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Endotoxin-stimulated human macrophages produce a factor that induces polymorphonuclear leucocyte infiltration and is distinct from interleukin-1, tumour necrosis factor α and chemotactic factors

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SUMMARY

Previously we reported that rabbit macrophages (Mϕ) in the presence of nanogram quantities of endotoxin (LPS) release factors that induce polymorphonuclear leucocyte (PMNL) infiltration into the skin of rabbits following i.d. injection. The predominant factor was a *de novo* synthesized protein of 45,000 MW on gel filtration that was distinguishable from IL-1 but not from TNFα. Here we examined human monocytes, *in vitro* monocyte-derived Mϕ and peritoneal Mϕ for the production of an analogous protein. Upon stimulation with LPS, they all rapidly (6 hr) produced a factor(s) that caused PMNL accumulation in the skin of rabbits when injected i.d. This activity, referred to as PMNL-recruiting activity (PRA), was heat labile and its production was blocked by cycloheximide. By Sephadex-G100 chromatography the major PRA of cultured Mϕ or peritoneal Mϕ had a molecular weight (MW) of 45,000–60,000. The active fractions were free of IL-1 (<0·2 U/ml) and Superose-12 FPLC chromatography separated the peak of PRA, which eluted at 45,000 MW, from TNFα, eluting at 20,000 MW. The peak PRA was not neutralized by antisera to IL-1α, IL-1β, TNFα, IL-6 or GM-CSF, indicating that it was distinct from these cytokines. The major PRA did not induce the migration of PMNL *in vitro* in a filter chemotaxis assay. In contrast to the Mϕ, the major PRA produced by LPS-stimulated monocytes eluted at 15,000–20,000 MW, contained IL-1 activity and was neutralized by antisera to IL-1α and IL-1β. Monocytes from a few donors also produced the 45,000–60,000 MW PRA simultaneously. We conclude that human peritoneal Mϕ and *in vitro* monocyte-derived Mϕ exposed to LPS secrete a protein of 45,000–60,000 MW, which is a potent inducer of PMNL infiltration but is distinct from IL-1, TNFα, IL-6, GM-CSF and PMNL chemotactic factors.

INTRODUCTION

Invasion of host tissues by bacteria usually incites a vigorous inflammatory reaction. The host and bacterial factors mediating polymorphonuclear leucocyte (PMNL) accumulation at sites of infection are not well understood. Live bacteria are known to secrete PMNL chemotactic factors, but killed Gram-negative bacteria, such as *Escherichia coli*, are also effective inducers of inflammation (Kopaniak, Issekutz & Movat, 1980). In Gram-negative bacteria the endotoxin (LPS) component seems likely to contribute to the inflammation induced because neutralization of the LPS on *E. coli* significantly diminished the acute inflammatory reaction, including the degree of PMNL infiltration (Issekutz, Bhimji & Bortolussi, 1982). Furthermore, LPS shed by *E. coli* or purified LPS causes intense PMNL infiltration of tissues in rabbits and other species, although LPS does not directly stimulate PMNL migration *in vitro* (Issekutz & Bhimji, 1982; Movat & Cybulsky, 1987).

Recently (Issekutz, Megyeri & Issekutz, 1987) we demonstrated that rabbit pleural macrophages (Mϕ), in the presence of nanogram quantities of LPS, release a protein factor(s) that induces PMNL infiltration into the skin of rabbits when injected intradermally. On gel filtration chromatography, one major peak of PMNL-recruiting activity (PRA) was observed eluting at a MW of 45,000–60,000 and a second minor peak appeared in the 14,000–18,000 MW region. Interleukin-1 (IL-1), which can induce PMNL infiltration *in vivo* (Granstein *et al.*, 1986; Movat & Cybulsky, 1987) was present only in the low molecular

Abbreviations: ACD, acid citrate dextrose; GM-CSF, granulocyte-macrophage colony-stimulating factor; HIS, heat-inactivated serum; HSA, human serum albumin; i.d. intradermal; IL-1, interleukin-1; LPS, endotoxin; Mϕ, macrophage; MEM, minimal essential medium; PB, polymyxin B; PBS, phosphate-buffered saline; PMNL, polymorphonuclear leucocyte; PRA, PMNL recruiting activity; RBC, red blood cells; TNF, tumour necrosis factor.

