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Effects of *Listeria monocytogenes* and *Yersinia enterocolitica* on Cytokine Gene Expression and Release from Human Polymorphonuclear Granulocytes and Epithelial (HEp-2) Cells

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The gene expression and cytokine release of the proinflammatory cytokines interleukin-1β (IL-1β), IL-6, and tumor necrosis factor alpha (TNF-α) after infection of human epithelial cells (HEp-2 cells) and polymorphonuclear granulocytes (PMNs) were investigated by using isogenic pairs of *Listeria monocytogenes* and *Yersinia enterocolitica* strains. By polymerase chain reaction-assisted mRNA amplification and RNA dot blot analysis, we showed that PMNs and HEp-2 cells expressed enhanced levels of mRNA encoding IL-1β, IL-6, and TNF-α after bacterial infection. Concomitant with the enhanced mRNA level, an increased secretion rate of IL-1β, IL-6, and TNF-α from PMNs as assessed by enzyme-linked immunosorbent assay was observed. HEp-2 cells after infection also released IL-6 and TNF-α into the cell supernatant, while no IL-1β release was detected. Cellular coincubation experiments were carried out with Transwell chambers. Our studies revealed that the coculture of PMNs and HEp-2 cells led to an increased IL-1β and IL-6 release. In contrast, after infection with the invasive bacteria, reduced levels of TNF-α were measured. Our data show that PMNs secrete the proinflammatory cytokines IL-1β, IL-6, and TNF-α within some hours after infection with *L. monocytogenes* and *Y. enterocolitica* and that cellular interactions with epithelial cells alone via soluble mediators influence the net amount of released proinflammatory cytokines.

During the onset of the acute-phase response, an influx of inflammatory cells, including monocytes and polymorphonuclear granulocytes (PMNs), to the sites of inflammation can be observed (29). Within the microenvironment of the inflamed tissues, these cells communicate with each other by direct cell-cell contact or a complex array of cytokines and inflammatory mediators. In the course of inflammation or microbial invasion, the proinflammatory cytokines interleukin-1β (IL-1β), IL-6, and tumor necrosis factor alpha (TNF-α) are responsible for either local or systemic effects (reviewed in reference 1).

The cytokines are functionally multipotent, with overlapping biological activities, e.g., they induce fever (33), stimulate hepatocytes to synthesize a wide spectrum of acute-phase proteins (44), and express immunomodulatory functions by increasing thymocyte proliferation (20). Small amounts of these proinflammatory cytokines produced locally within infected tissues play an important protective role in host defense prior to their secretion into the peripheral circulation (48).

Only few data exist for the expression of these cytokines into the microenvironment of the gastrointestinal tract tissue after infection with the facultatively intracellular bacteria *Listeria monocytogenes* and *Yersinia enterocolitica*. All pathogenic *L. monocytogenes* and *Y. enterocolitica* strains adhere to and penetrate the intestinal epithelium by means of specific gene products for invasion. The invasion process of *L. monocytogenes* is mediated by internalin as well as by a gene product called p60 (14, 28). Also, the *inv* gene locus, which encodes invasin and the attachment/invasion (*ail*) gene locus of *Y. enterocolitica*, is responsible for an invasive phenotype (reviewed in reference 38).

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The purposes of our study were to investigate (i) whether human epithelial (HEp-2) cells and PMNs transcribe the genes encoding IL-1β, IL-6, and TNF-α and release the mature cytokines after infection with *L. monocytogenes* and *Y. enterocolitica*; (ii) which role the invasion process plays with regard to cytokine gene expression and secretion; and (iii) whether paracrine cellular interactions between infected epithelial cells and PMNs modulate the net amount of released cytokines.

For bacterial stimulation, we used isogenic pairs of *L. monocytogenes* (invasive and noninvasive), *Y. enterocolitica* (plasmidless and plasmid bearing), and *Escherichia coli* (invasive and noninvasive) (18, 22, 24) strains.

MATERIALS AND METHODS

Buffers. The medium used for washing the bacterial cells was phosphate-buffered saline (PBS). Bacteria were grown in brain heart infusion (BHI) broth (Oxoid Ltd., London, England). The following electrophoresis buffers were used for gel electrophoresis: TBE buffer (Tris-borate) for analysis of amplified cDNAs and MOPS buffer [3-(N-morpholino-)propanesulfonic acid] for RNA gel electrophoresis.

Preparation of cells. (i) PMNs. PMNs were prepared from heparinized venous blood from healthy donors on a Ficoll-metrizoate gradient; preparation was followed by dextran sedimentation as described elsewhere (5). The contaminating erythrocytes were removed by hypotonic lysis of the obtained cell suspension. This method led to >98% pure PMNs (1 to 2% eosinophils). The cell preparations were routinely analyzed by morphological examination of Wright-stained smears and nonspecific esterase staining. Less than 1% mononuclear cells were present.

