Int. J. Exp. Path. (1994) 75, 191–196

Endotoxin is angiogenic

I. MATTSBY-BALTZER, A. JAKOBSSON, J. SÖRBO AND K. NORRBY

Departments of Clinical Bacteriology and Pathology, University of Göteborg, Göteborg, Sweden

Received for publication 12 November 1993
Accepted for publication 23 February 1994

Summary. The formation of new blood vessels, angiogenesis, is an important event in inflammation, wound healing and tumour growth. Mediators produced by various cells when exposed to endotoxin include cytokines (tumour necrosis factor, interleukins 1 and 6, and basic fibroblast growth factor) which, it has been suggested, stimulate angiogenesis. The angiogenic effect of endotoxin (lipopolysaccharide, LPS) was studied in rats using the quantitative mesenteric window assay. Adult rats were injected intraperitoneally with *Escherichia coli* LPS (5 pg/ml – 20 000 ng/ml) twice daily for 4.5 consecutive days and were sacrificed 14 days after the start of this treatment. An angiogenic response was observed at concentrations of > 2 ng/ml in a dose-dependent manner. No inflammatory cellular exudate was seen in the test tissue at the time of angiogenesis analysis. Suppressed body-weight gain, a marker of the systemic effect of LPS in the rat, was significant only at the highest dose tested. The data suggest that endotoxin-mediated neovascularization could be a component of inflammation and wound healing.

Keywords: angiogenesis, endotoxin, lipopolysaccharide, quantification, rat, mesentery, mast cells

Angiogenesis and the activation of macrophages appear to be inherent events in inflammation, wound healing and the growth of solid tumours. Endotoxin, which is a part of the cell wall of Gram-negative bacteria that is released during the growth or lysis of the bacteria (Mattsby-Baltzer *et al.* 1991), has the ability to elicit a wide spectrum of host-effector molecules such as TNF, IL-1, IL-6, TGF-β and bFGF by macrophages and various other cell populations (Morrison & Ryan 1987; Austgulen & Nissen-Meyer 1988). Since these factors have been reported to be angiogenic in various assays (Folkman & Klagsbrun 1987; Giulian *et al.* 1988; Motro *et al.* 1990; Fajardo *et al.* 1992) endotoxin is an angiogenic candidate. Very little, however, is, known about the angiogenic properties of endotoxin in normally vascularized mammalian tissue at concentrations that may occur *in vivo* in patients. The aim of the present study was to investigate whether endotoxin (lipopolysaccharide, LPS), within a concentration range of 5 pg/ml – 20 μg/ml saline is angiogenic when injected i.p. into healthy adult rats, using the quantitative mesenteric window assay.

Correspondence: Professor Klas Norrby, Department of Pathology, University of Göteborg, Sahlgren Hospital, S-413 45 Göteborg, Sweden.

Materials and methods

Lipopolysaccharide (LPS)

LPS was prepared and purified from *Escherichia coli* 018K1, a strain that was originally isolated from a patient (Mårild *et al.* 1989). The bacteria were grown on nutrient broth agar containing glucose at 37°C. The bacteria were harvested the following day and washed twice before freeze-drying. LPS was prepared by hot

phenol–water extraction (Westphal & Jann 1965). One ng of this LPS/ml corresponds to approximately 10.2 EU/ml (US FDA Guideline 1987).

Animals

Adult male Sprague–Dawley rats (B & K Universal AB, Sollentuna, Sweden), acclimatized for at least 5 days prior to experimentation to standardized conditions (Norrby *et al.* 1986), were used. Two animals shared each cage and were fed standard pellets and water *ad libitum*. Three experiments were performed. The animals weighed a mean of (± s.e.m.) 219.2 ± 1.6, 222.8 ± 1.9 and 214.07 ± 1.51 g at the start of the experiments and on average the saline treated controls gained approximately 46% in body weight from day 0 to sacrifice 14 days later.