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weight fraction, although PRA could not be unequivocally separated from tumour necrosis factor α (TNFα), another inflammatory Mϕ product (Movat & Cybulsky, 1987).

The results in the rabbit Mϕ system prompted us to study human monocytes and Mϕ. Our objectives were to determine if these human mononuclear phagocytes elaborate an analogous protein factor in response to LPS, to determine if it can be assayed across species barriers (human product in rabbit) and, by studying a human product, take advantage of the availability of neutralizing antibodies to human cytokines in analysing the relationship of PRA to other Mϕ products.

MATERIALS AND METHODS

Cell culture

Human blood mononuclear cells were isolated by Ficoll–Hypaque density sedimentation from heparin (10 U/ml) anti-coagulated blood of healthy adult donors. Mononuclear cells were resuspended to contain $7{\cdot}5 \times 10^5$ monocytes/ml, as determined by Coulter counter analysis, in RPMI-1640 medium (Gibco, Grand Island, NY), with 10% heat-inactivated autologous serum (HIS) with antibiotics, and plated in 24-well plates (Primaria) (Becton-Dickinson Labware, Oakland, CA). After incubation for 2 hr at 37° in 5% CO_2 in air, the non-adherent cells were removed by washing and, for stimulation, the adherent cells (≥90% monocytes) were refed with 1 ml medium without serum. Cultures set up to allow monocytes to differentiate to Mϕ were refed with RPMI-1640, 10% HIS and incubated for 2–10 days. Peritoneal Mϕ were collected from children undergoing peritoneal dialysis. These patients were not on antibiotics and were free of infection. The cells in the dialysis fluid were recovered by centrifugation, washed, and adhered as above in RPMI-1640-10% HIAB serum. The non-adherent cells were removed by washing prior to stimulation with LPS. The adherent cells were >90% Mϕ by morphology and non-specific esterase stain.

Stimulation of Mϕ with LPS was performed in 1 ml of fresh RPMI in the macrowells using *E. coli* 0111 LPS (List Biological, Campbell, CA), usually for 6 hr at 37°. Culture supernatants were harvested by centrifugation and frozen at −70°.

Leucocyte chemotaxis

PMNL chemotaxis was measured using 12-well 6·5 mm Costar Transwell culture plates equipped with 3 μm polycarbonate filters (PVP free, cat. no. 3415; Costar Inc., Cambridge, MA). Human PMNL were separated from acid-citrate-dextrose (ACD Formula A; Baxter Travenol, Malton, Ontario)-anti-coagulated blood using 6% dextran-saline (Abbott Labs., Mississauga, Ontario) (one volume to four volumes of blood) to sediment red cells, and centrifugation of the leucocyte-rich plasma on 62% Percoll (Pharmacia Fine Chemicals, Dorval, Quebec) to obtain purified PMNL. The PMNL were washed and resuspended at 2×10^6 in Minimal Essential Medium (MEM Microbiological Assoc., Walkersville, MD) containing 10 mM HEPES, 15 m Eq/l $NaHCO_3$, pH 7·2 and 0·25% pyrogen-free human albumin (HSA) (Connaught Laboratories, Toronto, Ontario). Rabbit PMNL chemotaxis was performed by harvesting PMNL from rabbit blood using hydroxyethylcellulose (Fluka Chemical Corporation, Ronkomkoma, NY) sedimentation of red cells and Percoll centrifugation (Issekutz *et al.*, 1987). For these cells rabbit albumin (0·25%) (Sigma Chemical Co., St Louis, MO) was used in the MEM. Chemoattractants (e.g. 1% zymosan-activated plasma, 10^{-8}M N f-norleu-leu-phe, column fractions) were diluted in MEM-HSA or MEM-RSA in the bottom chamber (600 μl). One-hundred microlitres of PMNL suspension were loaded above the filter and incubated for 40 min in humidified 5% CO_2 at 37°. The undersurface of the filter was then rinsed with cold 0·2% EDTA-PBS to detach PMNL and the number of PMNL that migrated through the filter was determined by cell counting (Coulter Electronics, Hialeah, FL). Results are expressed as percentage migration into the lower compartment of the PMNL added above the filter.

