

Leishmania major: Promastigotes Induce Expression of a Subset of Chemokine Genes in Murine Macrophages

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RACOOSIN, E. L., AND BEVERLEY, S. M. 1997. *Leishmania major*: Promastigotes induce expression of a subset of chemokine genes in murine macrophages. *Experimental Parasitology* **85**, 283–295. Recent studies suggest that *Leishmania major* promastigotes infect cultured macrophages in a stealthy fashion, activating little or no host gene expression and often interfering with the host's ability to respond to further stimulation. Here we examined macrophage transcription at early times following infection, when virulent parasites must execute steps required for survival. Stationary-phase promastigotes induced rapid and transient expression of transcripts of the chemokines JE (human MCAF/MCP-1) and KC (human GRO) in bone marrow-derived macrophages from BALB/c mice. JE and KC expression rose four- to sixfold shortly after infection and returned to uninduced levels by 4–24 hr. In contrast, chemokines MIP-1 α , C10, and RANTES were not induced, nor were TGF- β , IL-10, IL-12, or i-NOS. Chemokine induction did not occur following ingestion of latex beads, implicating a parasite-specific stimulus. Elevated expression of a subset of chemokines is the earliest known transcriptional response of macrophages to *L. major* infection and potentially may provide a signal for the initiation of downstream immunological responses which occur *in vivo*, such as cytokine induction and chemotaxis of monocytes and macrophages. Thus, *Leishmania* has a remarkable ability to take an active role in either inducing or preventing the expression of distinct sets of host genes during macrophage invasion and successful intracellular parasitism. © 1997 Academic Press

INDEX DESCRIPTORS AND ABBREVIATIONS: *Leishmania*; chemokines; MCP-1, monocyte chemoattractant protein-1; FBS, fetal bovine serum; BMM, bone marrow-derived macrophages; M-CSF, macrophage colony-stimulating factor; MIP, macrophage inflammatory protein; nt, nucleotide; iNOS, inducible nitric oxide synthase; LPG, lipophosphoglycan.

INTRODUCTION

Virulent metacyclic promastigotes of *Leishmania* are introduced into host tissues by the bite of an infected phlebotomine sand fly and are subsequently phagocytosed by tissue macrophages, where differentiation into the amastigote stage occurs. In order to survive within the host, *Leishmania* promastigotes express several abundant surface molecules, including a glycolipid termed lipophosphoglycan (LPG) and a surface protease, gp63 (Chang and Chaudhuri 1990; Turco and Descoteaux 1992). LPG, for example, participates in binding to the macro-

phage and phagocytosis and protects the parasite from lysis by complement, damage by reactive oxygen intermediates, and hydrolytic enzymes of the macrophage phagolysosome (Chen *et al.* 1989; Cooper *et al.* 1988; daSilva *et al.* 1989; Eilam *et al.* 1985; Talamas-Rohana *et al.* 1990).

Leishmania infection may also affect the pattern of gene expression in the macrophage. The effects reported vary greatly among different *Leishmania* species, source of macrophages, and laboratories. Many workers have found that, unlike most other pathogens, there is surprisingly little induction of macrophage gene expression immediately following infection with *Leishmania major* promastigotes *in vitro*. This included studies of IL-1, IL-10, IL-12, TNF- α , i-NOS, MIP-1 α , and TGF- β (Carrera *et al.* 1996; Reiner *et al.* 1994). Yet this

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“stealthy” mode of invasion may be misleading, as *Leishmania* infection actively interferes with the macrophage’s ability to respond to other immunological stimuli, such as LPS or other antigens. Bone marrow-derived macrophages (BMM) infected with *L. major* were defective in LPS-induced expression of IL-12 (Carrera *et al.* 1996), and macrophages infected with *L. donovani* were defective in LPS-induced *c-fos* and TNF- α gene expression (Descoteaux and Matlashewski 1989) and interferon- γ induced MHC class II antigen expression (Reiner *et al.* 1988). In some instances, gene expression in infected macrophages did not commence until 24 hr after infection. For example, in peritoneal macrophages isolated from BALB/c mice, expression of IL-1 was stimulated by *L. major* infection (Cillari *et al.* 1989) and expression of TGF- β was stimulated by *L. amazonensis* and *L. braziliensis* (Barral *et al.* 1993; Barral-Netto *et al.* 1992). In BMM, *L. donovani* promastigotes induced expression of GM-CSF, TNF- α , TGF- β , and IL-6 (Moore and Matlashewski 1994). Significantly, all of these effects on macrophage gene expression could affect the ability of the *Leishmania* to establish infection and induce host T-cell responses during infections *in vivo* (Reiner *et al.* 1994; Scharfen and Scott 1993).