Induction of angiogenesis

LPS diluted in isotonic saline (NaCl, 0.9% w/v) made for clinical use (Kabi Vitrum AB, Stockholm, Sweden) was injected i.p. (2 ml/100 g b.w.) twice daily for 4.5 days according to a previously described protocol (Norrby *et al.* 1986); controls received the same volume of the saline vehicle. LPS at doses of 20 000, 200, 2, 0.050 and 0.005 ng/ml were used. The saline was free from pyrogens (< 1pg/ml) as tested by the chromogenic *Limulus* assay (Coatest Endotoxin, Chromogenix, Mölndal, Sweden). Fourteen days after the start of the angiogenic treatment, the animals were killed and tissue samples were prepared for the quantification of angiogenesis. Each treatment group comprised eight animals.

Angiogenesis quantification in terms of the relative vascularized area

The true mesentery in the rat, which connects the small gut with the dorsal body wall, contains some 40–45 distinctly outlined connective tissue membranes or 'windows' which are framed by fatty tissue. These windows are natively extremely thin, measuring only 5–10 μm, and contain some 2–3% of mast cells and 1–2% of macrophages in addition to some 46–48% of fibroblasts and mesothelial cells respectively (Norrby & Enestrom 1984). The tissue is sparsely vascularized and lacks significant physiological angiogenesis (Norrby *et al.* 1986; 1988). In unsectioned intact window spreads on objective slides, the vasculature was measured morphometrically *en face* in terms of the vascularized area as a percentage of the whole area of the window examined (VA) as described elsewhere (Norrby *et al.* 1990). VA is a measure of the spatial extension of the virtually two-dimensional vasculature.

The vasculature in the test tissue was visualized by an ink–gelatin infusion which was performed by ligating the aorta above and below the superior mesenteric artery as soon as the animal had been killed by CO_2 inhalation and the right atrium of the heart had been opened. Through an incision in the aorta, a plastic tube (PE 90, Clay Adams intradermic polyethylene tubing; 0.89 mm inside diameter) was inserted about 10 mm into the superior mesenteric artery and secured with two ligatures. One hundred ml Perfadex (Kabi Vitrum AB, Stockholm, Sweden),infused manually for 3 minutes using a syringe connected to the tube with a blunt needle, was used as a blood wash-out medium. Ten ml of ink–gelatin solution (Norrby *et al.* 1990) was subsequently injected for about 30 seconds into the plastic tube from a connecting syringe and the carcass was placed in a freezer at −22°C for 1 hour to allow the gelatin to solidify. The mean size (± s.e.m.) of the windows, in cm^2 per treatment group, ranged from 1.23 ± 0.64 for the group treated with 200 ng LPS/ml to 1.37 ± 0.72 for the animals receiving LPS at 2 ng/ml. This difference was insignificant in statistical terms. Four windows per animal were analysed.

Histamine release as a measure of mast-cell secretion

The mast cell is the major, if not the only, source of histamine in the test tissue. The i.p. injections of LPS were given once daily for two consecutive days and the animals were killed 1 hour after the second injection. Three randomly selected, medium-sized mesenteric windows were excised per animal, pooled, immediately frozen and subsequently stored at −70°C. Histamine was quantified by a fluorimetric OPT reaction (Enerbäck & Wingren 1980), whereas the total protein content was quantified according to Lowry *et al.* (1951); the histamine content was expressed as the ng histamine base per μg protein.

Macrophotography

Individual mesenteric window spreads on objective slides were photographed using a Wild Macroscope M 240 equipped with an apochromatic 1:6 Apozoom objective and connected to a Wild MPS 46 Photoautomat. The macroscope features parallax-free imaging and large fields of view.

Statistics

The non-parametric Mann–Whitney *U*-rank sum test for unpaired observations (two-tailed) was used; $P \leqslant 0.05$