(ii) HEp-2 cells. Cells of the human laryngeal epithelium cell line HEp-2 were routinely grown in Dulbecco modified

TABLE 1. Strains used in this study and their characteristics

Strain	Serovar	Listeriolysin/ hemolysin phenotype	Invasion phenotype	Virulence
L. monocytogenes				
NCTC 7973	1/2a	Hly^+	Inv^+p60^+	++
SLCC 5779	1/2a	Hly^+	Inv^-p60^-	+
Y. enterocolitica				
108C (VP-less)	O:3		Inv^+	
108P (VP bearing)	O:3		Inv^+	++
E. coli				
HB101			Inv^-	
HB101(pRI203)			Inv^+	

Eagle medium containing 10% (vol/vol) heat-inactivated calf serum, 100 μg of streptomycin per ml, 100 IU of penicillin per ml, and 20 mM sodium hydrogen carbonate. The medium was replaced every 2 days until the monolayers were confluent. Confluent monolayers were harvested by repeated rinsing with ice-cold medium.

Culture conditions. All media and supplements were purchased from GIBCO Europe Ltd. (Karlsruhe, Germany). The buffers and cell media were prepared with pyrogen-free water. The lipopolysaccharide concentration ranged below the detection limit as determined by the *Limulus* amebocyte test (<100 pg/ml). The cells were cultured in a humidified incubator at 37°C under 5% CO_2.

Stimulation experiments. Prior to each experiment, the cells were washed three times with RPMI 1640 medium before adjustment to the desired concentration. Cell viability was tested by the trypan blue exclusion test. Only cell suspensions with a viability of more than 95% were used for the studies. The stimulation experiments were carried out with six-well Transwell cell culture plates (catalog no. 3412; Costar, Cambridge, Mass.) for up to 4 h. The pore size of the permeable polycarbonate membrane was 0.4 μm. HEp-2 cells (1×10^7) in a volume of 500 μl were added to the cluster plate. PMNs (4×10^7) in a volume of 500 μl were added to the Transwell cell culture chamber inserts. Washed bacterial cells (2.5×10^8) in 50 μl of RPMI 1640 medium were added to the cluster plates as well as to the chamber inserts. The final volume was adjusted to 2.5 ml on the cluster plate and to 1.5 ml in the chamber inserts. Controls of the coculture experiments were carried out by incubation of each cell type with only medium or invasive bacteria. All stimulation experiments were performed under gentle and constant agitation at 37°C. After the indicated coincubation times, the cells were immediately harvested, centrifuged, and lysed in 4 M guanidinium isothiocyanate solution. The supernatants were stored at −70°C.

Bacterial strains. The following bacterial strains were analyzed: *L. monocytogenes* NCTC 7973 (Hly^+ Inv^+) and SLCC 5779 (rough variant; Hly^+ Inv^-), *Y. enterocolitica* 108C (plasmidless) and 108P (plasmid bearing), and *E. coli* HB101 and HB101(pRI203) (Table 1). The *L. monocytogenes* strains were obtained from W. Goebel, Institute of Genetics and Microbiology, University of Würzburg, Würzburg, Germany (31). *Y. enterocolitica* 108C, serotype O:3, is plasmidless; *Y. enterocolitica* 108P bears a 42- to 46-MDa plasmid which encodes several virulence factors, e.g., *Yersinia* outer membrane proteins. Both isogenic strains are a kind gift from J. Heesemann, Institute of Medical Microbiology, Würzburg, Germany (18). In additional experiments, cells were infected with *E. coli* reference strain HB101 and *E. coli* HB101(pRI203). *E. coli* HB101 represents an *E. coli* K-12 strain which is noninvasive (Inv^-) and which does not have MS fimbriae (4). *E. coli* HB101(pRI203) is a K-12 derivative harboring the gene encoding the *inv* locus of *Y. pseudotuberculosis* (24). The invasive (Inv^+) *E. coli* strain is a generous gift from S. Falkow, Stanford University, Stanford, Calif.

Bacterial growth. The bacterial strains were grown in BHI medium overnight at 37°C on a shaker (150 rpm). BHI medium (10 ml) was inoculated with 100 μl of this overnight culture and allowed to grow for 3.5 h on a shaker at 37°C. Subsequently, the bacteria were centrifuged at 4,000 × *g* for 20 min, separated from the culture supernatant, and washed twice with PBS buffer. The bacterial concentration was determined microscopically. For stimulation experiments, bacteria were resuspended in RPMI 1640 medium to concentrations of 2.5×10^8 cells per 50 μl.

Phagocytosis and invasiveness. (i) PMNs and phagocytosis. [^{3}H]thymidine (555 kBq) was added to a 10-ml bacterial culture. PMNs (1×10^7 per 500 μl of PBS) were stimulated with 50 μl of the bacterial suspension and incubated at 37°C for 30 min. Subsequently, the cells were separated by centrifugation at 300 × *g* for 15 min. Adherent bacteria were removed from the cells by incubation on ice with 500 μl of PBS-EDTA-lysozyme (40 mM EDTA, 100 μg of lysozyme per ml) for 30 min. After the cells were washed, lysis in distilled water was carried out to determine the percent phagocytosis (46). After incubation with PBS-EDTA-lysozyme solution, control samples containing only bacteria revealed a loss of 80% of the radioactivity that could be centrifuged. Therefore, adherent gram-negative and -positive bacteria were effectively lysed by this method. Data shown (see Table 3) are mean values ± the standard errors of the means for four experiments.

