

INFLAMMATORY PROPERTIES OF RECOMBINANT TUMOR  
NECROSIS FACTOR IN RABBIT SKIN IN VIVO

BY M. RAMPART, W. DE SMET, W. FIERS, AND A. G. HERMAN

*From the Laboratory of Experimental Pharmacology, University of Antwerp (UIA), B-2610 Antwerp (Wilrijk); the Laboratory of Hematology, Innogenetics, B-3000 Leuven; and the Laboratory of Molecular Biology, State University of Ghent (RUG), B-9000 Ghent, Belgium*

A key phase in the emigration of neutrophils is the increased adherence of these cells to microvascular endothelial cells, initiated by the extravascular generation of chemical mediators. *In vitro* studies suggest that chemoattractants, such as C5a, FMLP, and leukotriene B<sub>4</sub> (LTB<sub>4</sub>), may act *in vivo* by stimulating the expression of the Mac I glycoprotein complex on the surface of the neutrophil within the venule lumen (1), whereas the macrophage-derived monokine, IL-1, may act by causing the expression of adhesive molecules (endothelial leukocyte adhesion molecule [E-LAM]) on the endothelial cell (2). *In vivo* and *in vitro*, the former process is rapid, whereas the latter is slow in onset and protein biosynthesis dependent (2, 3). Furthermore, neutrophil accumulation induced by C5a, FMLP, and LTB<sub>4</sub> is associated with a parallel time course of increased microvascular permeability, whereas little oedema formation is observed in response to IL-1, suggesting different mechanisms of action *in vivo* (4, 5).

Recent *in vitro* studies have demonstrated that another macrophage-derived monokine, TNF, enhances endothelial cell adhesiveness for neutrophils via the expression of the same membrane protein that is also induced by IL-1 (6). In contrast to IL-1, TNF has been reported to have stimulatory effects on neutrophils similar to C5a, FMLP, and LTB<sub>4</sub>, including increased adherence via expression of the Mac I glycoprotein complex (7). This unique spectrum of biological activities, plus the amounts of TNF that can be produced by macrophages in response to a microbial stimulus (up to 2% of their total protein biosynthesis), have implicated TNF as a putative mediator of the inflammatory reaction (8, 9).

Materials and Methods

*Animals.* New Zealand white rabbits (2.5–3.5 kg) were purchased from Janssen Pharmaceutica, Beerse, Belgium.

*Materials.* BSA, bradykinin (Bk), Evans blue dye, *N*-FMLP, 2-mercaptopyridine-*N*-oxide (MERC), Nitrogen mustard, and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) were from Sigma Chemical Co., Poole, UK. Nembutal (pentobarbitone sodium, 60 mg/ml) was from Abbott Laboratories, Paris, France. Plasmasteril (6% hydroxyethyl starch in 0.9% NaCl, sterile pyrogen free) was from Fresenius A. G., Homburg, FRG. Percoll was from Pharmacia Fine Chemicals, Upp-

M. Rampart is a Senior Research Assistant of the Belgian National Fund for Scientific Research. Address correspondence to M. Rampart, Laboratory of Experimental Pharmacology, University of Antwerp (UIA), B-2610 Antwerp (Wilrijk), Belgium.

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sala, Sweden. Sterile pyrogen-free solutions of acid-citrate-dextrose (ACD) and isotonic saline were from Travenol Laboratories, Lessines, Belgium.  $^{111}\text{In Cl}_3$  (2 mCi in 0.2 ml sterile pyrogen-free 0.04 N hydrochloric acid) and  $^{125}\text{I}$  human serum albumin (50  $\mu\text{Ci}/2$  mg albumin/ml sterile pyrogen-free isotonic saline) were from Amersham International, Amersham, UK.

Prostacyclin ( $\text{PGI}_2$ ) sodium salt was a gift from Dr. S. Moncada, Wellcome Research Laboratories, Beckenham, UK.

**Methods.** TNF preparations were kindly donated by Dr. J. Tavernier, Biogent, Belgium. Mouse and human recombinant TNF were expressed in *Escherichia coli* and purified to homogeneity as described previously (10). Using the L929 cytotoxicity assay the specific activity of mouse and human recombinant TNF preparations was  $7.5 \times 10^7$  U/mg protein and  $2.5 \times 10^7$  U/mg protein, respectively. LPS contamination was 36 ng/mg protein by Limulus assay.