Many studies of the effects of *Leishmania* on host gene expression examined periods well after macrophage invasion (usually more than 24 hr). However, here we showed that virulent *L. major* must successfully execute steps required for establishment of infection within 24 hr. This suggested that it would be important to examine macrophage responses during this critical period, especially for genes expressed only transiently. We used Northern blot analysis to follow gene expression at early times after infection and additionally compared the expression induced by avirulent vs. virulent *L. major* strains, since this could potentially assist in identifying key host pathways intercepted by virulent parasites. Significantly, we found that *Leishmania* induces rapid and transient expression of only a subset of macrophage chemo-

kines, specifically JE and KC (the murine homologs of human MCAF/MCP-1 and GRO, respectively). In addition, our results confirmed and extended studies showing that macrophages infected *in vitro* do not express a variety of genes shown under other circumstances to be involved in the host response to *Leishmania* infection (Carrera *et al.* 1996; Reiner *et al.* 1994).

MATERIALS AND METHODS

Macrophage culture. Murine BMM were obtained from bone marrow extruded from the femurs of female BALB/c mice (National Institute of Health, National Cancer Institute, Frederick, MD) as described (Racoosin and Swanson 1989). BMM were cultured in DME-10F, which is Dulbecco’s modified Eagle’s medium (DMEM; GIBCO BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT), and 100 units/ml penicillin and 100 μ g/ml streptomycin (GIBCO BRL). To stimulate BMM growth, DME-10F was further supplemented with 30% L929 fibroblast-conditioned medium, which contains macrophage-CSF. Twenty-four hours before infections, BMM were replated in either 100- or 60-mm tissue culture dishes (Falcon, Franklin Lakes, NJ) containing DME-10F but lacking L929-conditioned medium. To monitor *Leishmania* infection, dishes often contained one 12-mm-diameter, No. 1 glass coverslip (Fisher Scientific, Pittsburgh, PA) that had been washed in 95% ethanol and double-distilled water and autoclaved previously. RAW 264.7 cells (American Type Culture Collection; Rockville, MD) were grown in DME-10F. L929 fibroblasts (ATCC) were grown in MEM (GIBCO-BRL) + 10% FBS. All solutions and media used in growing murine macrophages were endotoxin-free, as reported by the manufacturers.

Leishmania strains and culture. The *L. major* strains Friedlin V1 (FV1), Friedlin A1 (FA1; (daSilva and Sacks 1987)), LV39 clone 5 (Lc5), and LV39 clone 79 (Lc79; (Marchand *et al.* 1987)) were maintained *in vitro* as promastigotes in M199 medium as described (Kapler *et al.* 1990). Promastigote cultures were routinely passaged while in the logarithmic phase of growth. Virulent strains were passaged periodically through BALB/c mice and were passaged no more than 10 times *in vitro*.

Infection of macrophages. Promastigotes in the stationary phase of growth were used to infect BMM at a final ratio of 10 parasites per macrophage. Parasites were pelleted at 500g in a SS-34 rotor (Sorvall) for 15 min at room temperature and then resuspended in DMEM containing 4% complement fragment 5-deficient serum obtained from strain B10.D2/oSnJ female mice (Jackson Laboratory, Bar Harbor, ME) at a concentration of 10^7 – 10^8 parasites/ml for 15 min at 37°C. Opsonization with complement has been shown to enhance phagocytosis of metacyclic promastigotes

(daSilva *et al.* 1989; Mosser and Edelson 1987). Parasites were then washed twice in DMEM alone and resuspended in DME-10F warmed to 37°C. Immediately prior to addition of parasites, the macrophages were washed once with DME-10F. To initiate infection, promastigotes were added to 3.6×10^6 or 10^7 BMM in 60- or 100-mm dishes, respectively. The 100-mm dishes were used in experiments with two time points, and 60-mm dishes were used for larger experiments. Where indicated, 6×10^6 4- μ m-diameter washed, sterile latex beads (Polysciences, Warrington, PA) were added to 3.6×10^6 macrophages plated in 60-mm dishes. After addition of parasites or beads, the macrophages were incubated at 37°C in 95% air, 5% CO₂. At times indicated, the glass coverslip was removed and stained with Diff-Quik (Baxter Scientific, McGaw Park, IL) to monitor the infection. The remaining cells were taken for RNA analysis. For infections longer than 2 hr, infected macrophages were washed three times with DME-10F to remove noninternalized parasites or beads, and fresh medium was added.

