Immunology 1990 **69** 548–553

IL-1 transcriptionally activates the neutrophil chemotactic factor/IL-8 gene in endothelial cells

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Accepted for publication 6 December 1989

SUMMARY

Leucocytes and vascular cells interact closely in inflammation and immunity and cytokines are important mediators of this interaction. The present study was designed to define the capacity of human endothelial cells (HEC) to produce a monocyte-derived neutrophil chemotactic factor (provisionally termed IL-8). IL-8 is a polypeptide chemotactic for neutrophils originally identified in the culture supernatant of lipopolysaccharide (LPS)-stimulated monocytes. IL-1 induced high levels of production of neutrophil chemotactic activity in culture supernatants of HEC. Optimal stimulation of activity was observed when HEC were cultured with 10–100 ng/ml IL-1β for 16 hr. Anti-IL-8 antibody blocked the chemotactic activity for neutrophils of IL-1-activated HEC supernatants. IL-1-treated HEC expressed high levels of IL-8 mRNA transcripts, as assessed by Northern blot analysis. Tumour necrosis factor (TNF) and LPS, unlike the inflammatory monokine IL-6, also induced IL-8 expression. Nuclear run-off experiments revealed that IL-1 activated transcription of the IL-8 gene. The production of IL-8 may represent a mechanism whereby endothelial cells, exposed to inflammatory signals, participate in the regulation of neutrophil extravasation.

INTRODUCTION

Inflammatory reactions and immunity require close interactions between leucocytes and the vessel wall (Mantovani & Dejana, 1989). The extravasation and localization of leucocytes at inflammatory sites depends upon adhesion to and passage through endothelial linings in response to tissue-derived signals. These processes are modulated by lymphokines, interleukin-1 (IL-1) and tumour necrosis factor (TNF) in particular. These polypeptide inflammatory mediators elicit a complex set of changes in endothelial cells, which include the production of prostacyclin (PGI_2), platelet-activating factor (PAF), factors acting on coagulation and fibrinolysis, membrane adhesion molecules and cytokines (reviewed by Mantovani & Dejana, 1989). Local inoculation of IL-1 and TNF in tissues causes rapid recruitment from the blood compartment of leucocytes, in particular polymorphonuclear leucocytes (PMN) (Sayers *et al.*, 1988; Averbrook *et al.*, 1987; Cybulsky, Movat & Dinarello, 1987; Movat *et al.*, 1987). Vasodilatation (Dejana *et al.*, 1984; Rossi *et al.*, 1985), expression of adhesion structures and, in the case of TNF, a direct chemotactic effect (Wang, Bersani & Mantovani, 1987; Figari, Mori & Palladino, 1987; Maestrelli *et al.*, 1988) are probably involved in leucocyte infiltration caused by monokines (Mantovani & Dejana, 1989). In addition, a novel lymphokine chemotactic for PMN has been identified in the culture supernatant of stimulated monocytes (Yoshimura *et al.*, 1987a, b; Matsushima *et al.*, 1988; Van Damme *et al.*, 1988; Schmid & Weissmann, 1987; Schroder, Mrowietz & Christophers, 1988; Peveri *et al.*, 1988). For this monocyte-derived neutrophil chemotactic factor the term IL-8 has been proposed. IL-8 belongs to an emerging family of polypeptide mediators that share the ability to modulate cell migration (Luster, Unkeless & Ravetch, 1985; Barone *et al.*, 1988; Holt *et al.*, 1986; Anisowicz, Bardwell & Sager, 1987; Richmond *et al.*, 1988). IL-8 could play a role as a mediator of inflammation and in particular, explain how IL-1, devoid of chemotactic activity *per se*, elicits leucocyte extravasation. This study reports that IL-1 induces production of IL-8 in human endothelial cells and transcriptionally activates the IL-8 gene.

