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Interaction of lipopolysaccharide with human small intestinal lamina propria fibroblasts favors neutrophil migration and peripheral blood mononuclear cell adhesion by the production of proinflammatory mediators and adhesion molecules

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Abstract

Fibroblasts are important effector cells having a potential role in augmenting the inflammatory responses in various diseases. In infantile diarrhea caused by enteropathogenic *Escherichia coli* (EPEC), the mechanism of inflammatory reactions at the mucosal site remains unknown. Although the potential involvement of fibroblasts in the pathogenesis of cryptococcus-induced diarrhea in pigs has been suggested, the precise role of lamina propria fibroblasts in the cellular pathogenesis of intestinal infection and inflammation caused by EPEC requires elucidation. Earlier we reported the lipopolysaccharide (LPS)-induced cell proliferation, and collagen synthesis and downregulation of nitric oxide in lamina propria fibroblasts. In this report, we present the profile of cytokines and adhesion molecules in the cultured and characterized human small intestinal lamina propria fibroblasts in relation to neutrophil migration and adhesion in response to lipopolysaccharide (LPS) extracted from EPEC 055:B5. Upon interaction with LPS (1–10 μg/ml), lamina propria fibroblasts produced a high level of proinflammatory mediators, interleukin (IL)-1α, IL-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α and cell adhesion molecules (CAM) such as intercellular cell adhesion molecule (ICAM), A-CAM, N-CAM and vitronectin in a time-dependent manner. LPS induced cell-associated IL-1α and IL-1β, and IL-6, IL-8 and TNF-α as soluble form in the supernatant. Apart from ICAM, vitronectin, A-CAM, and N-CAM proteins were strongly induced in lamina propria fibroblasts by LPS. Adhesion of PBMC to LPS-treated lamina propria fibroblasts was ICAM-dependent. LPS-induced ICAM expression in lamina propria fibroblasts was modulated by whole blood, PBMC and neutrophils. Conditioned medium of LPS-treated lamina propria fibroblasts remarkably enhanced the neutrophil migration. The migration of neutrophils was inhibited by anti-IL-8 antibody. Co-culture of fibroblasts with neutrophils using polycarbonate membrane filters exhibited time-dependent migration of neutrophils. These findings indicate that the coordinate production of proinflammatory cytokines and adhesion molecules in lamina propria fibroblasts which do not classically belong to the immune system can influence the local inflammatory reactions at the intestinal mucosal site during bacterial infections and can influence the immune cell population residing in the lamina propria. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Lamina propria fibroblast; Lipopolysaccharide; Proinflammatory cytokine; Adhesion molecule; Neutrophil migration

Abbreviations: HSILPF, human small intestinal lamina propria fibroblasts; CM, conditioned medium; EPEC, enteropathogenic *Escherichia coli*

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1. Introduction

Inflammatory responses generated by fibroblasts involve the secretion of proinflammatory cytokines and expression of adhesion molecules. Fibroblasts have been shown to be the target of lipopolysaccharide (LPS), and activation of these cells by LPS has been linked to the pathogenesis of variety of disorders. Fibroblasts are therefore thought to play an important role in the development of inflammation at a particular site of the host which is a prime step in bacterial infection. Skin, synovial and pulmonary fibroblasts are known to produce colony-stimulating factors (CSF) and interleukin (IL)-8 when stimulated with IL-1 or tumor necrosis factor (TNF)-α, thereby participating in the disease process [1–3]. Adhesion molecules such as intercellular cell adhesion molecule (ICAM) and vascular cell adhesion molecule (V-CAM) are expressed on the surface of the fibroblasts [4,5]. Moreover, studies with respiratory and synovial tissues have demonstrated that growth factors and cytokines released from cultured fibroblasts can support the growth, differentiation and activation of inflammatory cells [6]. It is also known that cytokine secretion by the fibroblasts is upregulated by bacterial LPS and viruses [7]. LPS has been recognized as a major triggering molecule for host inflammatory response observed during Gram-negative infections [8]. These observations suggest that following stimulation by bacteria or bacterial products such as LPS during infection, production of proinflammatory cytokines and expression of adhesion molecules by fibroblasts at an inflammatory site can optimize the inflammatory response.

The role of the specific cell population leading to inflammation in the intestinal diseases remain unclear. Elevated levels of IL-1α, G-CSF and transforming growth factor (TGF)-β, expression of ICAM in the lamina propria of patients with inflammatory bowel diseases [9–12], and cytokine gene and adhesion molecules in normal duodenal fibroblasts, have been detected [13]. Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of severe and persistent infantile diarrhea, claiming nearly millions of deaths per year [14], but the mechanism of EPEC-induced diarrhea is intriguing. However, the loss of the brush border of small intestine observed in EPEC infection is not the cause of diarrhea [15,16] and on the other hand, extensive infiltration of PMN in lamina propria and intestinal lumen was observed [17], which was suggested to be one of the factors responsible for the diarrhea [18]. The role of lamina propria fibroblasts has not been studied in detail with respect to the kinetics of cytokine profile and expression of adhesion molecules and their modulatory role in neutrophil migration in response to LPS of EPEC, one of the potent inflammatory stimuli of Gram-negative bacteria. We determined in our previous study that LPS-induced suppression of nitric oxide (NO) leads to the cell proliferation and collagen synthesis in human small intestinal lamina propria fibroblasts (HSILPF) [19]. However, it was of immense interest to investigate the inflammatory responses evoked in HSILPF by LPS. Therefore, in the present study we decided to illustrate whether LPS regulates the cytokine production and protein profile of adhesion molecules in lamina propria in a dose- and time-dependent manner, and if so, whether these cytokines and adhesion molecules modulate the neutrophil migration and PBMC adhesion.

In this study we report that LPS induced production of cell-associated IL-1α and IL-1β and soluble IL-6, IL-8 and TNF-α, and expression of adhesion molecules ICAM-1, vitronectin, A-CAM and N-CAM in lamina propria fibroblasts. The production of these inflammatory mediators coupled with neutrophil migration and PBMC adhesion suggest the possible involvement of lamina propria fibroblasts in the manifestation of inflammation due to EPEC infection.