Dermal inflammatory reactions

New Zealand white rabbits of either sex weighing 3–4 kg were used. On the shaved backs of the animals, 40–50 skin sites were designated and inflammatory stimuli, e.g. culture supernatants or chromatography fractions, were injected (0·2 ml) intradermally, as previously described (Issekutz *et al.*, 1987). Two hours later the animals were killed, the skin removed and the lesions punched out using a 16-mm leather punch. The radioisotope content of the lesions was analysed in an LKB gamma spectrometer (Fisher Scientific Company, Dartmouth, NS).

Measurement of leucocyte accumulation

For the quantification of leucocyte accumulation at skin lesions, the leucocyte labelling method described previously (Issekutz *et al.*, 1987) was employed. Briefly, 30 ml of blood were collected into ACD anti-coagulant. Hydroxyethylcellulose was added to sediment the red blood cells (RBC), the harvested leucocyte-rich plasma was centrifuged and the leucocyte-RBC pellet was resuspended in 4 ml of Ca^{2+}, Mg^{2+}-free Tyrode's solution-10% plasma and labelled with 100 μCi of sodium 51-chromate (Dupont Canada, Dorval, Quebec) during a 30-min incubation (37°). This leucocyte preparation gives results comparable to a highly purified PMNL preparation (Issekutz & Movat, 1980) when the leucocyte infiltrate consists of >90% PMNL, and haemorrhage is not observed, as was the case in these studies. The washed ^{51}Cr-labelled leucocytes were injected i.v. just prior to i.d. injection of the skin sites. A 5-ml blood sample was collected after 60 min, and processed using hydroxyethylcellulose and Percoll to obtain purified PMNL for determination of the blood ^{51}Cr-PMNL specific activity (number of blood PMNL/c.p.m.). Two hours after the i.d. injections the rabbit was killed. The accumulated ^{51}Cr in the skin lesions was converted to an accumulated number of PMNL ($\times 10^6$) in the lesions using the PMNL specific activity (Issekutz *et al.*, 1987).

Chromatography

Culture supernatants were chromatographed on a 1×40 cm Superose 12 FPLC column (Pharmacia Fine Chemicals, Dorval, Quebec) or on a Sephadex G-100 (Pharmacia) ($1{\cdot}5$ cm $\times$ 42·5 cm) column, equilibrated with pyrogen-free 0·01 M phosphate-buffered saline (PBS) (pH 7·0) (Issekutz *et al.*, 1987). Culture supernatants were concentrated 10 times by lyophilization and a small amount of HSA (2 mg) was added to improve recovery of activity. Superose-12 gel filtration was performed at a flow rate of 0·4 ml/min and 0·6-ml fractions were collected. Sephadex G-100 chromatography was performed at a flow rate of 5 ml/hr and 2-ml fractions were collected during 20-hr runs at 4°. Fractions were tested directly in the rabbit skin assay for PRA

following addition of 25 μg/ml Polymyxin B (Burroughs Wellcome Inc., Kirkland, Quebec) to neutralize any residual LPS.

TNF and IL-1 assays

TNF was assayed by cytotoxicity on actinomycin-D-treated L929 fibroblasts by a modification of the method of Aggarwal *et al.* (1984). L929 cells, at a density 5×10^3 per well in 96-well flat-bottomed microtitre plates (Nunc, Gibco), were cultured in 100 μl RPMI-1640 and 10% FCS (Gibco). Serial dilutions of TNFα, culture supernatants, or chromatography fractions were added to the wells together with 1 μg/ml actinomycin-D. After an 18-hr incubation, cell viability was assessed by trypan blue dye exclusion. Recombinant human TNF (kind gift from Dr M. Shepard, Genentech, San Francisco, CA) was used as a standard. The sensitivity of the assay was 0·5 U/ml.

IL-1 was assayed by the method of Simon, Laydon & Lee (1985) with minor modifications. Briefly, $1{\cdot}5 \times 10^4$ EL-4 mouse lymphoma cells suspended in 0·1 ml DMEM (Gibco) medium plus 10% FCS were added to each well of 96-well culture plates. Serial dilutions of recombinant human IL-1α or of test samples were then added in 0·1 ml. Finally, 0·05 ml of 1·25 μM A23187 (Calbiochem, San Deigo, CA) was added to the wells and incubated overnight at 37° in 5% CO_2. The medium from each well of the EL-4 cells was assayed for IL-2 by adding 0·1 ml of the supernatant to 10^4 CTLL-2 cells in each well of a second 96-well plate and determining [^{3}H]thymidine uptake after 24 hr. This assay measures IL-1 to a sensitivity of 0·02 U/ml.