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MATERIALS AND METHODS

Endothelial cells

HEC were isolated from umbilical veins and cultured in medium 199 (Gibco-Europe, Paisley, Renfrewshire, U.K.) supplemented with 20% fetal calf serum (FCS; Gibco) in the presence of an endothelial cell growth supplement (50 μg/ml; Sigma Chemical,

St Louis, MO) and porcine intestinal heparin (100 μg/ml; Sigma). Tissue culture plasticware was purchased from Cel-Cult (Flow Laboratories Ltd, Irvine, Ayrshire, U.K.). Cells were passaged by trypsinization and were used before the 8th passage. When cells were confluent, the HEC were stimulated with IL-1β, washed with phosphate-buffered saline (PBS) twice, and incubated for a further 20 hr with medium 199 containing 0·2% bovine serum albumin (BSA; Sigma), and the supernatant collected for assessing IL-8 activity.

Lymphokines

Recombinant human IL-1β (specific activity 10^6 U/mg) was obtained from Sclavo (Siena, Italy). Recombinant human TNF (specific activity $8{\cdot}1 \times 10^6$ U/mg) was a kind gift from BASF/Knoll (Ludwigs Hagen, FRG) and human recombinant IL-6 was obtained from Immunex, Seattle, WA, through the courtesy of Dr S. Gillis. LPS (E. coli 055: B5) was purchased from Difco Laboratories (Detroit, MA). Recombinant cytokines were endotoxin-free as measured by Limulus amebocyte lysate assay [M.A. Bioproducts, Walkersville, MD; sensitivity $\geq 1{\cdot}25$ endotoxin units (EU)/ml]. Human natural IL-8 was purified to homogeneity from supernatants of stimulated mononuclear cells as described elsewhere (Van Damme *et al.*, 1989). A specific, neutralizing antibody against natural pure IL-8 was prepared in goat (Van Damme *et al.*, 1989).

PMN separation

Human PMN were obtained from healthy volunteers by sedimentation of heparinized blood at 400 *g* for 30 min on Ficoll–Hypaque (Lymphoprep, Nyagaad, Oslo, Norway). The pellet containing erythrocytes and PMN was resuspended in PBS and mixed with a commercially available dextran solution (Eufusin; STHOLL Farmaceutici, Modena, Italy) at a concentration of 3 ml of Eufusin per milliliter of blood cells. The PMN-rich supernatant obtained after 30 min of 4° incubation was then centrifuged, and the cell pellet was mixed with cold distilled water for 30 seconds to lyse residual erythrocytes. Cells were then washed twice and resuspended at $1{\cdot}5 \times 10^6$/ml in Hanks' balanced saline solution (HBSS; Gibco) with 0·2% BSA. The final preparation contained 98% PMN.

PMN chemotaxis assay

PMN chemotactic activity was assessed by a microchamber chemotaxis technique (Falk, Goodwin & Leonard, 1980). Twenty-five microlitres of HEC supernatant were placed in the lower compartment of the microchamber and 50 μl of PMN suspension were seeded in the upper compartment. The two compartments were separated by a 5 μm pore-size polyvinylpyrolidone (PVP)-free polycarbonate filter (Nuclepore Corp., Pleasanton, CA). The chamber was incubated at 37° for 120 min. At the end of incubation, filters were removed, fixed and stained with Diff-Quik (Harleco, Gibbstown, NJ), and five oil immersion fields were counted after coding the samples. For assessment of migration with the leading front as the end-point, the upper and lower compartments of the chamber were separated by a 8-μm pore-size nitrocellulose filter (Nuclepore). After 120 min incubation, the filter was removed, fixed, stained and dehydrated by standard histological methods. Four fields were examined for each sample. Migration was expressed as the distance migrated by the two leading cells (in micrometers). Expected values of migration assuming no chemotactic response were calculated as described by Zigmond & Hirsch (1973). In each experiment, FMLP (Sigma) was used as a reference chemoattractant at the optimal concentration of 10 nM. The statistical significance of migration towards stimulus versus medium control was assessed by Dunnet's test; 1 U/ml was defined as the reciprocal of the dilution at which 50% of maximal chemotactic response compared to FMLP was obtained.

