular compartment (data not shown).

Role of myeloid cells in the LSR

An injection of 1 mg of monoclonal 5C6 (anti-CR3) intravenously 1 h before preparation with *M. vaccae* sonicate inhibited the LSR almost completely in mice that had not been pre-immunized with *M. vaccae*. Swelling and haemorrhage were significantly reduced (Fig. 2). In addition, when mice were given the antibody 1 h before provocation with TNF, weaker but still significant effects on both parameters were seen.

Effect of WEB2170 on the production of LSR

PAF may play an amplifying role in the final cascade of events leading to haemorrhagic tissue damage. Normal (not pre-immunized) C57Bl/GrFa mice were given a first dose of the PAF inhibitor WEB2170 (0·024 mg/kg) intravenously either 40 min before preparation or 40 min before TNF challenge. Levels were maintained by giving a second dose (10 mg/kg) orally, 1 h after preparation or 1 h after provocation, respectively. In both groups WEB2170 inhibited the haemorrhage in the foot-pads strikingly, but had no significant effect on the swelling (Fig. 3). However, the same dose schedule had no effect on the very strong LSR elicited in mice pre-immunised with *M. vaccae* (data not shown).

Effect of misoprostol on LSR

Misoprostol, a synthetic PGE_1 analogue, was administered in drinking water dispersed in HPMC. It was given continuously, starting either 3 h before the preparatory injection of *M. vaccae* antigen, or 3 h before the challenge injection of TNF. The mice were not pre-immunized with *M. vaccae*. Both protocols resulted in a partial inhibition of the haemorrhagic component

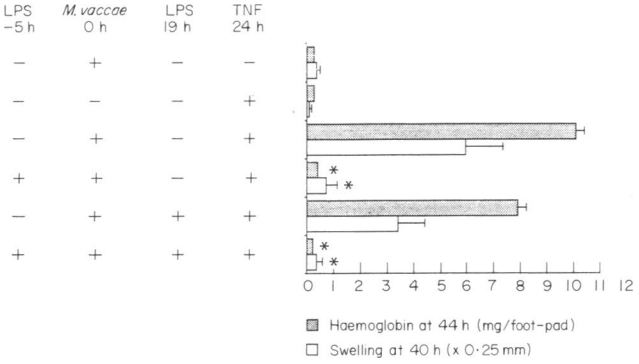

Fig. 1. The effect of pretreatment with lipopolysaccharide (LPS) 25 μg intraperitoneally, at the times shown, on the local Shwartzman reaction (LSR) induced in immunized mice by sequential injection into a foot-pad of soluble *M. vaccae* antigen (17 μg at 0 h) and recombinant murine tumour necrosis factor (TNF) (1 μg at 24 h). Mice had been pre-immunized with 10^9 autoclaved *M. vaccae* subcutaneously 10 days earlier. Results are expressed as mean ± s.d. *Significantly different (Mann–Whitney *U*-test) from the positive control.

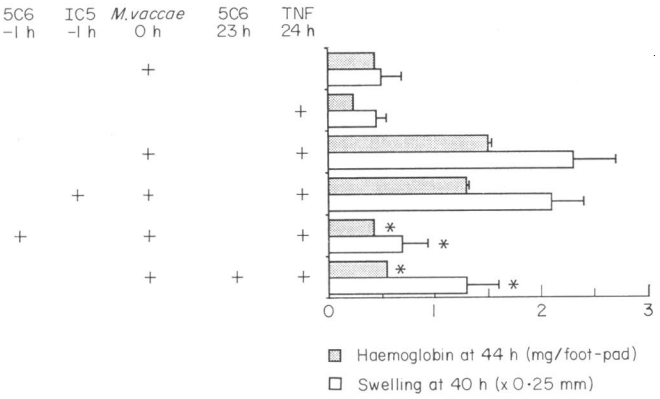

Fig. 2. The effect on the local Shwartzman reaction (LSR) in normal mice of pretreatment with MoAb to CR3 (monoclonal 5C6). *M. vaccae* followed by tumour necrosis factor (TNF) were used to elicit the LSR. IC5 is an isotype-matched control antibody. Mean ± s.d. *Significantly different (Mann–Whitney *U*-test) from the positive control.