2. Materials and methods

2.1. LPS preparation

LPS was extracted from enteropathogenic *E. coli* O55:B5 by hot phenol water extraction [20] and biochemically characterized [21]. The activity of LPS was found to be 100 000 EU/ml as measured by limulus amoebocyte assay kit (LAL kit, Sigma).

2.2. Human small intestinal lamina propria fibroblasts (HSILPF)

Small intestinal lamina propria fibroblasts were

established from human fetuses ($n = 48$, 18–22 weeks) obtained after medical termination of pregnancy (MTP) from the hospitals and used in accordance with the institutional guidelines as described previously [19]. HSILPF were cultured from 50 fetuses, characterized and cryopreserved at passage 2. Cells from 10 vials (one vial of each fetus) were randomly revived and plated separately. Preliminary experiments were carried out with individual samples in triplicate (data not shown) and no difference was observed between the cells of different fetuses. Henceforth, all 10 vials were pooled together and the cells in all the experiments were used at passage 3–10.

2.3. HSILPF stimulation

Cells at a density of 10^6/well were plated in 24-well plates supplemented with Dulbecco's modified Eagle's medium (DMEM) and 1% fetal calf serum (FCS). Different concentrations of LPS prepared in DMEM–1% FCS were added to the cultures. Depending upon the reaction conditions, the plates were incubated at 37°C/5% CO_2. After the indicated time interval, supernatant samples were collected, centrifuged and stored at −80°C until use. For cell-associated cytokine analysis, fresh medium was added to the cells followed by three cycles of freezing and thawing at −80°C. Samples were centrifuged briefly before assay. In certain experiments, 25 μg/ml polymyxin B (PMB) was incorporated in the culture medium along with LPS. Enzyme-linked immunosorbent assay (ELISA) kits for cytokine assay (IL-1α, IL-1β, IL-6, IL-8, TNF-α) from a commercial source (R&D Systems, Minneapolis, MN) were used according to the manufacturer's instructions.

2.4. Isolation of neutrophils and peripheral blood mononuclear cells (PBMC)

Neutrophils and PBMC were isolated from the blood collected from healthy volunteers as described previously [18]. Neutrophils and PBMC were washed and resuspended in RPMI.

2.5. ICAM assay

10^4 lamina propria cells per well were plated in 96-well microtiter plates. Cell layers were incubated with 10 μg/ml of LPS together with 10^6 whole blood cells, PBMC or neutrophils. In some experiments cells were incubated with PMB (25 μg/ml) and LPS. The plates were incubated for 8 h. At the end of the experiments the cells were washed five times with DMEM, and ICAM expressed by HSILPF was determined by direct cellular ELISA (CELISA) on the cell surface using first anti-ICAM mAb (Boehringer Mannheim) and a second peroxidase-conjugated goat anti-mouse IgG. Color was developed by *o*-phenylenediamine and H_2O_2 as substrate, and the plates were read at 490 nm (Dynatech ELISA reader).

2.6. Migration of neutrophils

Confluent monolayer of HSILPF was treated with 10 μg/ml of LPS for 24 h. The conditioned medium (CM) was collected, centrifuged and used as chemotactic medium. Neutrophils at a density of 10^6 were added to the upper chamber of cell culture insert (5 μm pore size, Coaster) and the inserts were placed in a 24-well plate containing various stimuli. As a positive control 1 μm *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma) was included in the experiments and the migration of neutrophils was determined. The plates were incubated for 8 h. Migration of neutrophils was assessed by quantitation of myeloperoxidase [22]. In brief, neutrophils which migrated onto the other side of membrane were lysed by 0.5% Triton X-100. Peroxidase assay was determined by addition of an equal volume of 1 mM 2,2′-di(3-ethyl)dithiazoline sulfonic acid and 10 mM H_2O_2 in 100 mM citrate (pH 4.2). The color reaction was quantitated spectrophotometrically at 410 nm (Dynatech ELISA reader). Standard curves were prepared by using a known number of neutrophils, and standard curves were prepared for each experiment. For some experiments, CM was incubated with anti-IL-8 antibody (30 μg/ml, Genzyme) and anti-ICAM antibody (30 μg/ml) at 37°C for 1 h prior to the experiment.

2.7. Co-culture of neutrophil and lamina propria fibroblasts

10^6 neutrophils were added to the upper cham-

ber of cell culture inserts and placed in 24-well plates with lamina propria fibroblasts exposed to various stimulants. The plates were incubated for various time intervals and the number of neutrophils which migrated were measured as described above.

2.8. Adhesion assay

Lamina propria fibroblasts were grown on glass coverslips and exposed to stimulants for different time periods. After the indicated time periods, PBMC (10^6 cells/well) were added and incubated for 2 h at 37°C/5%CO_2. The coverslips were gently washed with DMEM, fixed and stained with Giemsa. The number of PBMC adhering to the fibroblast layer was counted microscopically (100 microscopic fields).