Antibodies

Polyclonal rabbit anti-human IL-1α, anti-human IL-1β and anti-human TNFα antibodies were gifts from Dr C. Dinarello (Tufts University School of Medicine, Boston, MA) and were used in 1:100 dilution. Monoclonal mouse anti-human TNF antibody containing 8×10^6 neutralizing U/ml was a gift from Dr M. Shepard (Genentech, South San Francisco, CA). Culture supernatants or chromatography fractions were treated with the antibodies for at least 2 hr at 4° prior to further dilution and i.d. testing. Neutralization by antibodies was confirmed by IL-1 and TNF assays. Neutralizing sheep antiserum to IL-6 (B_2 interferon) was a gift from Dr J. Gauldie (McMaster University, Hamilton, Ontario) and neutralizing rabbit antiserum to human GM-CSF (recombinant) was a gift from Dr D. Stewart (Cangene Corporation, Toronto, Ontario).

RESULTS

Based on our previous findings with rabbit Mϕ, we initially studied *in vitro* matured human Mϕ. By 5 days in culture, the adherent mononuclear cells assumed a Mϕ phenotype. At this point addition of *E. coli* 0 111 LPS induced the time-dependent release into the supernatant of a factor(s) with PRA when tested in the skin of rabbits (Fig. 1a). Maximum PRA of 5×10^6 PMNL/site was reached in culture supernatants by 6 hr. In control experiments, addition of polymyxin B (25 μg/ml) completely inhibited the inflammatory effect of LPS in the skin (not shown) or of LPS added after culture to supernatants from unstimulated (LPS-free) Mϕ cultures. Supernatants from Mϕ stimulated with LPS remained active following polymyxin B treatment.

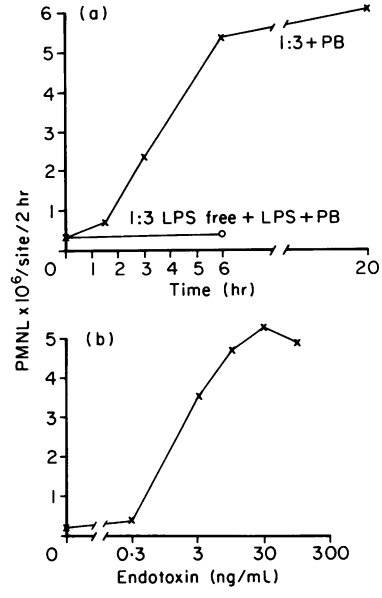

Figure 1. Effect of endotoxin concentration and kinetics of production of PMNL recruiting activity. In (a) 5-day-old monocyte-derived macrophages were stimulated for varying lengths of time with 30 ng/ml of *E. coli* 0111 LPS. The culture supernatants were harvested and tested for PMNL-recruiting activity (PRA) in the skin of rabbits following the addition of polymyxin B (25 μg/ml) to neutralize residual LPS activity. A dilution of 1:3 of culture supernatants was found to be optimal (×). Also shown in (a) is the activity of supernatants from LPS-free cultures to which LPS was added after culture (○). In (b) the 5-day macrophages were stimulated with varying concentrations of *E. coli* 0111 LPS for 6 hr and culture supernatants were again tested at 1:3 dilution following the addition of polymyxin B (×). Shown are representative results from at least three time–course and dose–response experiments.

Figure 1b shows the dose-dependent elaboration of PRA. Under LPS-free (< 0·05 ng/ml) conditions Mϕ supernatants did not induce PMNL accumulation upon i.d. injection.

PRA produced by 5-day Mϕ was heat labile, with > 95% of activity lost on boiling for 10 min. The production of PRA was inhibited by 95% by the protein synthesis inhibitor cycloheximide at 10 μg/ml when added at the time of LPS stimulation, although cell viability remained good (trypan blue exclusion > 90%).