**Preparation of  $^{111}\text{In}$ -labeled Neutrophils (4).** Rabbit neutrophils (>90% pure) were harvested from 72 ml of citrated blood by a two-layer discontinuous (40/60%) Percoll-plasma gradient after initial red cell sedimentation with hydroxyethyl starch (3% final concentration). Neutrophils ( $5 \times 10^7$ - $2 \times 10^8$  cells) were incubated with  $^{111}\text{In Cl}_3$  (50-200  $\mu\text{Ci}$ ) chelated to MERC (40  $\mu\text{g}$ ) for 15 min at room temperature. The labeled cells were washed twice and resuspended in autologous citrated plasma.

Neutrophil infiltration and oedema formation in the rabbit back skin were measured as the local accumulation of intravenously (marginal ear vein) injected  $^{111}\text{In}$  neutrophils and  $^{125}\text{I}$  human serum albumin (5  $\mu\text{Ci}$ , mixed with 2 ml of a 2.5% Evans blue dye solution) (4). Test agents were injected intradermally in 0.1-ml vol, each treatment having six replicates. At the end of the experiment, animals were killed by an overdose of pentobarbitone sodium, the back skin was removed, the injection sites were excised, and were then counted in a gamma counter with automatic spill-over correction (5005 Cobra; Packard Instrument Co. Inc., Downers Grove, IL).

Results were expressed in terms of number of  $^{111}\text{In}$  neutrophils by comparing skin sample  $^{111}\text{In}$  counts with  $^{111}\text{In}$  counts per cell in preparations before intravenous injection. Exudate volumes were expressed as microliters of plasma by dividing skin sample  $^{125}\text{I}$  counts by  $^{125}\text{I}$  counts in 1  $\mu\text{l}$  of plasma (4).

Intradermal injections were given according to a balanced site pattern and injection order was based on a Latin square design. Results are shown as mean  $\pm$  SEM for the number of rabbits indicated; one data unit being the mean of six replicates in each rabbit.

For neutrophil depletion studies, animals were injected with nitrogen mustard (1.75 mg/kg, i.v.) 2 d before experimentation (11).

## Results

Over a 30-min period mouse recombinant TNF (0.4-40 U/site,  $3 \times 10^{-16}$ - $3 \times 10^{-14}$  mol/site) induced very little plasma leakage and neutrophil accumulation when injected alone in rabbit skin (Figs. 1 and 2, *left panels, open symbols*). Coinjec-

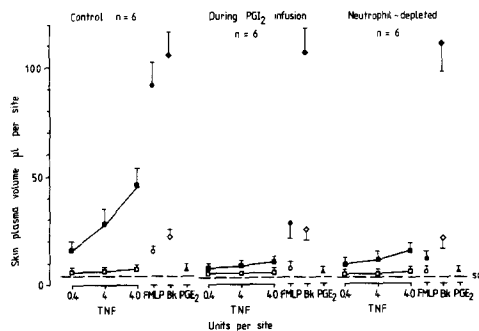


FIGURE 1. The effect of  $\text{PGI}_2$  infusion and neutrophil depletion on mouse TNF-induced plasma leakage. Open symbols are responses to mouse TNF, FMLP, and Bk alone. Closed symbols are responses to these mediators, but mixed with  $\text{PGE}_2$  ( $3 \times 10^{-10}$  mol/site). For experimental details, see Materials and Methods. Results are the mean  $\pm$  SEM for six rabbits.

tion of a vasodilator substance (PGE<sub>2</sub>,  $3 \times 10^{-10}$  mol/site) resulted in substantial oedema formation and neutrophil accumulation by these doses of mTNF (Figs. 1 and 2, *closed symbols*). Neutrophil emigration and their accumulation in extravascular tissue was confirmed by histological examination of sections of skin, injected with combinations of mTNF and PGE<sub>2</sub>. PGE<sub>2</sub> alone ( $3 \times 10^{-10}$  mol/site) did not induce leakage or cell accumulation.

Higher doses of mouse TNF (up to 4,000 U/site,  $3 \times 10^{-12}$  mol/site) did not result in any larger responses. In all experiments an established chemoattractant (FMLP,  $5 \times 10^{-11}$  mol/site) and a neutrophil-independent mediator of oedema formation (Bk,  $10^{-10}$  mol/site) were included for reference (11).

As previously documented for FMLP, oedema formation induced by mouse TNF was completely abolished in animals made neutropenic by previous injection of nitrogen mustard (Fig. 1, *right panel*). Responses to Bk were not affected.