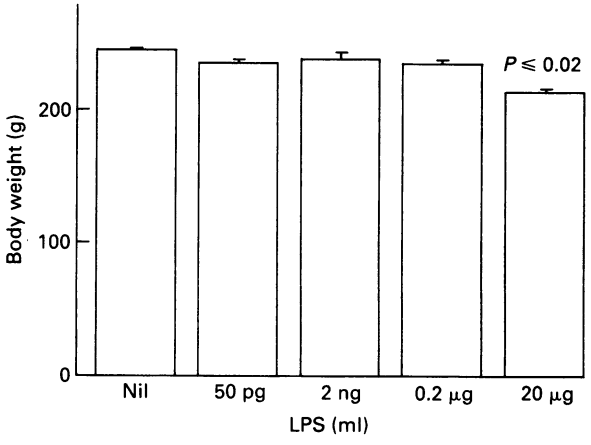

Figure 1. Body weight at the end of the i.p. treatment which lasted for 5 days. The highest dose of endotoxin used, i.e. LPS at 20 000 ng/ml, suppressed the normal body-weight gain significantly compared with the saline treated controls. Each bar represents eight animals and shows the mean± s.e.m. The *P*-value given is in comparison with the saline treated controls.

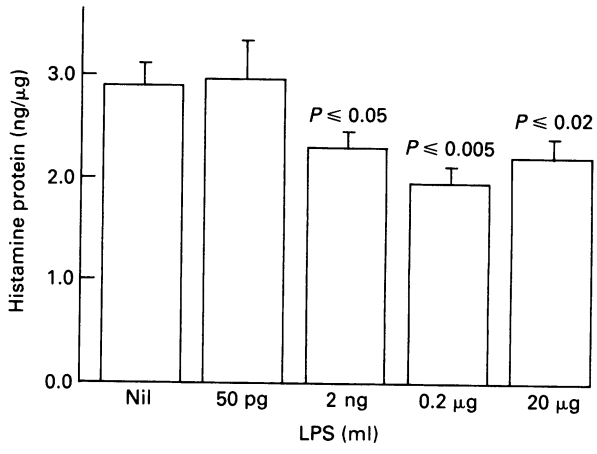

Figure 2. Histamine concentration in mesenteric windows following i.p. injections of endotoxin at different doses. A low concentration of histamine is a sign of a high degree of release from mast cells in the test tissue. The histamine content, in ng/μg protein, was almost the same in untreated controls as in animals receiving LPS 0.050 ng/ml (2.89 ± 0.22 and 2.95 ± 0.38). By contrast, LPS at concentrations of ⩾2 ng/ml caused a significant release of histamine. Note that there was no significant difference in effect between the LPS doses of 2, 200 and 20 000 ng/ml. Each bar represents eight animals. Mean ± s.e.m. The *P*-values shown are in comparison with the saline treated controls.

was considered significant. For VA the mean values of four windows per animal were used as independent data. There were therefore eight sets of independent data per treatment group throughout.

Results

Effect of LPS on body-weight gain

LPS at doses of ⩽200 ng/ml did not significantly affect the physiological body-weight gain as compared with untreated controls (Figure 1). However, at the highest dose tested, i.e. 20 000 ng/ml, LPS suppressed the body-weight gain significantly as recorded on the last day of the i.p. treatment ($P \leqslant$; 0.02; Figure 1) and at sacrifice on day 14 ($P \leqslant 0.05$; Table 1).

Table 1. Effect on body-weight gain of i.p. injections of LPS administered for 5 consecutive days (days 0–4). The body weights at the end of the experiments, on day 14, are given. At 20 000 g/ml, LPS suppressed the body weight significantly as measured on day 4 ($P \leqslant 0.02$; Figure 1) and at sacrifice ($P \leqslant 0.05$). The controls increased approximately 51% in body weight during this particular experimental period. Each group comprised eight animals.

Treatment i.p. LPS (ng/ml)	Body weight (mean ±s.e.m.
Saline	325.1 ± 4.5
0.05	316.5 ± 4.7
2	324.7 ± 5.9
200	316.0 ± 4.5
20 000	307.4 ± 4.4

Histamine-releasing effect of LPS

I.p. injections of LPS at 0.050 ng/ml did not release histamine in the mesenteric window compared with the untreated controls, suggesting that LPS at this concentration did not activate mast-cell secretion in the test tissue (Figure 2). The lowest concentration of LPS that released a significant quantity of histamine was 2 ng/ml ($P \leqslant 0.05$; Figure 2). It is interesting to note that there was no significant difference in statistical terms in the hista-