Analysis of PRA-produced by monocytes and Mϕ

Production of PRA on different days of culture from the stage of fresh monocytes (0 day) up to Mϕ at 10 days was examined (Table 1). PRA was produced at all stages in response to LPS with similar time–course and dose–response ($ED_{50} \approx 1$ ng/ml) as in Fig. 1. For further analysis, Mϕ at 4–5 days were used because IL-1 production by these cells was virtually nil (≤ 0·5 U/ml) and PRA production was comparable to older Mϕ. PRA produced by fresh monocytes was also examined. Histology of skin sites from representative test injections confirmed that the leucocytic infiltrates consisted of ≥ 95% PMNL (not shown). There was no haemorrhage or thrombosis in the lesions.

Gel filtration of supernatants from stimulated fresh monocytes and 5-day Mϕ was performed on Sephadex G-100 (Fig. 2).

Table 1. Production of PMNL-recruiting activity by LPS-stimulated human monocytes and cultured macrophages

Days of culture	PMNL × 10^6/SITE/2 hr
0	7·4±0·8*
4–5	5·3±0·6*
8–10	6·8±0·9

Fresh monocytes (0 day culture) or monocyte-derived (4–10 days) macrophages were stimulated with LPS at 30 ng/ml in serum-free RPMI-1640 for 6 hr. Culture supernatants were tested by i.d. injection at 1:3 dilution following addition of polymyxin B (25 μg/ml). Dose–response of monocytes to LPS stimulation was similar to that for macrophages with $ED_{50}=1$ ng/ml. Values are means±SEM of 4–13 experiments. Unstimulated, LPS-free supernatants induced only $0{\cdot}2\times10^6$ PMNL accumulation/site.

* $P<0{\cdot}05$.

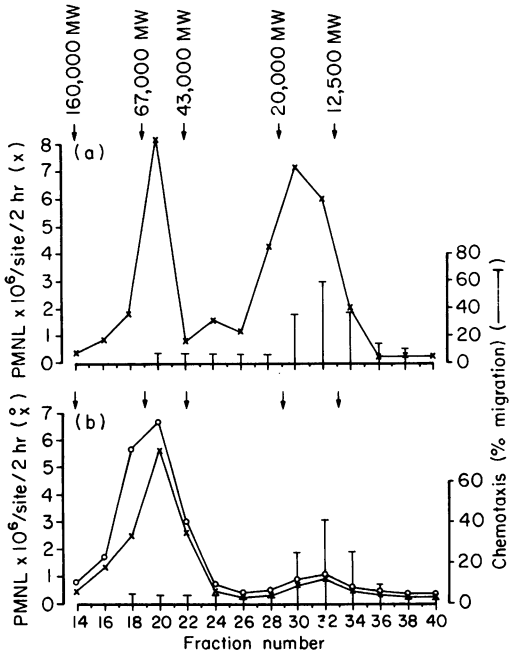

Figure 2. Gel filtration chromatography of fresh monocyte and 5-day cultured macrophage supernatants. Culture supernatants with PMNL-recruiting activity (PRA) were concentrated 10-fold by lyophilization and chromatographed on a Sephadex G-100 column. Column fractions were tested by i.d. injection into the skin of rabbits at a dilution of 1:3 following the addition of polymyxin B (25 μg/ml). In (a) the elution profile of PRA (×) in fresh monocyte supernatant is shown, while in (b) the elution profile of PRA for fresh peritoneal macrophage supernatant (○) and from 5-day monocyte-derived cultured macrophages (×) is shown. Also shown is the PMNL chemotaxis induced by column fractions expressed as percentage migration of PMNL through the chemotactic membrane filters (——|).