Infusion of PGI<sub>2</sub> (50 ng/kg/min, previously shown to have no effect on systemic blood pressure, reference 12) almost completely suppressed mouse TNF- and FMLP-induced neutrophil accumulation and oedema formation. Bk-induced leakage was not altered during PGI<sub>2</sub> infusion (Fig. 1, *middle panel*; Fig. 2, *right panel*). In the experiments shown in Fig. 3, the 30-min flux of albumin and neutrophils was measured in inflammatory lesions of different age (0–6 h). Mouse TNF (40 U/site,  $3 \times 10^{-14}$  mol/site) or FMLP ( $5 \times 10^{-11}$  mol/site) were injected intradermally at different time intervals (–360–0 min; Fig. 3, *x-axis*). At zero time, <sup>125</sup>I albumin and <sup>111</sup>In neutrophils were injected intravenously. At this time, all intradermal sites were re-injected with PGE<sub>2</sub> ( $3 \times 10^{-10}$  mol/site) and plasma leakage and neutrophil emigration were measured over a 30-min period. Fig. 3 shows that FMLP-induced leakage and cell accumulation were maximal in the first 30 min and then gradually declined to very low values by 4–6 h. The biological *t*<sub>1/2</sub> of FMLP (for both leakage and neutrophil emigration) was 50–60 min. Similarly, responses to mouse TNF were maximal in the first 30 min; after 30–60 min mouse TNF was no longer active. Based on a more detailed time course (Fig. 3 *inset*) the biological *t*<sub>1/2</sub> of mouse TNF for oedema formation and cell accumulation was estimated to be 6–7 min. Identical results were obtained with human recombinant TNF.

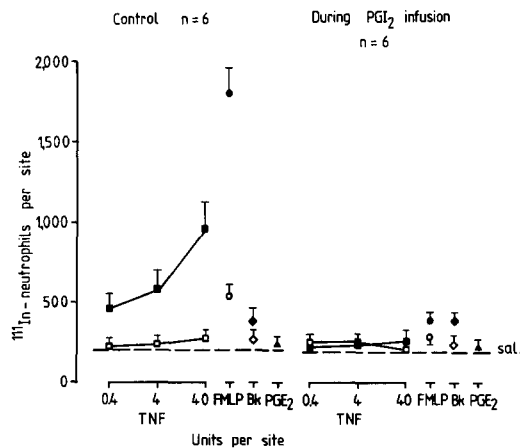


FIGURE 2. The effect of PGI<sub>2</sub> infusion on mouse TNF-induced neutrophil accumulation. For explanation of symbols, see legend to Fig. 1. Results are the mean  $\pm$  SEM for six rabbits.

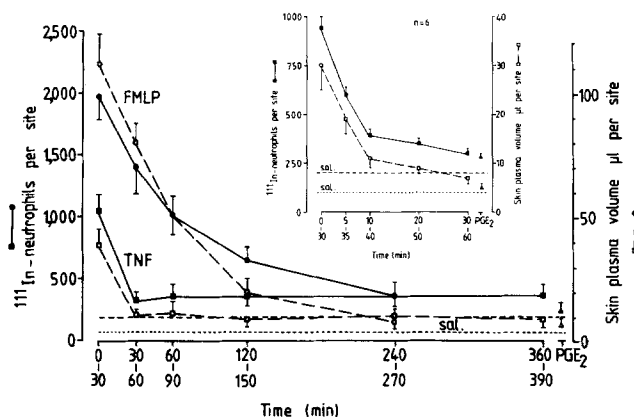


FIGURE 3. The 30-min flux of albumin and neutrophils in inflammatory lesions of different age (0–6 h). Mouse TNF ( $3 \times 10^{-14}$  mol/site) or FMLP ( $5 \times 10^{-11}$  mol/site) were injected intradermally at different times (0–360 min, x-axis) before intravenous injection of  $^{125}\text{I}$  albumin and  $^{111}\text{In}$  neutrophils (zero time). At this moment all intradermal sites were reinjected with  $\text{PGE}_2$  ( $3 \times 10^{-10}$  mol/site) and the reaction was allowed to proceed for 30 min. Animals were killed then by an overdose of pentobarbitone sodium and further processed as described in Materials and Methods. For saline-treated sites, the dotted line shows the background for plasma leakage, and the dashed line shows neutrophil accumulation. Results are the mean  $\pm$  SEM for six rabbits.