Controls for macrophage RNA transcripts. 10^7 BMM or RAW 264.7 cells plated in 100-mm dishes were treated with either 100 ng/ml lipopolysaccharide (LPS, *Escherichia coli* serotype 0127:B8 (SIGMA) or 100 ng/ml LPS + 500 units murine interferon- γ (Genzyme, Cambridge, MA). Total RNA from L929 fibroblasts and/or BMM treated with DME-10F containing 30% L929-conditioned medium containing macrophage-CSF (M-CSF) were used as negative controls.

Northern blots. Total RNA was extracted using 1 or 2 ml RNazol (Biotech, Houston, TX) for a 60- or 100-mm tissue culture dish, respectively, according to the manufacturer's instructions. RNA resuspended in diethyl pyrocarbonate-treated water was quantitated by UV absorbance and staining following gel separation. Samples containing 10 μ g total RNA were glyoxylated and electrophoresed at 4 V/cm on a 10 mM phosphate agarose gel, pH 7.0, with constant buffer circulation (Brown 1994). RNA was transferred using the Turbo Blot kit (Schleicher & Schuell, Keene, NH) onto Hybond N nylon membranes (Amersham, Arlington Heights, IL). Membranes were baked 2 hr at 80°C and then deglyoxylated by boiling 30 min in 20 mM Tris-HCl, pH 8.0. Prehybridization was performed at 67°C for 2 hr in $6 \times$ SSC ($1 \times$ SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 0.1–0.5% SDS, 200 μ g/ml denatured salmon sperm DNA, 0.04% Ficoll 400, 0.04% polyvinylpyrrolidone, and 0.04% bovine serum albumin (Brown 1994). For hybridization, denatured double-stranded cDNA probes (see below) labeled with [α -³²P]dCTP (Amersham) by the random primer method (Feinberg and Vogelstein 1983) were added directly to prehybridized membranes and incubated for at least 18 hr. Membranes were then washed at room temperature in $2 \times$ SSC + 0.5% SDS, then at 67°C in $0.1 \times$ SSC + 0.5% SDS, and were exposed first to X-LS or X-AR film (Kodak, Rochester, NY) and then to a phosphorimager screen (Molecular Dynamics, Sunnyvale, CA).

Quantitation of RNA levels was performed using a Molecular Dynamics Phosphorimager equipped with the Image Quant 3.2 program, using β -actin hybridization as an internal control.

cDNA probes. Probes used for Northern hybridization were as follows: murine KC cDNA (Oquendo *et al.* 1989), murine JE cDNA (Rollins *et al.* 1988), murine *i*-NOS (Xie *et al.* 1992), murine IL-1 β (Gray *et al.* 1986), rat TGF- β 1 (Qian *et al.* 1990), murine C10 (Orlowsky *et al.* 1994), murine RANTES (Heeger *et al.* 1992), and murine IL-10 (Moore *et al.* 1990). cDNA probes for murine actin and TNF- α were generated by polymerase chain reaction (PCR) using primer pairs purchased from Stratagene (San Diego, CA) and cDNA template prepared as described (Reiner *et al.* 1993) from total RNA prepared from BMM treated with LPS for 6 hr. cDNA probes for murine IL-12 p40 mRNA and MIP-1 α were generated by PCR using cDNA template from BMM treated with LPS and IFN- γ for 6 hr and the following primer pairs: for IL-12, (5') cgggatccaccATGTGTCCTCAGAAGCTAACCC (SMB 85) and (5') cgggatcCTAGGATCGGACCTGCAGGGAA (SMB 86) to generate a fragment of 1050 nt (Schoenhaut *et al.* 1992); for MIP-1 α , (5') GCCCTTGCTGTTCTTCTCTGT and (5') GGCAATAGTTCAGGTCAGT to generate a fragment of 300 nt (Davatelis *et al.* 1988). Lowercase letters denote nucleotides not present in the gene.

RESULTS

Macrophages phagocytose comparable numbers of avirulent and virulent L. major. We first compared the ability of BMM to phagocytose different strains of *L. major*. The virulent strains, Friedlin V1 (FV1) and LV39 clone 5 (Lc5), previously have been shown to replicate in macrophages *in vitro* and rapidly induce lesion formation following injection into BALB/c mouse footpads (daSilva and Sacks 1987; Shankar *et al.* 1993). In contrast, the avirulent Friedlin-derived clone A1 (FA1) derived by long-term passage *in vitro* does not form metacyclic promastigotes and cannot infect mice (daSilva and Sacks 1987). The attenuated LV39-derived clone 79 (Lc79) derived by passage and mutagenesis is similarly avirulent, although infections eventually occur after 6 months due to reversion to virulence *in vivo* (Shankar *et al.* 1993).