(ii) HEp-2 cells and invasiveness. Confluent stock monolayers were used to seed six-well tissue culture plates (Falcon 3046) at a concentration of 2×10^6 cells per well. After overnight incubation, the confluent monolayers were infected with bacteria at a multiplicity of infection of 10 to 20. After the addition of the bacterial suspension, the culture plates were centrifuged at 212 × *g* for 10 min (47). Infected monolayers were incubated for 2 h at 37°C, washed with PBS, and covered with Dulbecco modified Eagle medium containing gentamicin (100 μg/ml) for 1 h to kill extracellular bacteria. The monolayers were washed three times, and the infected cells were lysed by adding 1 ml of distilled water. Appropriate dilutions were plated on BHI agar plates, and viable intracellular bacteria were counted. The results are expressed as the mean CFU per well. The assay was repeated three times.

RNA extraction, RNA blotting, and polymerase chain reaction (PCR)-mediated mRNA amplification. (i) RNA extraction. Total cellular RNA was extracted essentially by the protocol of Chomczynski and Sacchi (6).

(ii) RNA blotting. (a) Northern (RNA) analysis. RNA electrophoresis was carried out under denaturing conditions in the presence of formaldehyde. For Northern blot analysis, 40 μg of total cellular RNA was subjected to electrophoresis on a 1.2% agarose-formaldehyde gel for 2 h at 120 V. The gels were transferred to GeneScreen Plus membranes (NEN, DuPont) by the method of Maniatis et al. (35). The RNA blots were prehybridized for at least 15 min at 42°C with a hybridizing solution containing 50% formamide, 10% dextran sulfate, 1% sodium dodecyl sulfate (SDS), and 1 M sodium chloride. After the addition of denatured salmon sperm DNA (100 μg/ml) and denatured radioactive probe

TABLE 2. Specific primers synthesized for PCR

mRNA	Size of PCR-amplified fragment (bp)	Synthesized primers (5′ sense and 3′ antisense)
β-Actin	661	5′-TGA-CGG-GGT-CAC-CCA-CAC-TGT-GCC-CAT-CTA-3′ 5′-CTA-GAA-GCA-TTG-CGG-TGG-ACG-ATG-GAG-GG-3′
IL-6	628	5′-ATG-AAC-TCC-TCC-TCC-ACA-AGC-GC-3′ 5′-GAA-GAG-CCC-TCA-GGC-TGG-ACT-G-3′
IL-1β	331	5′-CTT-CAT-CTT-TGA-AGA-AGA-ACC-TAT-CTT-CTT-3′ 5′-AAT-TTT-TGG-GAT-CTA-CAC-TCT-CCA-GCT-GTA-3′
TNF-α	325	5′-CAG-AGG-GAA-GAG-TTC-CCC-AG-3′ 5′-CCT-TGG-TCT-GGT-AGG-AGA-CG-3′

(<10 ng/ml), hybridization was performed overnight at 42°C under constant agitation. IL-6 mRNA was detected with an *Eco*RI cDNA fragment obtained from the clone pCSF309 (American Type Culture Collection). The cDNA fragment was ^{32}P radiolabeled by random priming (13). Standardization was performed with respect to 28S and 18S rRNA (21).

(b) RNA dot blot procedure. Total cellular RNA was dissolved in an appropriate volume of 50% deionized formamide–6% formaldehyde solution. This RNA solution was incubated for 60 min at 50°C to denature RNA. Up to 20 μg of total cellular RNA was distributed into wells of the used manifold. RNA dot blot analysis and Northern blot analysis were performed under the following stringent washing conditions: (i) twice for 30 min each with 2× SSC (0.3 M sodium chloride plus 0.03 M sodium citrate) and 1% SDS at 65°C; (ii) twice for 20 min each with 0.1× SSC at room temperature. The membranes were exposed to Kodak XAR 5 films at −40°C to obtain autoradiographs. The bound radioactivity of RNA dot blots representing 20 μg of total RNA was then determined (β Rack 1209; LKB, Turku, Finland). Background radioactivity was subtracted, and the specific binding was expressed as counts per minute.

PCR-mediated mRNA amplification. (i) Reverse transcription of total cellular RNA. RNA was reverse transcribed into cDNA as follows. Two micrograms of RNA was resuspended in 20 μl of DEPC-double-distilled water (ddH_2O) containing 2.5 μM oligo(dT) (16-mer; GIBCO-BRL), 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 mM each deoxynucleoside triphosphate (GIBCO-BRL), 20 U of placental RNase inhibitor (GIBCO-BRL), and 50 U of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL). The reaction mixture was overlaid with mineral oil to prevent evaporation and incubated in a thermocycler progammable heating block (Perkin-Elmer Cetus Corp.) for 1 cycle for up to 10 min at 18°C, for 60 min at 42°C, and for 5 min at 95°C. The cDNA samples were stored at −20°C.