2.9. Detection of IL-1α, IL-1β, vitronectin, A-CAM and N-CAM in HSILPF by Western blot

Lamina propria fibroblasts were seeded in a 35-mm petri dish. After 24 h, 10 μg/ml LPS was introduced into each petri dish and further incubated for the indicated time period. After each time period, cell layer was lysed by hot lysis buffer (2% SDS, 10% glycerol, 1% 2-mercaptoethanol). Protein concentrations in the lysates were determined by BCA kit (Pierce). Total proteins (20 μg per lane) present in the samples were resolved by 8% SDS–PAGE according to Laemmli [23]. The proteins were electrophoretically transferred to PVDF (Millipore) membranes by electroblotting [24]. The blots were blocked with 2% BSA and reacted with antibody to IL-1α (1:100 dilution, Genzyme), IL-1β (1:100 dilution, Genzyme), vitronectin (1:2500 dilution, Sigma),

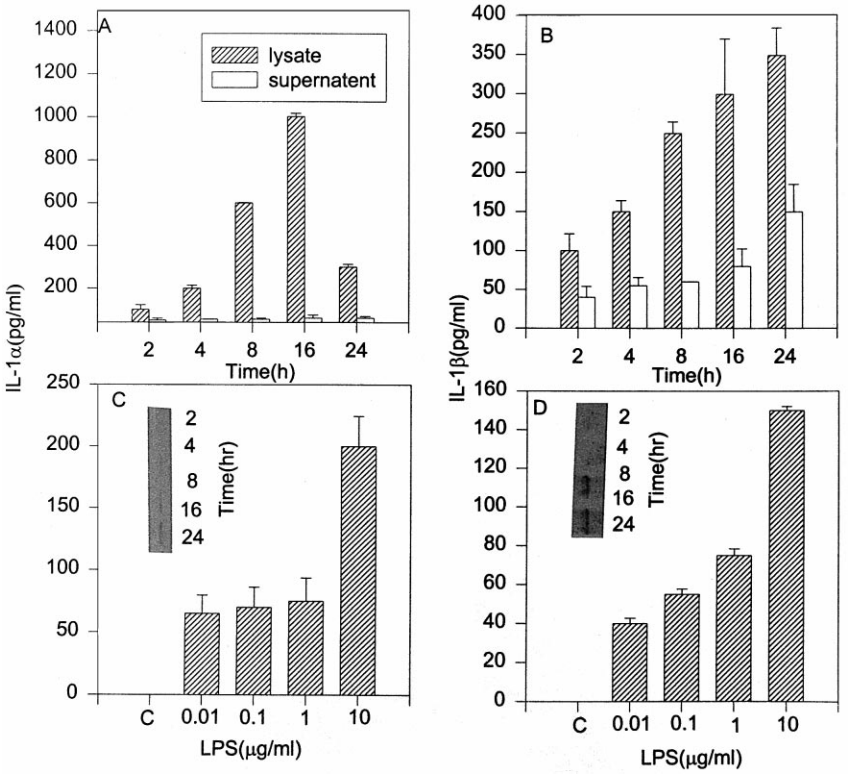

Fig. 1. Effect of LPS on soluble and cell-associated IL-1α and IL-1β production by lamina propria fibroblasts. (A,C) HSILPF (10^6 cells/well) were stimulated with LPS (10 μg/ml) for various time periods. IL-1α and IL-1β released into the supernatant and in cell-associated form were measured by immunoassay as described in Section 2.3. (B,D) Cells were stimulated with various concentrations of LPS (0.01–10 μg/ml) for 4 h. Cytokine assay in the cell-associated fraction was measured as described in Section 2.3. The results are derived from a single experiment in triplicate and are expressed as the mean values ± S.D. of triplicates. For cell-associated IL-1α and IL-1β, Western blot analysis was performed as described in Section 2.9 (insets in C and D).

A-CAM (1:100 dilution, Sigma) and N-CAM (1:100 dilution, Sigma), followed by peroxidase-conjugated goat anti-mouse IgG. The bands were detected by using an ECL kit as per manufacturer's instructions (Amersham).

2.10. Statistics

The statistical evaluation of the data was performed using paired Student's *t*-test. Results were expressed as mean values ± S.D. in triplicate of single or multiple experiments. Results were considered statistically significant at $P < 0.05$.

3. Results

3.1. Lamina propria fibroblasts express enhanced level of cell-associated IL-1α and IL-1β

IL-1α level in HSILPF as measured by ELISA in response to LPS (10 μg/ml) was elevated in a time-dependent fashion. The production of IL-1α was detected at 2 h, gradually increased by 16 h and then declined at 24 h (Fig. 1A). IL-1α was mostly associated with cell fraction than that present in the supernatant. Only a minimal quantity of soluble IL-1α (50 pg/ml) was released in the supernatants and the level remained constant throughout the study period. As shown in Fig. 1C, the basal level of cell-associated IL-1α without LPS stimulation was not detected in HSILPF by ELISA. LPS at the concentration range below 0.01–1 μg/ml did not show any difference in the IL-1α production, whereas with 10 μg/ml LPS there was a sudden increase in the cell-associated IL-1α level. Consistent with the ELISA results, Western blot analysis (Fig. 1C, inset) clearly showed time-dependent expression of cell-associated IL-1α.

An abundant quantity of IL-1β was detected in the supernatant of HSILPF exposed to LPS which remained elevated during the course of the study (Fig. 1B). However, in contrast to IL-1α, where the soluble IL-1α level remained constant, the level of soluble IL-1β was found to increase over the period of time. The quantity of IL-1β was twice the amount of soluble IL-1α at 24 h. As shown in Fig. 1D, the basal level of cell-associated IL-1β was not detected but upon LPS stimulation the production of cell-associated IL-1β level increased, which was dependent on concentration of LPS used (0.01–10 μg/ml). IL-1β protein expression in cell fraction as analyzed by Western blot was also gradually increased during the study period (Fig. 1D, inset).