### Discussion

TNF is a macrophage-derived peptide that was originally described as the mediator of spontaneous regression of certain tumor cells, discovered in the serum of endotoxin-treated animals (reviewed in references 8, 9, 13). However, several lines of evidence indicate that besides cytostatic and cytotoxic activities, TNF may be an important mediator of inflammation by increasing neutrophil-endothelial cell adherence (6, 7). The data presented here provide evidence that TNF (already in very low amounts) has potent pro-inflammatory properties *in vivo*. Under conditions where local blood flow was optimally enhanced by the simultaneous intradermal injection of a vasodilator substance ( $\text{PGE}_2$ ), both mouse and human TNF induced neutrophil accumulation and neutrophil-dependent oedema formation in rabbit skin. Comparable dose-response curves for oedema formation and neutrophil emigration were obtained with 0.4–40 U/site of both mouse and human TNF. In view of the three-fold higher specific activity of mouse TNF ( $7.5 \times 10^7$  U/mg vs.  $2.5 \times 10^7$  U/mg for human TNF), this means that on a molar base, mouse TNF is more effective than human TNF. Therefore, it is possible that TNF receptors on rabbit cells bind mouse TNF with higher affinity than human TNF.

The fast onset and the short duration of action of TNF in rabbit skin (especially the absence of responses after 4–6 h) make it highly unlikely that TNF-induced neutrophil accumulation involves protein biosynthesis-dependent mechanisms, as reported for IL-1 (4, 5). The immediate onset of TNF-induced neutrophil emigration, paralleled by an increase in microvascular permeability, more resembles the pro-inflammatory profile of chemoattractants ( $\text{C5a}$ , FMLP,  $\text{LTB}_4$ ) that are believed to act through activation of neutrophils (4, 11). This is further supported by following findings: (a) TNF-induced oedema formation was abolished by neutrophil depletion. (b) TNF-induced oedema formation and cell accumulation were greatly suppressed by infu-

sion of PGI<sub>2</sub>. We have previously provided evidence that in this model PGI<sub>2</sub> selectively inhibits neutrophil-dependent responses (12). (c) In vitro, TNF increased the expression of the CD11b/CD18 glycoproteins, known to be important for neutrophil-endothelial cell adherence (results not shown). Activation of neutrophils by TNF is consistent with a recent report from Rothstein and Schreiber (14), that TNF-induced hemorrhagic necrosis is critically dependent on circulating neutrophils.

It has also been suggested that activation of neutrophils by TNF may require the production of secondary mediators, such as LTB<sub>4</sub> and platelet-activating factor (PAF). It is unlikely, however, that LTB<sub>4</sub> or PAF are mediating the inflammatory effects of TNF in this model. The duration of action of LTB<sub>4</sub> in rabbit skin is much longer than that of TNF (*t*<sub>1/2</sub> 30 min vs. 6–7 min; Rampart and Williams, unpublished observations); and PAF is a weak chemoattractant in rabbit skin, and PAF-induced oedema formation is independent of neutrophils (15).

The short duration of action of TNF in vivo may be related to its rapid internalization and degradation after binding to its receptor (16). This short biological half-life of TNF in vivo may be compensated for by the high amounts of TNF that can be produced by macrophages (up to 2% of their protein biosynthesis). However, intradermal injection of much higher amounts of TNF (up to  $3 \times 10^{-12}$  mol/site) or repeated administration of TNF to the same skin site did not produce larger responses, suggesting that desensitization to TNF may occur very rapidly in vivo (unpublished observations).

Finally, intravenous administration of TNF to animals is associated with systemic activation of neutrophils, and intratumoral injection of TNF results in massive neutrophil infiltration in the tumor (8, 9, 13). To what extent neutrophils are involved in the elimination of tumor cells by TNF in vivo is unknown at the present time. There is a large body of in vitro evidence that TNF is directly cytotoxic for many tumor cell lines, thus, independent of neutrophils (13). Our present data indicate that activation of neutrophils by TNF can be prevented by an infusion of PGI<sub>2</sub>. It is tempting to speculate that a combination of antitumor treatment with TNF with an infusion of PGI<sub>2</sub> may suppress some of the undesired side effects of TNF. Studies are currently being undertaken to evaluate this hypothesis.

### Summary

We have investigated the ability of recombinant TNF (mouse and human) to produce acute inflammatory lesions in an established experimental model of inflammation. Upon intradermal injection in rabbit skin, TNF, in amounts as low as  $3 \times 10^{-14}$  mol/site, was found to be very potent at inducing local neutrophil accumulation and neutrophil-dependent oedema formation, thereby fulfilling two important criteria to be considered as an inflammatory mediator. Our findings further indicate that the pro-inflammatory properties of TNF are probably more related to its immediate stimulatory effects on neutrophils rather than to its slow (protein biosynthesis-dependent) effects on endothelial cells. Our data thus show that very low amounts of mouse and human recombinant TNF can initiate an acute inflammatory reaction in vivo in rabbit skin and that TNF is able to evoke two of the four cardinal signs of inflammation.

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