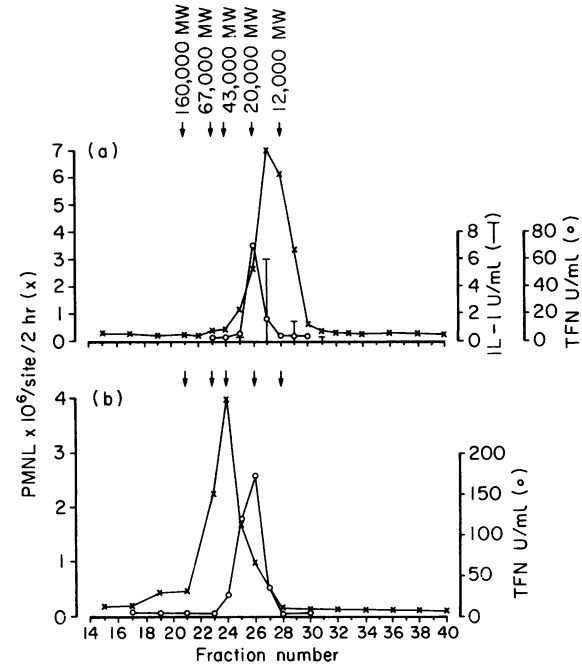

Figure 3. Superose-12 gel filtration FPLC of PMNL-recruiting activity from fresh monocyte and 5-day cultured macrophages. Supernatants from LPS-stimulated fresh monocytes (a) and from 5-day cultured macrophages (b) are shown. The elution profile of PRA (×) TNFα (○) and IL-1 (——|) are shown. In (b) only fractions 27 and 28 had detectable IL-1 activity, at 1 U/ml and 0·5 U/ml, respectively (not shown). PRA was measured as in Figs 1 and 2. The Superose-12 column was run at a flow rate of 0·4 ml/min in PBS, with 0·6 ml fraction being collected.

The major peak of PRA in supernatants from fresh monocytes eluted in a relatively broad range between 12,000 and 20,000 MW. In some experiments (two of five), activity was also detected in the 50,000–60,000 MW region (Fig. 2a).

Figure 2b shows that essentially all of the activity produced by 5-day Mϕ eluted in the 45,000–60,000 MW region. Because the Mϕ used in these studies were matured *in vitro*, we considered that these differences in PRA profile may have been the result of *in vitro* culture conditions. Therefore, peritoneal Mϕ from peritoneal dialysis patients were freshly isolated by adherence (2 hr) and stimulated with LPS. These cells also produced PRA and, on Sephadex G-100 chromatography, this PRA had the same molecular weight elution profile as that produced by the 5-day cultured Mϕ (Fig. 2b). Only a small amount of activity eluted in the 10,000–20,000 MW region.

The chromatography fractions with PRA *in vivo* were tested for capacity to induce PMNL chemotaxis or migration *in vitro*. The crude supernatants from LPS-stimulated monocytes or Mϕ induced similar PMNL migration responses of 15% and 17·5% at a 1:3 dilution, while unstimulated supernatants induced only 4·5% PMNL migration. The chromatography fractions containing the most PRA (45,000–60,000 MW region) in the *in vivo* assay did not induce PMNL migration *in vitro* over a wide concentration range (30–0·01%) (Fig. 2a, b) and were also lacking in chemokinetic activity (not shown). In contrast, fractions in the 10,000–15,000 MW region, which caused mild

Table 2. Effect of antibodies to cytokines on PMNL-recruiting activity

Test material	Antibodies	Inhibition (%)
Monocyte		
15,000–20,000 MW PRA*	Anti-IL-1α & IL-1β	98
45,000–60,000 MW PRA†	Anti-IL-1α & IL-1β	0
45,000–60,000 PRA	Anti-IL-1α, IL-1β & TNFα	0
5-day Mϕ		
Supernatant‡	Anti-IL-1α & IL-1β	10
Supernatant	Anti-TNFα	0
Supernatant	Anti-IL-1α, IL-1β & TNFα	0
Supernatant	Anti-GMCSF	0
Supernatant	Anti-IL-6	3
5 day cultured Mϕ or peritoneal Mϕ		
45,000–60,000 MW PRA§	Anti-TNFα	9
45,000–60,000 MW PRA	Anti-IL-1α, IL-1β & TNFα	8

Culture supernatants or gel filtration fractions of designated molecular weight range were treated with neutralizing dilutions (1 : 100) of polyclonal antisera or appropriate control serum, followed by testing for PRA by i.d. injection. A neutralizing monoclonal antibody to human TNFα was also used. None of the antibodies alone, at the dilutions used, induced PMNL accumulation directly. Values are means of at least two experiments performed with quadruplicate replicates.

After treatment with non-immune serum baseline activities were:

* $6{\cdot}8 \times 10^6$ PMNL/site.