Table 2. Angiogenesis in terms of the relative vascularized area (VA) following i.p. injections of saline and LPS at 0.005 ng/ml and 0.050 ng/ml for 5 consecutive days. The LPS treatment at either dose, or when combined, did not affect angiogenesis significantly in statistical terms as compared with the saline treated controls. Each group comprised 32 specimens

Treatment i.p. LPS (ng/ml)	VA (mean ± s.e.m.)
Saline	14.63 ± 2.49
0.005	9.09 ± 1.22
0.050	9.10 ± 1.27

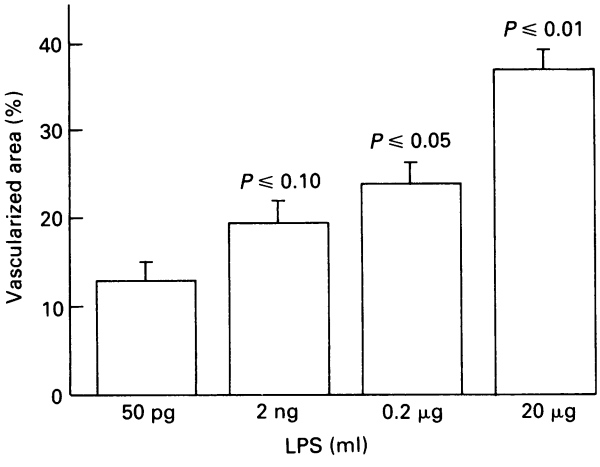

Figure 3. Angiogenic response in terms of the relative vascularized area (VA) in mesenteric windows following i.p. injections of endotoxin at different concentrations. LPS at concentrations of $\geqslant 2$ ng/ml was angiogenic in an approximately linear, dose-dependent manner as compared with the LPS 0.050 ng/ml dose which was angiogenically inert (Table 2). The *P*-values shown in the Figure are based on eight sets of independent data per group and relate to the difference as compared with the LPS 0.050 ng/ml dose. The differences between 2 and 20 000 ng/ml and between 0.050 and 20 000 ng/ml were also significant ($P \leqslant 0.025$ and $P \leqslant 0.02$). Each bar represents 32 specimens from eight animals. Mean ± s.e.m.

mine releasing effect of LPS within the range of 2–20 000 ng/ml.

Angiogenic effect of LPS

LPS at 0.005 and 0.050 ng/ml did not affect the angiogenesis significantly in statistical terms compared with saline treated controls (Table 2). LPS was, however, angiogenic in a roughly linear, dose-dependent manner at concentrations of $\geqslant 2$ ng/ml (Figure 3). No inflammatory cellular exudate was seen in toluidine blue stained window spreads at the time of angiogenesis analysis.

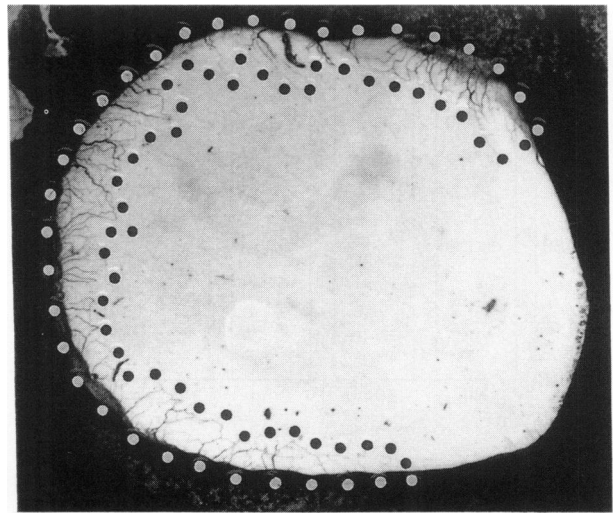

Figure 5. Macrophotograph of a mesenteric window spread from an animal given intraperitoneal injections of LPS at a dose of 2 ng/ml. The total window area is 101.8 mm^2, the vascularized area (within the dotted line) is 19.4 mm^2 or 19.1%. This value is close to the mean of the treatment group receiving LPS 2 ng/ml (see Figure 3).