3.2. LPS induces soluble IL-6 level in lamina propria fibroblasts

An abundant amount of IL-6 was produced in the

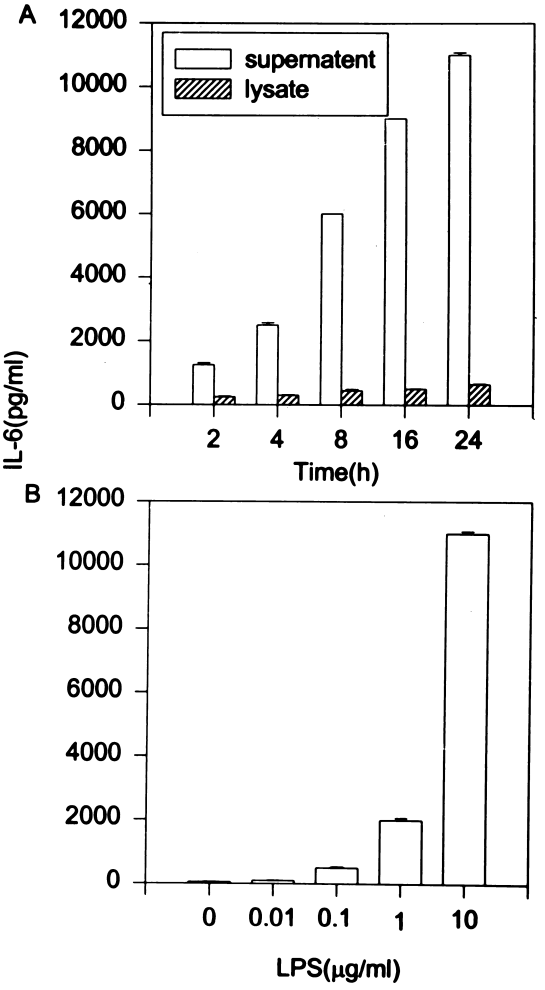

Fig. 2. Release of IL-6 by LPS in response to LPS. (A) HSILPF were stimulated with LPS (10 μg/ml) and incubated for different time periods. IL-6 in the supernatant and cell fraction was measured as described in Section 2.3. (B) Cells were stimulated with LPS (0.01–10 μg/ml) for 24 h. IL-6 in the supernatants was measured as described in Section 2.3. The results are derived from a single experiment in triplicate and are expressed as the mean values ± S.D. of triplicates.

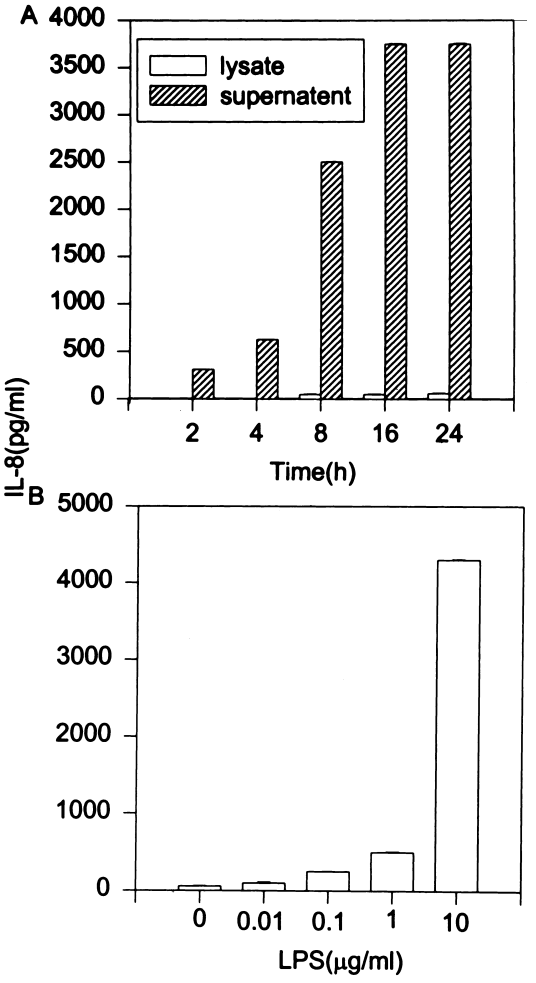

Fig. 3. Effect of LPS on IL-8 release by lamina propria fibroblasts. (A) HSILPF were stimulated with LPS (10 μg/ml) and incubated for different time intervals. IL-8 in the supernatant and cell fraction was measured as described in Section 2.3. (B) Cells were stimulated with LPS (0.01–10 μg/ml) for 24 h. IL-8 in the supernatants was measured as described in Section 2.3. The results are derived from a single experiment in triplicate and are expressed as the mean values ± S.D. of triplicates.

supernatant by HSILPF upon LPS stimulation (Fig. 2A). The production of IL-6 was time-dependent and gradually increased from 2 h to 24 h. The amount of cell-associated IL-6 remained constant throughout the time period studied. The basal level of soluble IL-6 in HSILPF was found to be 50 pg/ml (Fig. 2B). The dose–response pattern exhibited a gradual increase in the level of soluble IL-6 in response to different concentrations of LPS, and reached a maximum at 10 μg/ml LPS.

3.3. Production of soluble form of IL-8 from lamina propria fibroblasts by LPS

When stimulated with LPS (10 μg/ml), soluble IL-8 was detectable in the supernatant of HSILPF by 2 h and was elevated gradually up to 16 h (Fig. 3A). No difference in the IL-8 level was observed at 24 h as compared to 16 h. Cell-associated IL-8 was detected only at 8 h and the level remained constant up to 24 h. A gradual increase in the IL-8 level was