Northern blot analysis

Northern blot analysis was carried out according to standard procedures (Sambrook, Fritsch & Maniatis, 1989). Total RNA was isolated by guanidine isothiocyanate method (Chirgwin *et al.*, 1979). Fifteen micrograms total RNA were analysed by electrophoresis through 1% agarose formaldehide gels, followed by Northern blot transfer to Gene Screen Plus membranes (New England Nuclear, Boston, MA). The plasmid containing a human IL-8 cDNA clone (Matsushima *et al.*, 1988) was nick-translated with α-^{32}PdCTP (5000 Ci/mmol; Amersham, Amersham, Bucks, U.K.). Membranes were pretreated and hybridized in 50% formamide (Merck, Rahway, NJ) with 10% dextran sulphate (Sigma) and washed twice with 2 × SSC (1 × SSC: 0·15 M sodium chloride, 0·015 sodium citrate) at room temperature for 10 min, then twice with 2 × SSC and 1% sodium dodecyl sulphate (SDS; Merck) at 60° for 30 min and finally twice with 0·1 × SSC at room temperature for 30 min. The membranes were exposed for 12–24 hr at −80° with intensifying screens. The IL-8 probe was removed from membrane according to manufacturer's instructions and then hybridized to α-actin probe under the same experimental conditions to ascertain that comparable amounts of RNA were transferred to filters.

Nuclear run-off

Nuclear run-off experiments were performed essentially as described by Greenberg & Ziff (1984) with some modifications. To isolate nuclei, 2×10^6 HEC were washed twice with ice-cold HBSS with Ca^{2+} and Mg^{2+} and then resuspended in 0·5 ml lysis buffer (Tris-HCl 10 mM, pH 7·4, $MgCl_2$ 3 mM, NaCl 10 mM, NP-40 0·5%). After 5 min incubation on wet ice, tubes were centrifuged at 400 *g* at 4° and cells resuspended in 250 μl ice-cold freezing buffer (Tris-HCl, pH 8·3, glycerol 40%, $MgCl_2$ 5 mM, 0·1 mM EDTA, pH 8). Then 60 μl run-off buffer, 5 × (25 mM Tris-HCl, pH 8, $MgCl_2$ 12·5 mM, KCl 750 mM and 1·25 mM each of dGTP, dCTP and dATP) and 100 μCi α[^{32}P]UTP (Amersham; 6000 Ci/mmol) were added to 230 μl of nuclei suspension and incubated at 30° for 30 min. Elongated transcripts were then isolated using the guanidine/cesium chloride procedure as described above adding 50 μg yeast tRNA as carrier. The RNA pellet was resuspended in 180 μl ice-cold TNE (Tris-HCl, 100 mM, EDTA 10 mM, pH 8, NaCl 0·1 M; Merck, Darmstadt, FRG) and denatured adding 20 μl NaOH 2N on ice for 10 min. The solution was neutralized by the addition of HEPES, pH 7·2 (final concentration 0·48 M). RNA was then precipitated adding 880 μl ethanol; the pellet was resuspended in 100 μl hybridization solution (TES 10 mM, SDS 0·2%, EDTA 10 mM, NaCl 3000 mM; N-Tris[Hydroxymethyl]methyl-2-aminoethanesulphonic acid; Sigma, St Louis, MO) and radioactivity checked with a β-counter. RNA solution was hybridized at 65° for 48 hr to DNA immobilized to nitrocellulose filters. In a given experiment, each filter was hybridized with the same number of c.p.m. The filters were then washed with several changes of 0·2 × SSC at 65° for 30

min and incubated at 37° in 0·2 SSC × with 10 μg/ml RNase A for 30 min. Filters were then exposed for autoradiography as described above. For immobilization of DNA to filters, 5 μg of plasmid containing IL-8 cDNA were denatured with 0·3 M NaOH at 60° for 30 min, neutralized with ammonium acetate (final concentration 4 M) and spotted onto nitrocellulose filter (Schleicher and Schuell, Dassel, FRG) using a slot blot apparatus (Scheleicher and Schuell). pBR 322 DNA (Bochringer-Mannheim, Mannheim, FRG) was used as a negative hybridization control.