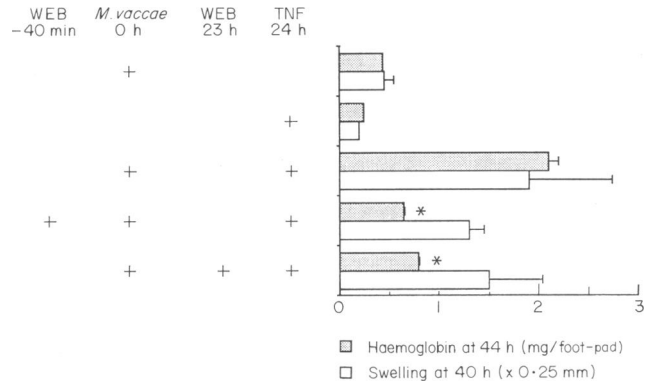

Fig. 3. The effect of an inhibitor of platelet-activating factor (WEB2170) on the local Shwartzman reaction (LSR) in normal mice. Administration of WEB2170 was started at the time indicated, and continued according to the dose schedule described in the text. Mean ± s.d. *Significantly different (Mann–Whitney *U*-test) from the positive control.

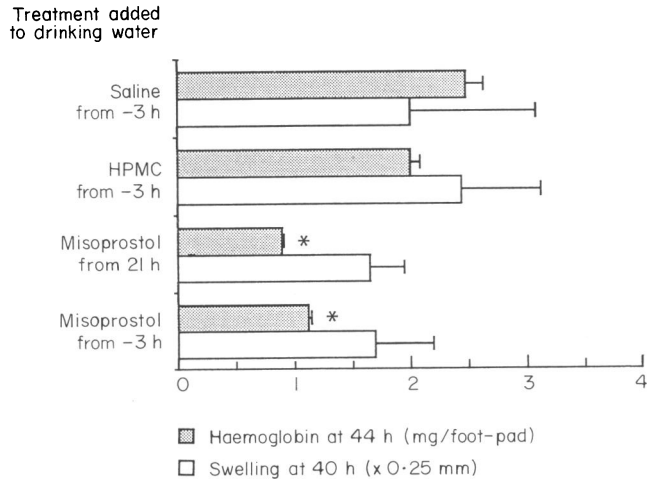

Fig. 4. The effect of the prostaglandin E_1 analogue, misoprostol, on the local Shwartzman reaction (LSR) in normal mice. Control mice received saline or the HPMC (methyl cellulose) used to suspend the misoprostol. Mean ± s.d. *Significantly different (Mann–Whitney *U*-test) from the positive control.

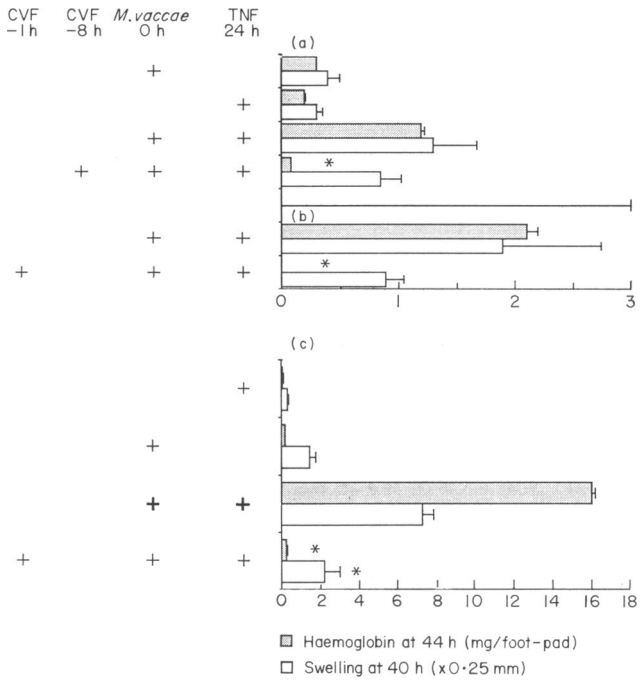

Fig. 5. The effect of cobra venom factor (CVF) pretreatment on the local Shwartzman reaction (LSR) in unimmunized (a, b) and *M. vaccae*-immunized mice (c). Mean ± s.d. *Significantly different (Mann–Whitney *U*-test) from the positive control.

of the reaction when compared with saline-treated and HPMC-treated groups, but had no effect on the swelling (Fig. 4). We did not seek this effect in pre-immunized mice.