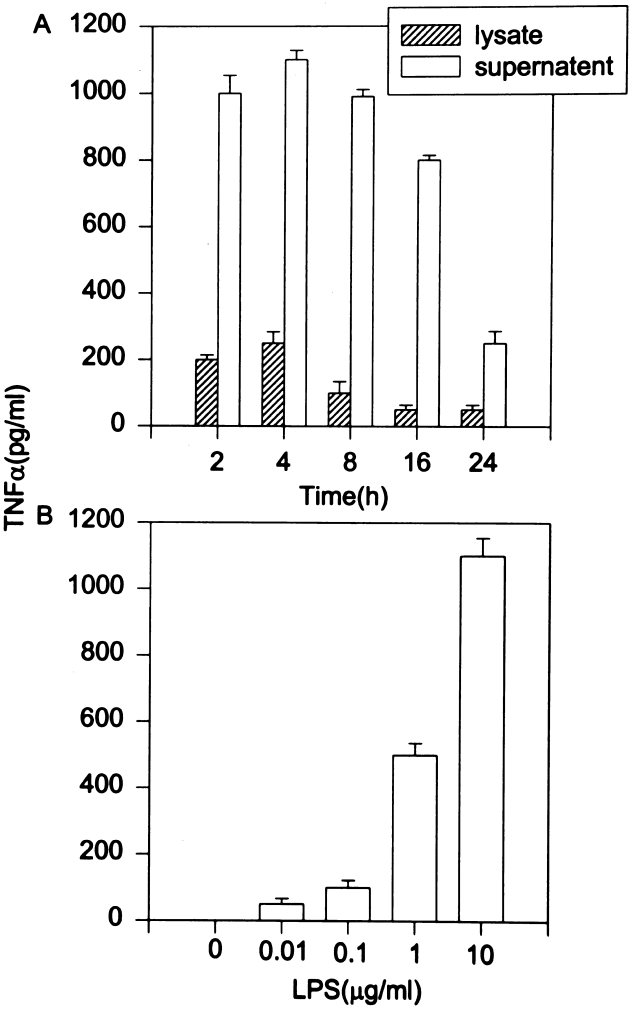

Fig. 4. Effect of LPS on TNF-α release by lamina propria fibroblasts. (A) HSILPF was stimulated with LPS (10 μg/ml) and incubated for different time periods. TNF-α in the supernatant and cell fraction was measured as described in Section 2.3. (B) Cells were stimulated with LPS (0.01–10 μg/ml) for 24 h. TNF-α in the supernatants was measured as described in Section 2.3. The results are derived from a single experiment in triplicate and are expressed as the mean values ± S.D. of triplicates.

Table 1
Effect of polymyxin B sulfate (PMB) on cytokine production by lamina propria small intestinal fibroblasts treated with LPS

Stimulus	PMB	Cytokine production (pg/ml)				
		IL-1α	IL-1β	IL-6	IL-8	TNF-α
None	−	ND	ND	10(±1)	60(±2)	ND
None	+	ND	ND	9.5(±5)	57(±1)	ND
LPS (10 μg/ml)	−	200(±1)	150(±1)	11 000(±5)	3750(±9)	250(±1)
LPS (10 μg/ml)	+	10(±1)	8.5(±2)	100(±7)	50(±4)	5(±3)

Cells were treated with LPS (10 μg/ml) alone or LPS and PMB (25 μg/ml) for 24 h. Cytokines were measured as described in Section 2.3. ND, not detected.

observed with various concentrations of LPS (Fig. 3B). An approximately 70-fold increase in the IL-8 level was observed when the cells were treated with 10 μg/ml LPS as compared to untreated cells (Fig. 3B).

3.4. LPS induces TNF-α production at an early phase by HSILPF

The amount of TNF-α secreted in the culture supernatant was rapidly elevated at 2 h in response to LPS, in contrast to all other soluble cytokines measured, and the level of TNF-α gradually decreased after 24 h (Fig. 4A). The level of cell-associated TNF-α exhibited the same pattern as that of soluble TNF-α. The basal level of TNF-α was not detected in HSILPF and was induced upon different concentrations of LPS used (Fig. 4B).

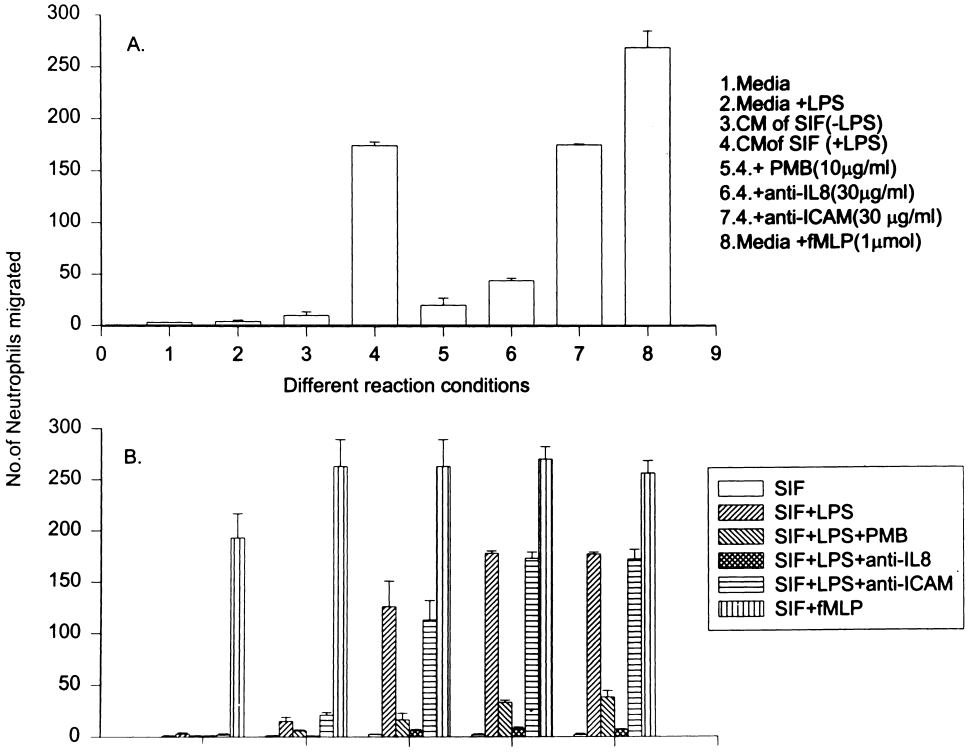

Fig. 5. Migration of neutrophils. (A) Neutrophils were isolated as described in Section 2.4. 10^6 neutrophils (seeded in cell culture inserts) were stimulated with various stimulant and with 1 μm fLMP as a positive control. Migration of neutrophils was measured as described in Section 2.6. (B) Fibroblasts were co-cultured with neutrophils (separated by 5 μm pore size membrane) under different reaction conditions. Neutrophil migration was measured as described in Section 2.6. The results are derived from the six individual experiments performed in triplicate and are expressed as the mean values ± S.D. of six experiments.