RESULTS

Upon exposure to IL-1β, HEC released considerable amounts of IL-8 activity, as assessed by induction of PMN migration across polycarbonate filters. Figure 1 shows one representative experiment of three performed, in which HEC were exposed to IL-1β (100 ng/ml) for 16 hr, and the supernatant was obtained 20 hr later. In this series of three experiments, IL-1-stimulated HEC released about 11 U/ml (median; range 9–16 U/ml) of activity compared to 0·5–3 U/ml of unstimulated HEC. Several lines of evidence indicate that the IL-1 molecule *per se* was not responsible for PMN chemotaxis. When HEC were exposed to IL-1β (100 ng/ml) for 16 hr, washed and then incubated with serum-free medium for 20 hr, they released high levels of chemotactic activity. Moreover, in agreement with previous reports (Yoshimura *et al.*, 1987a), IL-1, tested over a wide range of concentrations and experimental conditions, had no effect on leucocyte migration, nor did it affect the chemotactic activity of reference chemoattractants (data not shown).

In a series of experiments, the kinetics of appearance of activity and the effect of different IL-1 doses were examined. The maximal induction of PMN chemotactic activity in HEC by IL-1 was observed after 16 hr incubation and the optimal doses for the stimulation ranged between 10 and 100 ng/ml in different experiments (data not shown).

It was important to evaluate whether chemotactic activity induced in HEC by IL-1 elicited in PMN a gradient-dependent locomotory response or an augmentation of gradient-independent motility. To examine this point, checkerboard experiments were performed using polycarbonate (Table 1) or nitrocellulose (Table 2) filters. As shown in Table 1, maximal induction of

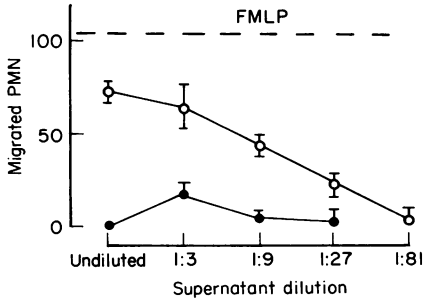

Figure 1. Induction of neutrophil chemotactic activity in HEC by IL-1. HEC were cultured for 16 hr with 100 ng/ml IL-1β, washed and culture supernatants were collected after 20 hr. Results are presented as number of migrated PMN (mean ± SD) after subtraction of spontaneous migration (74 ± 6 cells). FMLP (10 nM) served as a reference chemoattractant. (○) IL-1-treated HEC supernatant; (●) control HEC supernatant.

Table 1. Checkerboard analysis of PMN migration across polycarbonate filters induced by IL-1-stimulated HEC supernatant*

	Above			
		Supernatant dilution		
Below	Medium	1:27	1:9	1:3
Medium	53 ± 8	59 ± 8	52 ± 11	44 ± 5
1:27	85 ± 9	73 ± 5	68 ± 2	58 ± 7
1:9	110 ± 9†	91 ± 7	77 ± 9	75 ± 6
1:3	129 ± 12†	125 ± 28†	75 ± 9	90 ± 4

* Different dilutions of HEC supernatant were seeded in the upper and/or lower compartments of the chemotaxis chamber. Results are number of migrated PMN (± SD) in five oil fields with three replicates.

† $P < 0.05$ versus migration to control medium (above and below the filter).