(ii) PCR amplification of cDNA. Ten microliters of reaction mixture was mixed with 40 μl of DEPC-ddH_2O containing 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.25 U of *Taq* polymerase (GIBCO-BRL), 150 nM downstream primer, and 150 nM upstream primer. The final mixture volume of 50 μl was overlaid with 30 μl of mineral oil to prevent evaporation and then transcribed to double-stranded DNA in a DNA thermocycler in a three-temperature cycle which included 5 min of denaturation at 95°C, 60 s of annealing at 60°C, and 180 s of transcription at 72°C. Unless stated otherwise, the double-stranded DNA was amplified 25 times in a repeated three-temperature cycle which included 60 s of denaturation at 95°C, 60 s of annealing at 60°C, and 120 s of extension at 72°C.

(iii) Primer synthesis for PCR. Primer pairs specific for IL-1β, IL-6, TNF-α, and β-actin were synthesized on a DNA synthesizer (391 DNA synthesizer; Applied Biosystems). The specific primer sequences used are depicted in Table 2. The downstream and upstream primers are complementary to sequences in the first and last exons, respectively. The synthesized oligonucleotide sequences spanned exon-exon connections, so they are mRNA specific. The sequences of the primers specific for IL-1β, IL-6, and β-actin were recently described by Ehlers and Smith (12).

Analysis of IL-1β, IL-6, and TNF-α release. In addition to mRNA analysis, the cell supernatants of infected and cocultured cells were analyzed by enzyme-linked immunosorbent assay (ELISA) for secreted IL-1β, IL-6, and TNF-α (Medgenix, Brussels, Belgium). The secretion rate of the cocultured cells was compared with the amount of the secreted cytokines in the control experiments.

RESULTS

Invasiveness and phagocytosis of bacteria. Prior to the cytokine gene expression studies, we analyzed the bacterial strains (Table 1) with respect to their invasiveness and their phagocytosis pattern.

Isogenic pairs of *L. monocytogenes*, *Y. enterocolitica*, and *E. coli* (Table 1) were studied with regard to their invasiveness in cultured HEp-2 cells. As can be seen in Table 3, after 2 h of infection all invasive bacteria entered the epithelial cells by means of their invasive gene products. *E. coli* HB101 and the noninvasive rough mutant strain of *L. monocytogenes* (SLCC 5779) were not able to invade the cultured epithelial cells. The phagocytosis data for the bacterial strains after 30 min of incubation with PMNs are also shown in Table 3. The data demonstrate that all bacterial strains were phagocytosed by PMNs irrespective of their virulence factors. The degrees of phagocytosis differed from 3.7 to 22.0%. Thus, the bacterial strain specificities were not able to inhibit the uptake by neutrophils; they only modulated the degree of uptake, resulting in an altered phagocytosis rate.

Cytokine gene expression and secretion by HEp-2 cells and PMNs after bacterial infection. (i) HEp-2 cells. In subsequent experiments, HEp-2 cells were used as a model system for infection. We investigated the release of TNF-α, IL-6, and IL-1β and gene expression by analyzing the cytoplasmic levels of mRNA encoding these cytokines.

TABLE 3. Rates of bacterial invasion and percent phagocytosis by PMNs

Strain	Invasion of HEp-2 cells[a] (CFU) (10^6)	% Phagocytosis by PMNs[b]
L. monocytogenes		
NCTC 7973	11 ± 4	3.7 ± 0.6
SLCC 5779	0	17.3 ± 2.1
Y. enterocolitica		
108C (VP-less)	9 ± 4	22.0 ± 3.5
108P (VP bearing)	14 ± 4	22.2 ± 4.1
E. coli		
HB101	0	20.7 ± 4.0
HB101(pRI203)	13 ± 6	14.3 ± 3.8

[a] Rates of invasion 2 h after infection. Values are means ± standard errors of the means for three experiments.
[b] Patterns of phagocytosis by PMNs after 30 min of incubation. Values are means ± standard errors of the means for four experiments.

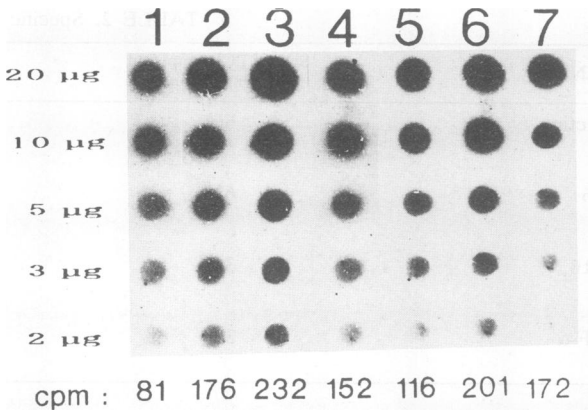

FIG. 1. RNA dot blot analysis of IL-6 mRNA expression in HEp-2 cells after 60 min of infection. Total cellular RNA was isolated from HEp-2 cells cultured in medium (lane 1) or in the presence of *E. coli* HB101 (lane 2), *E. coli* HB101(pRI203) (lane 3), *L. monocytogenes* (Hly^+ Inv^+) (lane 4), *L. monocytogenes* (Hly^+ Inv^-) (lane 5), *Y. enterocolitica* (108C) (lane 6), or *Y. enterocolitica* (108P) (lane 7). Up to 20 μg of RNA was spotted onto GeneScreen Plus membranes. The row at the bottom shows the quantitation of the respective dot blots representing 20 μg of total RNA. Shown is an experiment representative of three performed, with similar results.