Discussion

LPS endotoxin induced angiogenesis in a dose-dependent manner at a concentration of $\geqslant 2$ ng/ml and the increase was statistically significant at concentrations of $\geqslant 200$ ng/ml, as assessed quantitatively in the mesen-

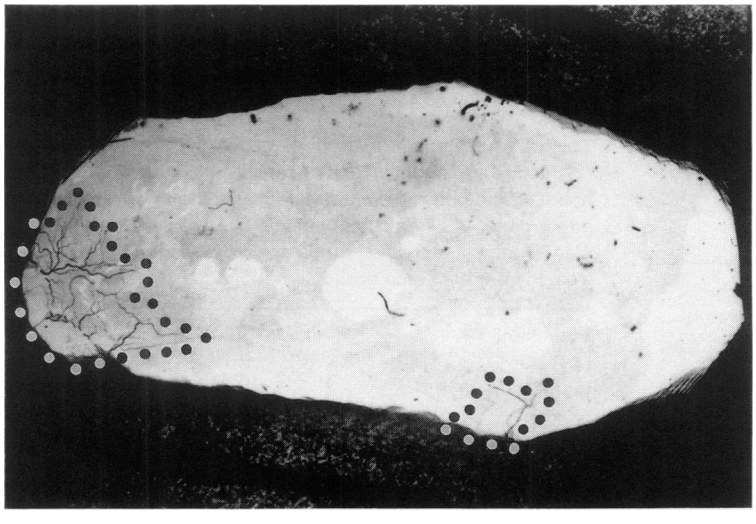

Figure 4. Macrophotograph of a mesenteric window spread from an animal which received intraperitoneal injections of LPS at a dose of 0.050 ng/ml. The demarcation between the light window and the dark surrounding fatty tissue is clear cut. The total area of the mesenteric window is 90.4 mm^2 and the vascularized area, delimited by the dotted line, is 11.0 mm^2 or 12.2%, which is close to the mean value of the treatment group receiving LPS 0.050 ng/ml (see Figure 3).

teric windows. The histamine releasing effect in the test tissue of LPS at these concentrations did not run in parallel to the angiogenic response. This indicates that LPS was angiogenic over and above any mast-cell mediated angiogenesis (Norrby *et al.* 1986; 1989; 1990) which, if it occurred at all, may have been a component of the LPS mediated angiogenic response studied.

I.p. injections of LPS at doses of $\leqslant$200 ng/ml did not significantly affect body-weight increase, suggesting that LPS at these doses was non-toxic to the adult animals. Furthermore, LPS at a concentration of 0.050 ng/ml neither released histamine nor induced angiogenesis in the mesenteric windows, as compared with saline treated controls. Thus, LPS injected i.p. at 0.050 ng/ml appeared to be inert in terms of general toxicity, as well as mast-cell activation and angiogenesis in the test tissue used.

One of the unique features of LPS is its capacity to elicit a wide spectrum of host effector molecules by different cell populations, although macrophages appear to be a major target. Endotoxin tolerance, that is a diminished febrile response, reduction in the levels of inflammatory mediators and increased resistance to a lethal LPS challenge, may develop upon repeated injections of endotoxin (Greisman 1983) so it cannot be excluded that a reduction in the inflammatory response in fact occurred during the course of the LPS treatment given here. Nevertheless, a dose-dependent angiogenic response was observed from a dose as low as 2 ng/ml. This LPS induced angiogenesis, observed 10 days after the end of the i.p. treatment, was not accompanied by an inflammatory cellular exudation in the test tissue. It is probable, however, that a transient inflammatory cellular response preceded the angiogenic response, because on the second day of the LPS treatment with a dose of 2 ng/ml/day, a marked cellular inflammatory reaction was seen in the mesenteric window (unpublished observation). It is generally believed that a cellular response is an early stage in any LPS induced action.