Table 2. Checkerboard analysis of PMN migration into nitrocellulose filters elicited by IL-1-stimulated HEC supernatant*

	Above			
		Supernatant dilution		
Below	Medium	1:27	1:9	1:3
Medium	45·1 ± 14·1	44·2 ± 13·0 (52·6)	40·0 ± 10·0 (41·4)	35·8 ± 8·6 (57·7)
1:27	67·4 ± 11·2† (46·6)	54·1 ± 12·1	41·6 ± 8·8 (46·1)	35·6 ± 7·5 (67·2)
1:9	67·3 ± 10·8† (47·6)	57·9 ± 10·3 (52·5)	44·5 ± 10·6	39·1 ± 9·2 (55·4)
1:3	97·7 ± 12·9† (45·3)	70·2 ± 14·5† (48·4)	57·7 ± 17·7 (46·9)	58·3 ± 11·6

* Different dilutions of HEC supernatant were seeded in the upper and/or lower compartments of the chemotaxis chamber. Migration is expressed as leading front distance (micrometers ± SD), and numbers in parentheses are the expected values of migration calculated according to the method of Zigmond & Hirsch (1973), assuming no chemotactic response.

† $P < 0.05$ versus spontaneous migration with medium seeded above and below the filter.

PMN migration across polycarbonate filters occurred in the presence of a positive concentration gradient between lower and upper compartments of the chemotaxis chamber. In the presence of a negative gradient (higher concentration in the upper compartment), no enhanced migration of PMN occurred. With equal concentrations of supernatant above and below the filter, little or no enhancement of migration was observed. Evaluation of leading front distance in nitrocellulose filters allows a quantitative estimate of the contribution of chemotaxis versus chemokinesis in the induction of leucocyte migration by a given signal (Zigmond & Hirsch, 1973). Observed values (Table 2) consistently exceeded the expected levels in the presence of positive gradient and were less than expected under negative-

Table 3. Effect of anti-IL-8 antibody on the PMN chemotactic activity of IL-1-stimulated HEC supernatant*

Stimulus	Antibodies	No. migrated PMN	% inhibition‡
Medium	Control	72±7	—
	Anti-IL-8†	61±9	—
FMLP 10 nM	Control	152±7	—
	Anti-IL-8	170±6	—
Unstimulated HEC sup.	Control	85±20	—
	Anti-IL-8	81±13	—
Stimulated HEC sup.	Control	155±13	
	Anti-IL-8	84±11	86
Purified IL-8 10 U/ml	Control	165±13	—
	Anti-IL-8	70±4	100

* Supernatants were at a final dilution of 1:10.

† The stimuli were incubated for 2 hr at 37° with specific anti-IL-8 goat antiserum at a final dilution of 1:20, before being tested for chemotaxis.

‡ After subtraction of spontaneous migration, the percentage inhibition was calculated as:

$$1-\frac{\text{migration to stimulated HEC sup. in the presence of anti-IL-8}}{\text{migration to stimulated HEC sup. in the absence of anti-IL-8}}\times 100.$$

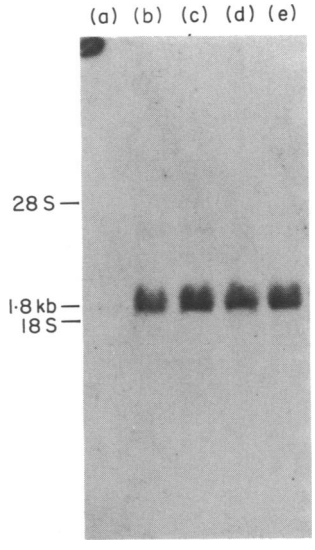

Figure 2. Northern blot analysis of the kinetics of IL-8 mRNA induced by IL-1 in HEC. Total RNA extracted from HEC either unstimulated (a) or incubated with IL-1 (100 ng/ml) for 1 hr (b), 4 hr (c), 8 hr (d) and 16 hr (e).

gradient conditions, suggesting a chemotactic response of PMN to the stimulus employed.