† $7{\cdot}1 \times 10^6$ PMNL/site.

‡ $6{\cdot}1 \times 10^6$ PMNL/site.

§ $5{\cdot}2 \times 10^6$ PMNL/site.

leucocyte accumulation in the skin, were also active in the chemotaxis assay when rabbit or human PMNL were used (Fig. 2).

FPLC gel filtration chromatography

Monocytes and tissue Mϕ produce cytokines such as IL-1, TNFα and chemotactic factors which may mediate PMNL infiltration of tissues during inflammation (Hunninghake *et al.*, 1980; Movat & Cybulsky, 1987; Schroder *et al.*, 1987; Valone *et al.*, 1980; Wankowicz, Megyeri & Issekutz, 1988; Wolpe *et al.*, 1988; Yoshimura *et al.*, 1987). To help analyse the contribution of TNFα and IL-1 to the PRA, we employed a high resolution FPLC–Superose-12 column. As shown in Fig. 3b, one major peak of PRA eluted in the 40,00 region in the case of the 5-day Mϕ product. Here the peak of TNFα was clearly separated from the peak of PRA, although Fractions 23 and 24, which were most active in PRA, did contain small amounts (5–20 U/ml) of TNFα activity.

The 5-day Mϕ and fresh peritoneal Mϕ secreted very little IL-1 (≤0·5 U/ml) On Superose-12 chromatography a small amount of IL-1 (0·5–1 U/ml) was present in Fractions 27 and 28, corresponding to the 15,000–20,000 MW region. Fractions 23 and 24 were free of IL-1 and of IL-1 inhibitor as indicated by mixing experiments (not shown). In contrast, IL-1 was produced in appreciable amounts (20–40 U/ml) by LPS-stimulated monocytes. The IL-1 from monocytes co-eluted with the major PRA of monocytes (15,000–20,000 MW), as shown in Fig. 3a. The monocytes from the donor studied in Fig. 3a did not make the 40,000–60,000 MW PRA.

To exclude the possibility that minute amounts of IL-1 and TNF present in the 40,000–60,000 MW gel filtration fractions contributed to the PRA observed, perhaps due to synergistic actions (Wankowicz *et al.*, 1988), we treated these fractions simultaneously with neutralizing polyclonal anti-IL-1α and anti-IL-1β and neutralizing monoclonal anti-TNFα antibodies. These antibody combinations did not inhibit the PRA of the 40,000–60,000 MW fractions (Table 2). In contrast, anti-IL-1 treatment alone completely neutralized the 15,000–20,000 MW PRA produced by monocytes. Table 2 also shows that antibody to IL-6 or to human GM-CSF, two additional LPS-induced secretory products of monocytes and Mϕ (Gauldie *et al.*, 1987; Nathan, 1987), did not neutralize the PRA.

DISCUSSION

There is growing evidence that at least some Mϕ products, such as IL-1, TNFα, LTB_4 and chemotactic factors, may be responsible for PMNL accumulation during inflammation (Granstein *et al.*, 1986; Hunninghake *et al.*, 1980; Issekutz *et al.*, 1987; Movat & Cybulsky, 1987; Russo, 1980; Valone *et al.*, 1980; Wankowicz *et al.*, 1988). Recently we reported (Issekutz *et al.*, 1987) that rabbit Mϕ, upon exposure to LPS, secrete a protein factor, which induces PMNL migration into the skin of rabbits. That factor co-chromatographed with rabbit TNFα. In the present work we observed the release of an analogous product by human Mϕ and employed antibodies to human cytokines to help distinguish this protein from some other inflammatory cytokines.

The kinetics of PRA generation by monocytes and Mϕ, its sensitivity to heat and the nearly complete inhibition of its production by cycloheximide, indicate that the PRA is likely to be due to newly synthesized protein(s). It is clearly separable from contaminating LPS, which is heat stable and the activity of which was effectively neutralized by polymyxin B (Fig. 1). Both the monocytes and Mϕ produced TNFα (60–200 U/ml) in response to LPS but gel filtration on Superose-12 distinguished PRA in the 35,000–45,000 MW region from TNFα activity in the 17,000–20,000 MW region (Fig. 3). There are conflicting reports as to whether TNFα is chemotactic for PMNL *in vitro* (Figari & Palladino, 1987; Ming *et al.*, 1987; Mrowietz *et al.*, 1988), but *in vivo* TNFα is only a weak inducer of PMNL infiltration into the skin of rabbits (Wankowicz *et al.*, 1988) or the peritoneum of mice (Sayers *et al.*, 1988). The failure of neutralizing polyclonal or monoclonal anti-human TNFα antibodies to effect PRA (Table 2) helps to further exclude a role for TNFα in the observed PMNL recruitment by the 35,000–45,000 MW fractions (Fig. 3).