Endotoxin most probably mediates its biological actions indirectly by inducing macrophages to secrete factors such as TNF, IL-1, IL-6 and basic FGF (bFGF), all of which have been described as promoting angiogenesis (Austgulen & Nissen-Meyer 1988; Giulian *et al.* 1988; Motro *et al.* 1990; Fajardo *et al.* 1992). The fact that all nucleated cells appear to possess TNF receptors (Piquet *et al.* 1990) suggests that this cytokine may elicit a host of significant effects *in vivo*. It is, moreover, interesting to note that the three-dimensional structure of human IL-1 closely resembles the structure of bFGF and together these factors might comprise a large cytokine family (Partanen *et al.* 1992). Furthermore, cultured human pulmonary artery endothelial cells are stimulated both to produce increased amounts of PDGF mRNA and to release PDGF-like protein after exposure to LPS at doses of 100–10 000 ng/ml (Albelda *et al.* 1989). As recently shown, PDGF, and in particular the PDGF-BB homodimer, stimulates organogenic angiogenesis in the chick chorioallantoic membrane assay (Risau *et al.* 1992). When stimulated, endothelial cells can also produce and secrete IL-6 (May *et al.* 1989). The hypothetical contribution of each cytokine and growth factor to the angiogenic response observed would probably depend on the hierarchy, the concentration and the kinetics of the factors.

As recently reported, angiogenesis in the rabbit cornea can be induced by implanting into that tissue a 1-mm^3 pellet of sustained release polymer containing *E. coli* LPS (Li *et al.* 1991). Vessel growth was documented by measuring the longest vessels as the perpendicular distance between the limbus and the growing vessel tip, using slit-lamp examination. It was found that the rate of neovascularization induced by the 7.5% (75 ng) LPS loads was greater on average than that induced by the 15% LPS loads used. However, vessel density was significantly greater with the 15% LPS load. It was speculated that bFGF, TNF and TGF-β were the actual angiogenic effectors, as they were probably produced by inflammatory cells stimulated by the LPS treatment. Since the cornea is normally strictly avascular, its regulation of angiogenesis may not be representative of that of vascularized tissues (Blood & Zetter 1990). It has recently been observed in this laboratory that i.p. injections of low doses of TNF accelerate angiogenesis in the rat mesentery (J. Sörbo, to be published).

The angiogenesis assay used in the present study demonstrated that the rat is sensitive to low doses of endotoxin, even though it is one of the most resistant species when it comes to endotoxin induced lethality (McCuskey *et al.* 1984). The lowest dose at which an angiogenic response emerged, 2 ng/ml, corresponds to approximately 10^5–10^6 *E. coli* bacteria per ml, as based on the *Limulus* activity test (Mattsby-Baltzer *et al.* 1991 and unpublished observations). These data suggest that the present finding of LPS induced angiogenesis may be of clinical relevance. Since native LPS, that is, cell wall fragments shed from the bacteria, is known to be 5–50 times stronger in *Limulus* activity based on the β-hydroxymyristic acid content of LPS as compared with whole bacterial cells or purified LPS, as used here, it is conceivable that angiogenesis could be induced by even lower doses of the native type of LPS.

In previous experiments using the mesenteric window assay, we have observed that the i.p. injection of saline alone may induce angiogenesis, the potency of which

varies from one experiment to another. All the saline solutions that were used previously were made for clinical use and should therefore contain less than 0.25 EU/ml (US FDA Guideline 1987), corresponding to 24.5 pg/ml of the 018K1 LPS, which was below the angiogenically inert concentration of 50 pg/ml of the 018K1 LPS used in the present study. Our own endotoxin analyses using the *Limulus* test on saline solution of three different brands of isotonic saline, including the one used in the present experiments, revealed no endotoxin activity whatsoever (sensitivity 1 pg/ml). It would thus appear that some factor(s) other than endotoxin accounts for the differences in angiogenic responsiveness occurring in saline treated rats from one time to another, such as uncontrollable variations in the angiogenic response of the animals.

In conclusion, endotoxin is reported here to induce de-novo angiogenesis in adult mammalian, normally vascularized tissue at doses that appear realistic in a clinical setting. This finding suggests that endotoxin mediated neovascularization could be a component in inflammation and wound healing since macrophages, which are apparently the main target cell for endotoxin, can be activated by endotoxin in these diseased states.

Acknowledgements

This work was supported by grants from the Swedish Medical Research Council, project nos. 5942 (K.N.) and 9488 (I.M.-B.). We thank Gunvor Jefferth and Ann Nehlmark for their skilful technical assistance.

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