3.5. Polymyxin B abrogates LPS-induced cytokine expression

As described in Table 1, incorporation of PMB (25 μg/ml) along with LPS completely diminished the IL-6, IL-8 and TNF-α in the supernatants and cell-associated IL-1α and IL-1β cytokine production. PMB alone did not affect the cytokine production but ablated the LPS-induced enhanced cytokine production in HSILPF.

3.6. Conditioned media (CM) of LPS-treated HSILPF induces migration of neutrophils

Media harvested from LPS-treated HSILPF induced the migration of neutrophils in a convincing manner. In fact, neutrophil migration induced by CM of LPS-treated HSILPF was 65% as effective as that which occurred in response to the potent chemoattractant, fMLP (Fig. 5A). LPS alone or conditioned medium of fibroblasts not stimulated with LPS failed to induce migration of neutrophils. Addition of PMB decreased the LPS-induced neutrophil migration by 8-fold. Incubation of CM with anti-IL-8 antibody (30 μg/ml) inhibited the neutrophil migration by 75%, whereas anti-ICAM antibody (30 μg/ml) was not effective in inhibiting the neutrophil migration (Fig. 5A).

Co-culture of neutrophils with lamina propria fibroblasts showed a time-dependent migration of neutrophils. As indicated in Fig. 5B, the migratory response of neutrophils was observed at 4 h and was increased by 24 h. Lamina propria fibroblasts alone could not induce neutrophil migration, but when treated with LPS gradually increased the migration of neutrophils. Incorporation of fMLP into the lower chamber having a fibroblast monolayer exhibited prompt migration of neutrophils at 2 h. PMB and antibody to IL-8 greatly abolished the migration of neutrophils. Anti-ICAM did not alter the status of neutrophil migration.

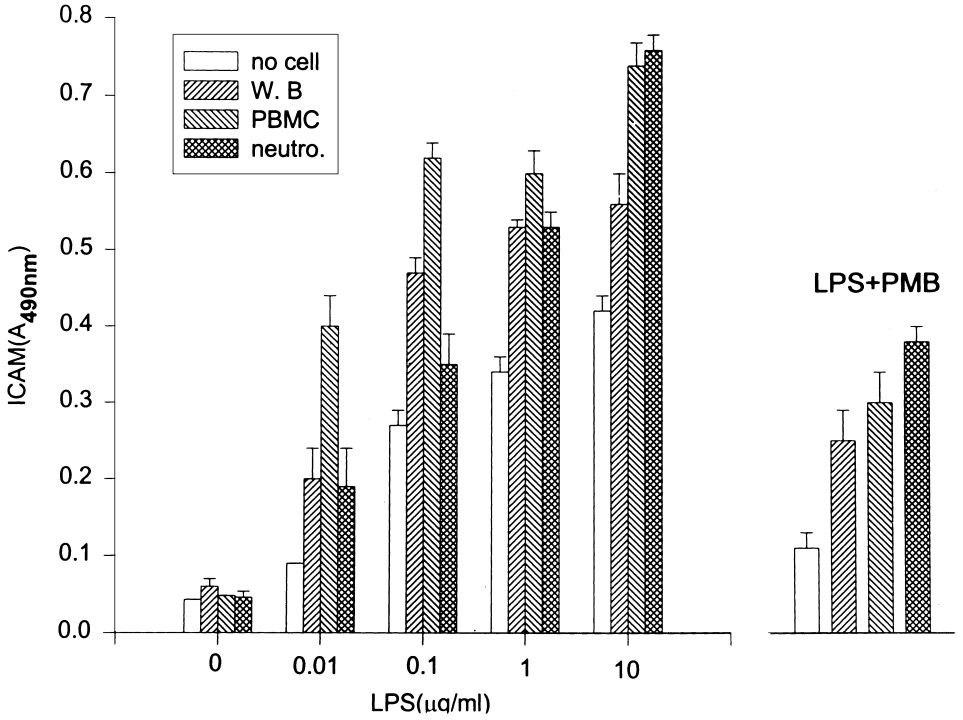

Fig. 6. Effect of LPS on ICAM expression by lamina propria fibroblasts and its modulation by blood cells. HSILPF (10^4 cells/well) was plated in 96-well plates and incubated with LPS (10 μg/ml) or LPS and PMB (25 μg/ml) alone or in the presence of blood cells. ICAM expression in the fibroblasts was detected as described in Section 2.5. The results are derived from five individual experiments performed in triplicate and are expressed as the mean values ± S.D. of five experiments.

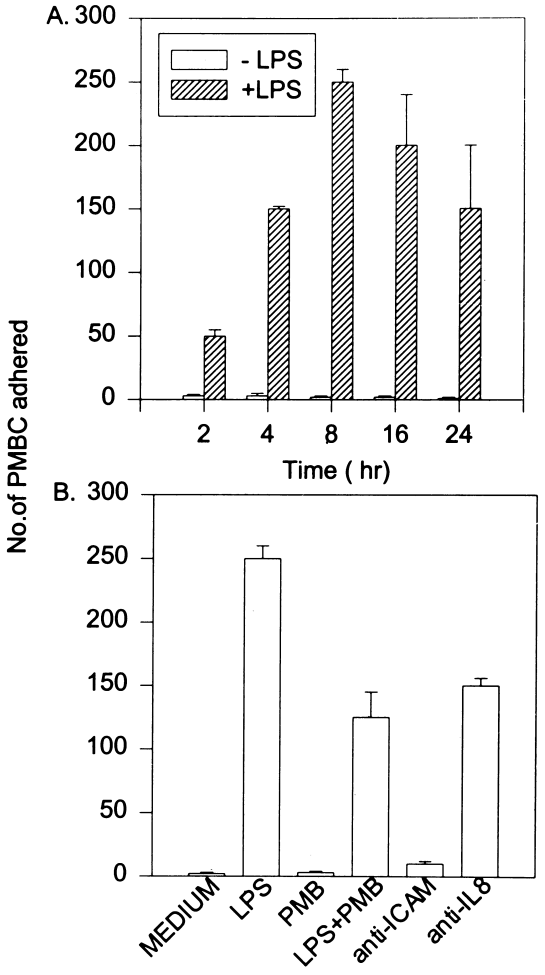

Fig. 7. Adhesion of PBMC to LPS-treated fibroblast layer. (A) Fibroblast layers were treated with LPS (10 μg/ml) or left untreated and incubated for various time periods. After each time period, PBMC were added to the fibroblasts and incubated for a further 2 h. The PBMC adhering to the fibroblasts were measured as described in Section 2.8. (B) Fibroblasts were incubated with LPS, LPS+PMB (25 μg/ml), anti-IL-8 and anti-ICAM antibody (30 μg/ml). Adhesion of PBMC was measured as described in Section 2.8. The results are derived from five individual experiments performed in triplicate and are expressed as the mean values ± S.D. of five experiments.