The role of complement in the production of local tissue damage

Unimmunized animals received 10 μg of CVF intravenously, either 1 h (Fig. 5b) or 8 h (Fig. 5a) prior to the preparatory injection of *M. vaccae*. Neither protocol suppressed the swelling significantly, but haemorrhage was completely eliminated in the animals treated 1 h before preparation, and almost completely eliminated in those treated 8 h before. This minimal degree of recovery in the latter group may be attributable to a partial recovery of C3 levels. Thus in mice pretreated with CVF 8 h prior to the preparatory injection, C3 was reduced to 33·3% of the normal level at the time of preparation. However, their C3 level had increased to 47% of the normal at the time of TNF challenge. However, mice injected with CVF just 1 h before preparation had a higher complement level (58·8%) 1 h later at the time of the preparatory injection, but a lower level (35%) at the time of TNF challenge (data not shown).

Similar experiments were then performed in mice that had been pre-immunized with *M. vaccae*, in order to find out whether CVF would block the much more powerful and destructive LSR evoked in such animals. In the example shown (Fig. 5c), treatment with CVF 1 h before the preparatory injection of *M. vaccae* eliminated the haemorrhagic component, but again had a less marked though significant effect on swelling.

Histological comparison of LSR sites in the foot-pads of control and of CVF-treated mice showed a similar increase in the thickness of the dermis, with similar oedema and prominent infiltration of the tissue by inflammatory cells (neutrophils and

macrophages) but in contrast to the lesions in animals not given CVF, there was no tissue damage or erythrocyte extravasation (data not shown).

DISCUSSION

It is clear that at least three types of event can cause a tissue to become sensitive to TNF: there is the inflammation induced by local injection of LPS [1,2] which is likely to involve local cytokine release, cellular infiltration, and complement activation; there are the poorly understood characteristics of the microvasculature in some, but not all tumours [3]; and we have shown that T cell-dependent inflammation induced by mycobacterial antigens can prepare the skin of mice for particularly violent necrotic responses to TNF [4, and Al Attiyah & Rook, manuscript submitted]. Thus the Shwartzman reaction can contribute to the explanation of phenomena as diverse as the adult respiratory distress syndrome [24]; the haemorrhagic rash in meningococcal septicaemia, and the necrosis in tuberculosis lesions [5–9]. We need to understand how the necrosis is regulated.

Our findings are summarized in Table 1. Pretreatment with sublethal doses of LPS has been shown to exert anti-inflammatory effects in a variety of systems [21–23]. The mechanisms of this effect are complex and seem to involve factors produced by the liver [22], and the pituitary/adrenal axis [25] as well as changes in numbers and migratory properties of neutrophils [26,27]. These effects may be secondary to sub-lethal release of cytokines. In the LSR studied here, pretreatment with LPS profoundly inhibited both the swelling and the haemorrhagic necrosis, although in order to achieve this it had to be given 5 h before the preparatory injection. The other treatment which profoundly modulated both the swelling and the haemorrhage was that with MoAb 5C6. This antibody binds to an epitope on murine CR3 and inhibits recruitment of macrophages and polymorphonuclear cells into sites of inflammation [19,20]. Our results suggest that myeloid cells are essential mediators of swelling and necrosis. Some confirmation of this view was obtained in a single experiment in which phagocytes were depleted by an i.v. injection of liposomes containing the toxic drug dichloromethylene diphosphonate (Cl_2MDP) 24 h prior to their preparation for a LSR (a gift from Dr N. van Rooijen, Amsterdam). Administration of the toxic liposomes, but not of control PBS-containing ones, led to complete elimination of the synergy between preparatory and challenge injections (unpublished observations).

The involvement of complement in the LSR in mice was reported previously [19,20]. The model studied here allows the swelling and haemorrhagic components to be studied separately. We show that depletion of complement with CVF preferentially reduces the haemorrhage in both normal and preimmunized mice, and histologically the cellular infiltration seemed undiminished by CVF, although erythrocyte extravasation was eliminated. Separation of the two components of the reaction was also seen using the PAF inhibitor, WEB2170, which greatly reduced haemorrhage but not the swelling. It could do this even when given only 1 h before the challenge dose of TNF. These compounds are known to protect from the lethal effects of endotoxin shock [28,29] and as swelling occurs before haemorrhage, our results suggest that they may do so by inhibiting a late stage in the cascade of events. It is possible that at this stage, stasis is limiting the rate at which the deacetylase present in serum can gain access to the PAF, and so stop its actions on microvasculature, though it was without effect on the enormous haemorrhagic reaction evoked in pre-immunized mice. Similarly, the protective effect of misoprostol was similar whether it was given from before the preparatory injection, or only before challenge with TNF, but the effect was confined to the haemorrhagic component. Some prostaglandins have been shown to be useful in combating tissue damage during sepsis [30], and we suggest that this may reflect an influence on late events.

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