The results discussed so far indicate that exposure to IL-1 elicits production in HEC of chemotactic activity for PMN. In an effort to define the molecular basis of chemotactic activity, the effect of anti-IL-8 antibody and expression of IL-8 mRNA

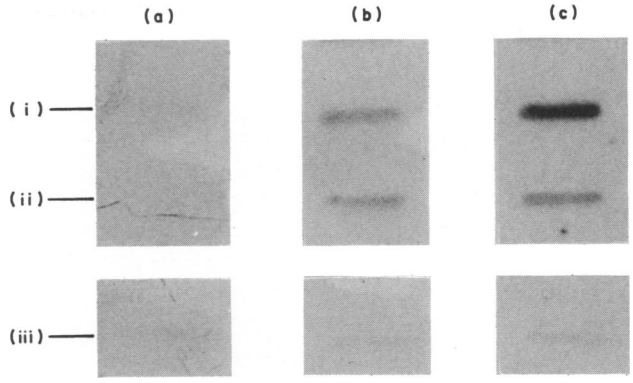

Figure 3. Nuclear run-off analysis of IL-8 gene expression induced by IL-1 in HEC. DNA samples sticked to filters are as follows: (i) human IL-6 cDNA (Sironi *et al.*, 1989); (ii) human IL-8 cDNA; (iii) pBR 322. Radioactive transcripts were from: (a) untreated HEC; (b) IL-1 (100 ng/ml, for 1·5 hr)-treated HEC; (c) IL-1 (100 ng/ml for 3·5 hr)-treated HEC.

transcripts in HEC were examined by Northern blot analysis. As shown in Table 3, anti-IL-8 antibody blocked the chemotactic activity of purified IL-8 with no effect on the unrelated chemoattractant FMLP. Anti-IL-8 antibody also blocked most of the chemotactic activity of IL-1-stimulated HEC supernatants. As shown in Fig. 2, unstimulated HEC expressed undetectable or relatively low levels of IL-8 transcripts. Upon exposure to IL-1, high levels of IL-8 mRNA were detected as early as 1 hr and these were maintained throughout the 16 hr observation period. It was important to establish whether induction of high levels of steady-state IL-8 transcripts by IL-1 was dependent upon transcriptional activation of the gene. To address this issue, nuclear run-off tests were performed. As shown in Fig. 3, nuclear run off assays confirmed that the IL-8 gene is indeed transcriptionally activated in HEC following IL-1 treatment.

IL-1 is a polypeptide mediator produced by monocytes that shares properties with IL-6 and TNF. It was therefore of interest to investigate whether the functionally related monokines IL-6 and TNF induced IL-8 transcripts. As shown in Fig. 4, TNF induced appreciable levels of IL-8-related mRNA, whereas IL-6 was inactive. As expected, LPS induced IL-8 expression (Fig. 4).

DISCUSSION

The results presented here demonstrate that endothelial cells, upon stimulation with IL-1, produce high levels of PMN chemotactic activity. The chemotactic activity for PMN of IL-1-stimulated HEC supernatants was inhibited by anti-IL-8 antibodies. Moreover, release of IL-8 activity induced by IL-1 in HEC was associated with expression of specific mRNA transcripts. Nuclear run-off experiments revealed that IL-1 transcriptionally activates the IL-8 gene. While this study was being completed, it has been reported that the monokines IL-1 and TNF, as well as LPS, induce production of chemotactic activity for PMN and expression of IL-8 mRNA (Schroder & Christophers, 1989; Strieter *et al.*, 1989). It is also of interest that Wen *et al.* (1989) recently reported that activated endothelial cells express the MGSA/gro gene, which belongs to the same family as IL-8. The present study, by identifying IL-8 as being involved