After infection of the epithelial HEp-2 cell line with *L. monocytogenes* (Hly^+ Inv^+) and *Y. enterocolitica* 108P, TNF-α in the cell supernatant was measured (Table 4). In the absence of a bacterial stimulus, the HEp-2 cells released up to 30 pg of IL-6 per ml after 120 min of incubation. This constitutive secretion was further enhanced after infection with the isogenic pairs of bacteria. The adhesion of the noninvasive *E. coli* HB101 strain induced an elevated IL-6 release, but, after invasion by *E. coli* HB101(pRI203), the IL-6 release increased to 235 pg/ml. This effect was also observed after infection with the two *L. monocytogenes* strains. The noninvasive mutant induced a lower rate of IL-6 secretion than the invasive *L. monocytogenes* strain. Infection of the cells with *Y. enterocolitica* 108C and 108P resulted in similar rates of secretion of IL-6 (Table 4). These data suggest that virulence factors encoded by the virulence plasmid (VP) do not influence the pattern of IL-6 secretion by HEp-2 cells.

IL-1β was not released from infected HEp-2 cells. This cytokine secretion profile was also obtained 4 h after infection. Prolonged incubation decreased the viability of the cells. Therefore, data for cytokine release presented in this study were obtained 2 and 4 h after infection.

In order to elucidate whether the pattern of IL-6 cytokine secretion by infected HEp-2 cells is accompanied with elevated cytoplasmic IL-6 mRNA levels, we performed IL-6 RNA dot blot analysis. Figure 1 shows a characteristic RNA dot blot probed with a radioactively labeled IL-6 cDNA fragment. After 60 min of incubation, HEp-2 cells expressed a detectable amount of IL-6 mRNA without any further cellular stimulation, demonstrating a constitutive IL-6 gene expression in HEp-2 cells (Fig. 1, lane 1). The infection of cultured HEp-2 cells with all three isogenic bacterial strain pairs increased the IL-6 mRNA level compared with that of HEp-2 cells cultured in medium alone (lanes 2 to 7). When the radioactivity of the RNA dot blots representing 20 μg of total RNA was counted, it was shown that HEp-2 cells expressed an increased IL-6 mRNA level after infection with the invasive *E. coli* or *L. monocytogenes* strain in comparison with the corresponding noninvasive isogenic bacterial strains (lanes 2 to 5).

Infection of HEp-2 cells with the VP-less *Y. enterocolitica* strain 108C induced a more pronounced IL-6 mRNA accumulation (lane 6) than infection with VP-harboring strain 108P (lane 7). However, no differences in the IL-6 secretion

TABLE 4. Secretion of cytokines by HEp-2 cells and PMNs[a]

Strain	PMN secretion of:			HEp-2 cell secretion of:	
	TNF-α	IL-6	IL-1β	TNF-α	IL-6
Control	123 ± 31	9 ± 4	10 ± 5	0	30 ± 23
L. monocytogenes					
NCTC 7973	528 ± 80	190 ± 18	277 ± 36	61 ± 12	110 ± 15
SLCC 5779	292 ± 73	107 ± 20	195 ± 25	ND[b]	83 ± 13
Y. enterocolitica					
108C (VP-less)	2,286 ± 381	213 ± 30	382 ± 51	ND	187 ± 23
108P (VP bearing)	1,508 ± 280	253 ± 24	267 ± 42	18 ± 4	198 ± 20
E. coli					
HB101	1,680 ± 243	208 ± 23	338 ± 50	ND	93 ± 21
HB101 pRI203	2,631 ± 371	307 ± 32	483 ± 45	ND	235 ± 11

[a] The incubation time was 2 h. Cytokine release by 10^7 cell per ml was analyzed by ELISA. Values are means ± standard errors of the means for three experiments and are expressed as picograms per milliliter.
[b] ND, not done.

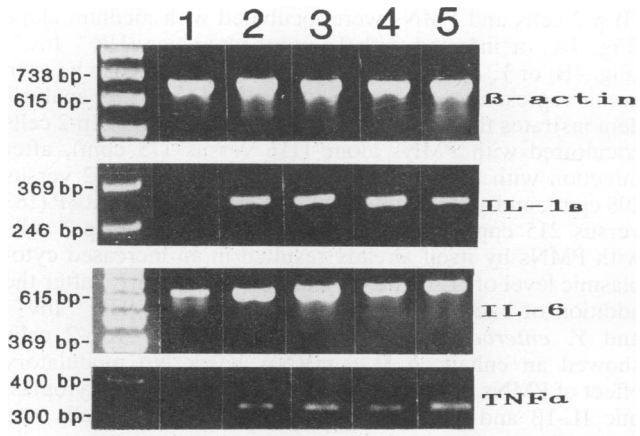

FIG. 2. PCR amplification of HEp-2 cells derived mRNA for β-actin, IL-1β, IL-6, and TNF-α. Total cellular RNA was reverse transcribed, and the resulting cDNAs were PCR amplified for 25 cycles. HEp-2 cells were incubated with medium (lane 1), *L. monocytogenes* (Hly^+ Inv^+) (lane 2), *L. monocytogenes* (Hly^+ Inv^-) (lane 3), *Y. enterocolitica* (108C) (lane 4), or *Y. enterocolitica* (108P) (lane 5). Shown is an experiment representative of three.