IL-1 was secreted in significant amounts by LPS-stimulated monocytes, but cultured Mϕ and fresh peritoneal Mϕ secreted only minute amounts of IL-1 (≤0·5 U/ml). These findings are in keeping with those reported by Northoff *et al.* (1987) and Becker *et al.* (1986), with *in vitro* monocyte-derived and fresh peritoneal Mϕ, respectively. The analysis of the PRA from monocytes indicated that LPS-stimulated monocytes from all donors tested secreted IL-1 and this was the predominant PRA of these cells, as indicated by the elution on gel filtration (15,000–20,000 MW) and neutralization by anti-IL-1 antibody (Table 2). Furthermore, although monocytes from some donors (2/5) also secreted

a 45,000–60,000 PRA, *all Mϕ* tested secreted predominantly this higher molecular weight form of PRA. The transition from IL-1 secretion to the high molecular weight PRA may be a maturational process, since monocytes from the same individual, during the initial few hours after isolation, produced IL-1 as the predominant PRA but shifted to producing the 45,000–60,000 MW PRA a few days later when secretion of IL-1 decreased dramatically. The analogous results with fresh peritoneal Mϕ, i.e. little IL-1 production and the production of 45,000–60,000 MW PRA (Fig. 2), suggest that this finding is not related to *in vitro* culture conditions during Mϕ differentiation. The variable production of the high molecular weight PRA by monocytes from some donors might indicate a heterogeneity in the maturational state of circulating blood monocytes.

It is unlikely that an interaction between IL-1 and TNFα, as reported previously (Wankowicz *et al.*, 1988), contributes to the high molecular weight PRA, because simultaneous treatment of the Mϕ PRA with neutralizing anti-IL-1 and TNFα antibodies had no inhibitory effect (Table 2). Furthermore, IL-1 activity in the 40,000–45,000 MW region was undetectable (<0·2 U/ml) and an inhibitor of the IL-1 bioassay was excluded by mixing experiments (not shown).

It is of interest that the major PRA at the 45,000–60,000 MW region, depending on gel filtration system used (Fig. 2 or 3), does not stimulate the migration of PMNL across filters *in vitro*, even over a wide concentration range (30–0·01%), although lower molecular weight (10,000–15,000) fractions did induce PMNL migration and did induce mild PMNL accumulation *in vivo* (Fig. 2). This low molecular weight chemotactic activity may be related to the newly described monocyte and Mϕ protein chemotactic factors designated by various names, e.g. monocyte-derived chemotactic factor, Mϕ inflammatory protein, neutrophil activating protein-1, and most recently IL-8 (Peveri *et al.*, 1988; Schroder *et al.*, 1987; Wolpe *et al.*, 1988; Yoshimura *et al.*, 1987). However, the major 45,000–60,000 MW PRA is clearly distinguishable from these low molecular weight chemotactic factors, as well as from other chemotactic factors such as platelet-derived growth factor (Mornex *et al.*, 1986) and LTB_4 (Valone *et al.*, 1980) by its gel elution profile and lack of chemotactic activity. The major PRA is unrelated immunologically to GM-CSF or IL-6 (Table 2), and both GM-CSF and IL-6 are of smaller molecular weight (22,000–26,000) (Gauldie *et al.*, 1987; Nathan, 1987). Since this PRA has no demonstrable effect on PMNL migration *in vitro*, this raises the possibility that its *in vivo* mechanism of action may involve other cells or mediator systems. A prime candidate for involvement in this process is the vascular endothelium, which is already known to play an important role in the inflammatory response to such cytokines as IL-1, TNFα and interferon (Cotran, 1987). This is under investigation.

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