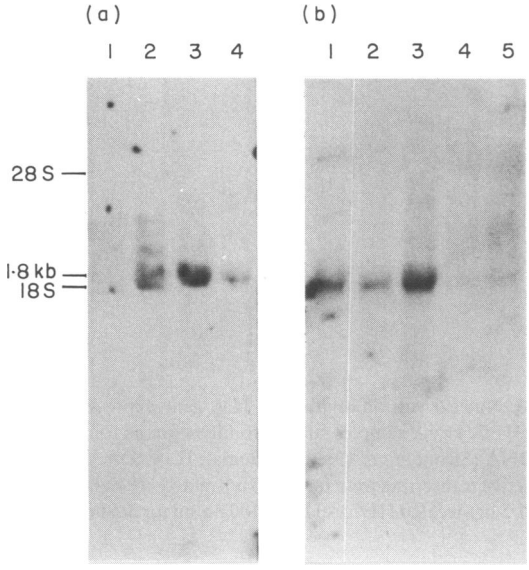

Figure 4. Induction of IL-8 mRNA expression by LPS and cytokines in HEC. Incubation with stimuli indicated below were carried out for 4 hr. RNA are as follows. (a): lane 1, unstimulated HEC; lane 2, IL-1-treated (100 ng/ml) HEC; lane 3, TNF-treated (400 U/ml) HEC; lane 4, IL-6-treated (200 U/ml) HEC. (b): lane 1, LPS-treated (10 μg/ml) HEC; lane 2, IL-1-treated (100 ng/ml) HEC; lane 3, LPS-treated (10 μg/ml) human circulating monocytes; lanes 4 and 5, untreated HEC and monocytes, respectively.

in HEC chemotactic activity using specific antibodies and by showing activation of IL-8 gene transcription, confirms and extends these observations.

TNF activates endothelial cells and elicits a spectrum of responses largely overlapping with that induced by IL-1. Activation of endothelial cell function by IL-1 and TNF is associated with expression of the immediate early genes c-fos and jun (Colotta *et al.*, 1988; F. Colotta and A. Bertani, unpublished data). It was therefore not surprising that TNF induced IL-8 expression, as did IL-1. IL-6 is a pleiotropic cytokine involved in inflammation and immunity, produced mainly by monocytes, among circulation leucocytes, and active on a spectrum of cells and tissues partially overlapping with that of IL-1 and TNF (Wong & Clark, 1988). It has been reported recently that IL-1 induced production of high levels of IL-6 in endothelial cells (Sironi *et al.*, 1989). It was therefore important to ascertain whether IL-6 affected expression of IL-8 in HEC. It was found that IL-6, unlike IL-1, TNF and LPS, did not induce IL-8 in HEC. Along the same line, it has previously been reported that IL-6 did not affect the adhesion properties of HEC for leucocytes, their proliferation and production of PGI_2 and procoagulant activity (PCA) (Sironi *et al.*, 1989). Based on these observations one would infer that IL-6, unlike IL-1 and TNF, is not an important mediator directly involved in regulating the recruitment of leucocytes at sites of inflammation.

IL-1 and TNF elicit a complex set of modifications of the properties of endothelial cells. These include changes in shape and interaction with the extracellular matrix, production of PGI_2, PCA, PAF and plasminogen activation-inhibition (PA-I), expression of leucocyte adhesion molecules, and secretion of colony-stimulating factor (CSF), IL-6, and IL-8 (reviewed by Mantovani & Dejana, 1989). Collectively these alterations favour vasodilation (PGI_2), thrombosis (PCA, PAF and PA-1) and leucocyte recruitment (CSF, leucocyte adhesion molecules, chemotactic peptides). Recently, it has been reported that IL-1-stimulated HEC also produce a polypeptide inhibitor of leucocyte adhesion (Wheeler *et al.*, 1988) and one of monocyte chemotaxis (Wang *et al.*, 1989). These factors could serve as down-regulation signals of the HEC responses to monokines, which have the potential to be highly disruptive of vessel wall and tissue integrity. The production of IL-8 by monokine-stimulated HEC probably represents a mechanism whereby endothelial cells, strategically located at the interface between tissues and the blood compartment, participate in the regulation of leucocyte extravasation.

ACKNOWLEDGMENTS

This work was supported by project Biotechnology from CNR, Italy. The contribution of the Italian Association for Cancer Research and of the Italian Ministry for Scientific Research (legge 46, consorzio CITI) is gratefully acknowledged.

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