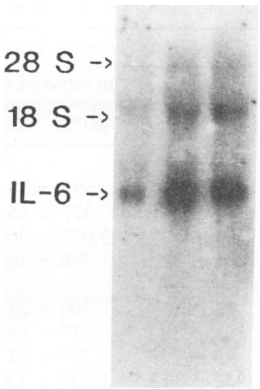

FIG. 3. Northern blot analysis of IL-6 mRNA in PMNs. Thirty micrograms of total cellular RNA was subjected to gel electrophoresis under denaturing conditions in the presence of formaldehyde. PMNs were stimulated with medium (lane 1), *L. monocytogenes* (Hly^+ Inv^+) (lane 2), or *Y. enterocolitica* (108P) (lane 3) for 120 min.

rates after infection with these two strains were seen (Table 4).

Because of the fact that both the invasive and noninvasive *E. coli* strains were able to enhance the IL-6 mRNA level, virulence factor invasiveness is not solely responsible for the increased IL-6 mRNA accumulation. Therefore, additional virulence factors of *L. monocytogenes* and *Y. enterocolitica* besides the *inv* and *ail* loci and p60 led to IL-6 mRNA accumulation.

We examined IL-1β, IL-6, and TNF-α gene expression by HEp-2 cells by PCR mRNA amplification. Figure 2 shows that the genes encoding IL-6 and IL-1β are constitutively expressed in HEp-2 cells (Fig. 2, lane 1). In contrast, TNF-α mRNA was not detected. After infection with the invasive and noninvasive strains of *L. monocytogenes* (lane 2 and 3) and *Y. enterocolitica* 108C (lane 4) or 108P (lane 5), an accumulation of mRNA encoding IL-1β, IL-6, and TNF-α was detected. The changes of the corresponding β-actin bands for every amplified RNA probe are minor in comparison to the observed accumulation of mRNA encoding IL-1β, IL-6, and TNF-α. This means that equal amounts of RNA were amplified and assessed by gel electrophoresis. Furthermore, the pronounced IL-6 gene expression after bacterial infection analyzed by PCR-amplified cDNA fragments was in good agreement with the results by the RNA dot blot technique (Fig. 1). Therefore, elevated TNF-α and IL-6 secretion was accompanied by elevated cytoplasmic levels of mRNA encoding these cytokines.

(ii) PMNs. It was shown that virulence factor invasiveness modulates cytokine expression by HEp-2 cells. Therefore, we investigated the influence of this virulence factor on cytokine expression by human PMNs.

After phagocytosis of the above-mentioned bacterial strains (Table 3), we observed that PMNs secreted TNF-α, IL-6, and IL-1β (Table 4). The noninvasive *L. monocytogenes* strain (SLCC 5779) induced a lower release of TNF-α, IL-6, and IL-1β than the invasive *L. monocytogenes* strain (NCTC 7973). In addition, the invasive *E. coli* strain, HB101(pRI203), induced an elevated TNF-α, IL-6, and IL-1β release from PMNs in comparison to the noninvasive *E. coli* strain. Thus, as was observed with epithelial cells, the individual virulence factors which encode invasiveness triggered an increased cytokine release in granulocytes. The noninvasive *L. monocytogenes* strain tends to form cell chains and becomes more readily phagocytosed by PMNs (Table 3), but smaller amounts of cytokines were released after phagocytosis compared with the amounts released in response to the invasive *L. monocytogenes* strain. Therefore, invasin receptor-mediated phagocytosis (uptake) is carried out differently from the unspecific phagocytosis of bacterial aggregates.

The uptake of the two invasive *Y. enterocolitica* strains led to differences in the release of TNF-α and IL-1β. As is shown, VP-bearing *Y. enterocolitica* strain 108P led to a reduced cytokine release (Table 4). Therefore, the individual virulence factors which encode invasiveness are not solely responsible for cytokine gene expression in granulocytes. Additional virulence factors, e.g., outer membrane components and adhesion and secretory factors specific for the individual bacteria, may be responsible for the differences in TNF-α and IL-1β release from PMNs. As was shown for HEp-2 cells, IL-6 release was not modulated by determinants encoded by the VP.

Within 1 h after uptake of the bacterial strains, the TNF-α, IL-6, and IL-1β mRNA levels of PMNs were increased as assessed by PCR (data not shown). The cytokine mRNA levels remained elevated for up to 5 h. After this time period, the cell viability and therefore the cytoplasmic mRNA amounts decreased. An IL-6 Northern blot analysis of total cellular RNA isolated from human PMNs (Fig. 3) is shown. A significant signal is apparent. The constitutive IL-6 mRNA level in uninfected PMNs may be a result of cell adherence to the plastic wells and resulted in a constitutive IL-6 release of 10 pg/ml (Table 4). The size of the IL-6 gene transcripts was in agreement with published results (21) demonstrating a specific hybridization of IL-6 cDNA in the RNA dot blot studies.