3.7. ICAM expression by LPS in HSILPF

ICAM expression in lamina propria fibroblasts was not only upregulated upon LPS exposure, but also considerably modulated by whole blood cells, neutrophils and PBMC. As shown in Fig. 6, whole blood cells, neutrophils and PBMC enhanced the LPS-induced ICAM expression in HSILPF. The expression of ICAM in HSILPF was dependent on the concentration of LPS used and was markedly enhanced by PBMC as compared to neutrophils or whole blood cells alone. There was a 2-fold increase in the ICAM level in HSILPF when incubated with PBMC as compared to the ICAM level observed in HSILPF without PBMC. Neutrophils in the presence of a higher concentration of LPS (10 μg/ml) elevated ICAM expression in HSILPF similar to PBMC, but LPS at lower concentrations (0.01–1 μg/ml) was less effective in inducing ICAM than PBMC. ICAM expression was partly abrogated by PMB.

3.8. LPS-induced ICAM expression in HSILPF favors PBMC adhesion

Fibroblasts treated with LPS (10 μg/ml) favored the PBMC to adhere to the fibroblasts in a time-dependent manner. The maximum number of adhered PBMC was at 8 h of LPS exposure and thereafter there was decrease in the adhesion at 16 and 24 h (Fig. 7A). Incorporation of PMB abolished the adhesion of PBMC to LPS-treated fibroblasts by 50%. When the cells were incubated with anti-ICAM antibody after stimulating the cells with LPS, there was remarkable inhibition of PBMC adhesion to fibroblasts. Similarly, there was 40% inhibition in the adhesion of PBMC to the LPS-treated HSILPF when the cells were incubated with anti-IL-8 antibody (Fig. 7B).

3.9. Expression of vitronectin, A-CAM and N-CAM in lamina propria fibroblasts in response to LPS

As shown in Fig. 8, vitronectin expression was maximum at 8 h and gradually declined at 24 h in

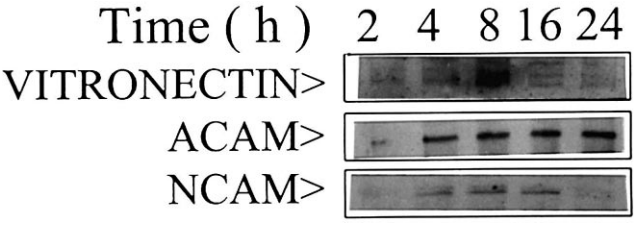

Fig. 8. Expression of vitronectin, A-CAM and N-CAM by lamina propria fibroblasts upon LPS exposure. Vitronectin, A-CAM and N-CAM were detected in LPS-stimulated HSILPF lysates as described in Section 2.9. The detection system consisted of an ECL kit and was used according to manufacturer's instructions (Amersham).

HSILPF upon LPS treatment. Vitronectin was observed as a 75 kDa band in lamina propria fibroblasts and was strongly induced upon LPS treatment. A-CAM was not detected in untreated HSILPF, but was expressed by LPS. The expression pattern of A-CAM remained constant throughout the study period. N-CAM, which was barely detected in the lamina propria fibroblasts, was upregulated upon LPS treatment. The expression of N-CAM was maximum at 16 h and thereafter it declined.

4. Discussion

Mucosal surfaces are lined by epithelial cells forming a potentially effective barrier between the pathogenic organism and the underlying cells. However, breaching of epithelial barrier may allow the bacteria and the bacterial antigens to translocate to the underlying tissue. Infection with Gram-negative bacteria results in inflammation, which is in part caused by LPS [25]. Proinflammatory mediators produced by the host cells in response to bacteria and bacterial antigenic stimuli are known to determine the outcome of the inflammatory reactions occurring at the local site in the host. Evidence shows that non-immunocompetent cells (epithelial cells, endothelial cells and fibroblasts) generate many potent proinflammatory cytokines capable of modulating local inflammatory reactions. Earlier studies have reported the extensive infiltration of PMN in the lamina propria layer [17], but the mechanism of such an inflammatory response, the cell types involved and the role of LPS in inflammation remain unknown. We hypothesized that the lamina propria fibroblasts, upon LPS stimulation, secrete certain proinflammatory cytokines and adhesion molecules and induce neutrophil migration and PBMC adhesion, thereby contributing to the intestinal inflammation.

Our findings demonstrate that lamina propria fibroblasts expressed proinflammatory cytokines, IL-1α, IL-1β, IL-6, IL-8 and TNF-α and adhesion molecules ICAM-1, vitronectin, A-CAM and N-CAM. The synthesis of chemokine IL-8 and adhesion molecule ICAM-1 further led to neutrophil migration and PBMC adhesion to lamina propria fibroblasts, respectively. In a similar line of research, Pang et al. also suggested the role of intestinal fibroblasts in intestinal inflammatory disorders and studied the expression of IL-1α, IL-1 β, IL-6, IL-8, IL-10, ICAM-1 and V-CAM in adult duodenal fibroblasts in response to LPS, IL-1α and TNF-α [13]. However, the culture method adopted by Pang et al. was different from that of ours (in preparation).