TNF-α, IL-6, and IL-1β release from cocultured HEp-2 cells and PMNs after bacterial infection. In order to determine whether cocultured PMNs and HEp-2 cells show different releases of cytokines, we performed coculture experiments for 2 and 4 h with Transwell chambers. We analyzed the TNF-α, IL-6, and IL-1β secretions of the cocultures and compared the results with the cytokine amounts released in the control experiments.

TABLE 5. Secretion of cytokines by PMNs and HEp-2 cells[a]

Cytokine, expt type, and incubation time	Amt (pg/ml) of cytokine secreted:		
	In medium	In response to *L. monocytogenes* NCTC 7973	In response to *Y. enterocolitica* 108P
TNF-α			
Control, 2 h	123 ± 31	589 ± 78	1,526 ± 284
Coincubation, 2 h	193 ± 24	427 ± 65	768 ± 94
Control, 4 h	194 ± 19	3,095 ± 365	4,158 ± 401
Coincubation, 4 h	362 ± 71	786 ± 163	1,026 ± 205
IL-6			
Control, 2 h	32 ± 9	300 ± 21	450 ± 30
Coincubation, 2 h	362 ± 28	637 ± 58	707 ± 47
Control, 4 h	92 ± 22	1,205 ± 81	941 ± 42
Coincubation, 4 h	942 ± 113	1,603 ± 139	1,412 ± 93
IL-1β			
Control, 2 h	10 ± 9	277 ± 36	267 ± 42
Coincubation, 2 h	119 ± 48	373 ± 76	447 ± 81
Control, 4 h	55 ± 20	1,185 ± 287	1,833 ± 252
Coincubation, 4 h	955 ± 154	1,877 ± 360	2,433 ± 351

[a] PMNs and HEp-2 cells were cocultured in Transwell chambers. Cytokine release was assessed by ELISA. Values are means ± standard errors of the means for three experiments.

Table 5 shows the cytokine release in these coculture experiments. As can be seen, coincubation of PMNs and HEp-2 cells led to an increased IL-6 and IL-1β secretion. This increased cytokine release was also shown in coculture experiments performed without any further bacterial stimulation. For TNF-α release, a different pattern was observed. The release was enhanced in coculture experiments without bacterial stimulation and decreased in coculture experiments in which HEp-2 cells and PMNs were simultaneously infected with either *L. monocytogenes* (Hly^+ Inv^+) or *Y. enterocolitica* 108P (Table 5).

We wished to analyze whether the increased IL-6 secretion was accompanied by elevated IL-6 mRNA levels in HEp-2 cells. Therefore, we performed RNA dot blot studies with cocultured HEp-2 cells. Figure 4 shows the cytoplasmic level of IL-6 mRNA of HEp-2 cells cocultured with PMNs.

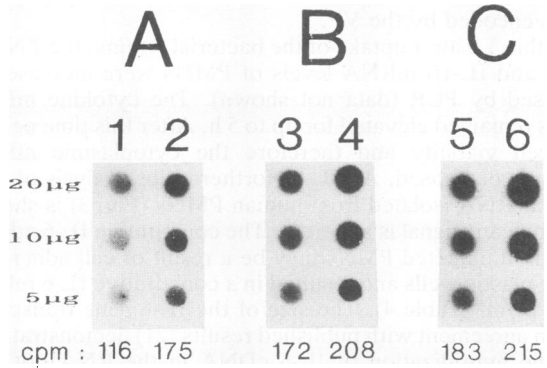

FIG. 4. RNA dot blot analysis of IL-6 mRNA of HEp-2 cells cocultured with PMNs in Transwell coculture chambers for 60 min. Both HEp-2 cells and PMNs were incubated with medium (lanes 1 and 2), *L. monocytogenes* (Hly^+ Inv^+) (lanes 3 and 4), or *Y. enterocolitica* (108P) (lanes 5 and 6) in each chamber compartment. HEp-2 cells were cocultured with PMNs (lanes 2, 4, and 6) or incubated alone (lanes 1, 3, and 5). The autoradiographs were exposed for 4 (A), 3 (B), or 5 (C) days. The row at the bottom shows the quantitation of the respective dot blots representing a total RNA amount of 20 μg.

HEp-2 cells and PMNs were incubated with medium alone (Fig. 4A) or infected with *L. monocytogenes* (Hly^+ Inv^+) (Fig. 4B) or *Y. enterocolitica* 108P (Fig. 4C). As can be seen in this representative experiment, the RNA dot blot analysis demonstrates the elevated IL-6 mRNA levels of HEp-2 cells cocultured with PMNs alone (116 versus 175 cpm), after infection with *L. monocytogenes* (Hly^+ Inv^+) (172 versus 208 cpm), and after infection with *Y. enterocolitica* 108P (183 versus 215 cpm). Therefore, coincubation of HEp-2 cells with PMNs by itself already resulted in an increased cytoplasmic level of IL-6 mRNA expression. Similarly, after the addition of bacteria, e.g., *L. monocytogenes* (Hly^+ Inv^+) and *Y. enterocolitica* 108P, the cocultured HEp-2 cells showed an enhanced IL-6 mRNA level. No modulatory effect of PMNs cocultured with HEp-2 cells on the cytoplasmic IL-1β and TNF-α mRNA levels of HEp-2 cells was detected.

DISCUSSION

The results presented show that the facultatively intracellular bacteria *L. monocytogenes* and *Y. enterocolitica* induce a cytoplasmic mRNA accumulation and secretion of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in PMNs.

Epithelial (HEp-2) cells infected with *L. monocytogenes* or *Y. enterocolitica* accumulate mRNA encoding TNF-α, IL-1β, and IL-6 and release TNF-α and IL-6 into the cell supernatant. No IL-1β release was observed.

The fact that epidermal cells secrete not only cytokines which regulate growth and differentiation, such as granulocyte macrophage colony-stimulating factor, but also proinflammatory cytokines, e.g., IL-8 and IL-6 (8, 26), suggests that epithelial-cell-derived cytokines are obviously important for the regulation of inflammatory cell recruitment and activation. The cytokines TNF-α and IL-1 activate PMNs (27, 45) and as a consequence of the induced IL-8 release are chemotactic for PMNs (36). The migration of PMNs through fibroblast layers (39), which is highly dependent on the function of CD11b/CD18 (MAC-1) as was reported for the migration across intestinal epithelia (42), is further mediated by TNF-α and IL-1β.

Therefore, with respect to our in vitro data, one may suggest that epithelial cells of the gastrointestinal tract infected with *L. monocytogenes* or *Y. enterocolitica* have the ability to recruit and activate PMNs by the release of proinflammatory cytokines. It appears that the induction of mRNA accumulation and secretion from HEp-2 cells is obviously not solely dependent on the penetration step (25, 32). Additional virulence factors which trigger the increased accumulation of IL-1β, IL-6, and TNF-α mRNA after stimulation with *Listeria* spp. (10, 16, 37) and *Yersinia* spp. (3, 15, 19, 43) must exist.

It is well documented that the presence of systemic TNF-α and IL-6 plays an important and protective role during murine listeriosis (17, 40, 41), and Iizawa et al. reported the cytokine mRNA response in the spleen of *L. monocytogenes*-infected mice during the first few hours of infection (23). They showed by qualitative PCR analysis that mRNA encoding TNF-α, IL-1β, and IL-6 accumulated during the first 4 h after infection with viable listeriae. Recently, it was shown that PMNs are the dominant effector cells during the early nonspecific phase of murine listeriosis in the liver and spleen (30) and that PMNs are able to synthesize IL-1, IL-6, and TNF-α after phagocytosis of different pathogens (2, 7, 9, 11, 34).

In this study, we demonstrated the effect of invasive bacteria, e.g., *L. monocytogenes* and *Y. enterocolitica*, on cytokine synthesis and secretion by PMNs. Thus, PMNs are not only endstage effector cells but they are also able to synthesize and secrete proinflammatory cytokines, e.g., TNF-α, IL-1β, and IL-6, after uptake of *L. monocytogenes* or *Y. enterocolitica*. The fact that human PMNs are stimulated by these intracellular bacteria for cytokine release suggests that PMNs recruited to the sites of infection (liver, spleen, gastrointestinal tract) modulate the inflammatory and immune response in vivo during the onset of infection.

In addition, our data obtained from coculture experiments confirmed that epithelial cells (HEp-2) and PMNs regulate their cytokine expression via paracrine cell communication.

Epithelial cells cocultured with PMNs expressed increased cytoplasmic levels of IL-6 mRNA resulting in an elevated IL-6 secretion. Furthermore, the IL-1β release of cocultured PMNs is further enhanced. Since HEp-2 cells (i) did not release IL-1β into the cell supernatant after bacterial infection, perhaps because of the tumor cell status, and (ii) release only small amounts of TNF-α, it appears that soluble factors released from HEp-2 cells are responsible for the increased IL-1β and diminished TNF-α release by infected PMNs.

The increased TNF-α release by cocultured PMNs incubated in medium without bacterial infection suggests that an altered pattern of soluble factors, e.g., cytokines or soluble cytokine receptors, is spontaneously secreted from HEp-2 cells, which then results in an elevated TNF-α release from PMNs.

Conclusively, our coculture studies suggest that the redundancy of the cytokine release often observed in in vitro experiments may be under the control of a paracrine regulation network. Obviously HEp-2 cells behave differently from gastrointestinal tract epithelial cells. The model, however, provides a means to investigate the modulatory effect of cell-cell interaction via direct cell-cell contact or soluble mediators. It is evident that the model system may be useful in interpreting pathophysiological changes occurring during infection in vivo.

Further studies are needed to determine the soluble cellular factors and the microbial virulence components of *Listeria* spp. and *Yersinia* spp., besides the virulence factor invasiveness, which are involved in cytokine expression, secretion, and modulation.

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