Kinetic analysis showed that LPS in a time- and dose-dependent manner induced the synthesis of cytokines by HSILPF in the order IL-6 > IL-8 > TNF-α > IL-1β > IL-1α. All these cytokines reached peak levels at different time periods, i.e., IL-6 at 24 h, IL-8 at 16 h, TNF-α at 4 h, IL-1β at 16 h and IL-1α at 24 h. To avoid the ambiguity in the kinetic analyses, all the cytokines were measured from a single sample. As observed in our study, the synthesis of these cytokines by HSILPF upon LPS exposure at different time periods may be depicting the intricate network among the cytokines for generating the optimal immune response in the gut. IL-1α and IL-1β remained as cell-associated cytokines. Although duodenal fibroblasts stimulated with LPS expressed mRNA for IL-1α and IL-1β, the secreted proteins were not detected [13]; however, we were able to detect IL-1α and IL-1β produced in the supernatant by ELISA, though the majority of IL-1α and IL-1β was found to be cell-associated. It is possible that cell-associated IL-1α is responsible for activation of lamina propria lymphocytes, as earlier studies have shown that cell-associated IL-1α in FS-4 fibroblasts retained the capacity to stimulate thymocytes [26]. The low amount of IL-1α in the supernatant of LPS-stimulated lamina propria fibroblasts may be sufficient for evoking the response in other cells residing in lamina propria. Production of IL-6, a multifunctional cytokine, further suggests that the abundant quantity of IL-6 produced by lamina propria fibroblasts may activate T cells and mucosal B cells to differentiate into antibody-producing cells, thereby responding to the EPEC infection in the gut. As it is evident that IL-6 also downregulates the production of inflammatory cytokines such as TNF-α [27], the production of IL-6 in lamina propria fibroblasts may be orchestrating the cytokine balance for an enhanced and efficient immune response in gut. As reported in our previous study [19], the enhanced collagen synthesis in lamina propria fibroblasts by LPS may be related to enhanced IL-6 production, as IL-6 is known to inhibit the metalloproteinases responsible for degrading ex-

tracellular matrix molecules such as collagen [28]. TNF-α is a key mediator of inflammation and the mammalian host cell response to invasion by microbes, and is known to be produced by mast cells, macrophages, basophils, keratinocytes and astrocytes [29]. Up to now, fibroblasts have not been known to secrete TNF-α. We detected the TNF-α in the supernatant of HSILPF stimulated by LPS at 2 h. TNF-α reached a peak at 4 h and the level declined thereafter. Possibly the downregulation of TNF-α may be due to the gradual increase in IL-6 production which is known to downregulate TNF-α. The production of TNF-α by lamina propria fibroblasts may be involved in the activation of lamina propria lymphocytes, and also may be exerting a paracrine effect on intestinal epithelial cells. TNF-α acts on chloride transport of intestinal epithelial cells via fibroblasts in cryptococcus-induced diarrhea, thereby suggesting the involvement of fibroblasts in epithelial functioning [30]. IL-8 is a chemokine responsible for migration and recruitment of neutrophils during inflammation [31]. The production of IL-8 by lamina propria fibroblasts can be expected to have a major impact on the lamina propria lymphocytes, neutrophils and monocytes. Production of IL-8 by lamina propria fibroblasts and enhanced migration of neutrophils as observed in our study probably may suggest the cell type involved and the mechanism of the accumulation of PMN in the lamina propria layer in EPEC-induced diarrhea, as reported earlier [17]. ICAM is a glycoprotein expressed by variety of cell types and is involved in leucocyte aggregation [4,32–34]. Enhanced expression of ICAM-1 was also observed in the lamina propria cells of small intestine from a patient with celiac disease, but the cell types involved were not detected [35]. Our study demonstrates increased expression of ICAM-1 by HSILPF in response to LPS and increased adhesion of PBMC to HSILPF which is ICAM-dependent. Further enhanced expression of ICAM-1 in presence of PBMC and LPS suggests that the residing lamina propria lymphocytes may modulate the ICAM expression for attaining rapid and effective but controlled response to inflammatory stimuli such as LPS during EPEC-induced diarrhea. We have investigated the expression pattern of other adhesion molecules such as vitronectin, A-CAM and N-CAM in HSILPF upon LPS interaction. Vitronectin is an adhesion-promoting glycoprotein that is involved in the coagulation, fibrinolysis and complement systems with activities that point to a role in inflammation [36]. Interestingly, whether LPS can modulate the vitronectin in HSILPF has not been discovered. We found that vitronectin was enhanced upon LPS exposure in HSILPF. The possibility of serum vitronectin being adsorbed onto the cell surface cannot be ruled out. However, we minimized the serum concentration to 1% in our cell culture experiments, and the control cells at the same time period did not exhibit vitronectin protein band in the Western blot experiment. Addition of anti-vitronectin to fibroblasts or CM of LPS-treated HSILPF did not affect the neutrophil migration or PBMC adhesion (data not shown). Although A-CAM and N-CAM are identified as major cell adhesion molecules [37,38], and we have also observed the enhanced expression of A-CAM and N-CAM in HSILPF upon LPS treatment, neutralizing antibodies to these adhesion molecules did not inhibit PMN migration or PBMC adhesion (data not shown). Further investigation is required to determine whether the increased expression of these adhesion molecules has any specific role in LPS-induced inflammation.

Taken together, these results strongly suggest that LPS interaction with lamina propria fibroblasts may contribute to the inflammatory reactions in the gut by producing these proinflammatory cytokines in a regulated manner, thus leading to the accumulation of neutrophils in the gut lumen. Moreover, these proinflammatory cytokines and adhesion molecules produced by lamina propria fibroblasts can mediate their action on lamina propria lymphoid cells and maintain their proliferation, differentiation and activation states. Hence, the lamina propria fibroblasts form one of the vital cell populations responsible for the inflammatory reactions in the intestine and can act in an autocrine and paracrine manner to enhance the local immune response at the site of EPEC infection.

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