BMM were infected with opsonized stationary-phase promastigotes from these four strains, and the numbers of intracellular parasites determined after 1 or 24 hr. At 1 hr, 83–95% of the

TABLE I
Murine Bone Marrow-Derived Macrophages Are Infected Equally by Avirulent and Virulent *L. major* Promastigotes

Time	<i>L. major</i> strain			
	FA1	FV1	Lc79	Lc5
1 hr				
Percentage macrophages infected ^a	86%	83%	95%	92%
Parasites/macrophage	3.0	2.8	3.7	3.4
24 hr				
Percentage macrophages infected ^b	2%	57%	23%	67%
Amastigotes/macrophage	0.3	1.2	0.5	2.2

Note. Data from one representative experiment of three total are shown.

^a The ranges for the three experiments were FA1, 86–95%; FV1, 76–87%; Lc79, 88–98%; Lc5, 86–97%.

^b For the 24-hr time point, the percentage of cells infected and the number of parasites per macrophage were based only on amastigotes; promastigotes (if present) were not counted. The ranges of percent cells infected for the three experiments were FA1, 0–2%; FV1, 55–60%; Lc79, 19–23%; Lc5, 65–72%.

macrophages contained 2.8–3.7 promastigotes, regardless of the infecting strain (Table I). After 24 hr of infection, parasites from the virulent FV1 and Lc5 strains differentiated into amastigotes and survived in 57–67% of the BMM, whereas only 23% of the macrophages contained Lc79 parasites and 2% contained FA1 parasites. Similar results were obtained in two other independent experiments not shown. Thus, initially both virulent and avirulent parasites are rapidly ingested by macrophages, but within 24 hr avirulent strains are destroyed. This suggests that during this interval virulent parasites must execute steps critical to survival.

Macrophages infected with L. major promastigotes exhibit rapid transient induction of chemokine gene expression. BMM were incubated for increasing times in medium alone or in medium containing opsonized stationary-phase promastigotes. Subsequently, total RNAs extracted from uninfected and infected macrophages from the same experiment were subjected to Northern blot analysis with a number of cDNA probes. Hybridization of the same filters with a β -actin probe was used as a control.

A comparison of cDNA probe hybridization to RNA from uninfected and infected macrophages showed that only JE and KC transcripts were expressed at significantly higher levels following *Leishmania* invasion of BMM. This is shown in Fig. 1 for the Lc5 and Lc79 lines. In this experiment, expression of both chemokines was detectable by 45 min postinfection and was maximal at 60 and 120 min for JE and KC, respectively. For JE, the maximal stimulation was 4-fold for Lc5 and 12-fold for Lc79, and expression returned to baseline levels by 3 hr (Figs. 1 and 2). For KC, maximal stimulation was 12-fold for Lc5 and 30-fold for Lc79 and returned to baseline values by 4 hr (Figs. 1 and 2). These studies were repeated numerous times with both of the LV39 strains as well as the virulent FV1 and avirulent FA1 *L. major* strains (Northern blots not shown; Table II). On average, the magnitude of JE and KC expression was higher with the avirulent than virulent strains (Table II). For the LV39 lines, peak expression of both JE and KC transcripts was about 2–3 times higher following infection with the avirulent clone Lc79 than with virulent Lc5 (Figs. 1B and 1C).

We observed considerable variability in the magnitude of the induction among experiments with all strains, a finding also evident in the JE and IL-10 data of Carrera *et al.* (1996). This was apparent in comparisons between individual experiments, with some showing generally low and some showing generally high induction of both genes with all parasite lines tested. We attribute this to differences in BMM, which are primary cell cultures, or to differences among parasite preparations. Nonetheless, in each experiment, there was greater induction of JE and KC expression in infected BMM than in uninfected BMM.

In order to determine if chemokine induction was a general phenomenon following *Leishmania* infection, we examined the expression of several other members of the β -chemokine family. MIP-1 α was expressed at a low constitutive level but was not elevated by *Leishmania* infection of BMM (Figs. 1A, 2A, and 2F). Expression of murine RANTES was undetectable except in the control, LPS-treated macrophages

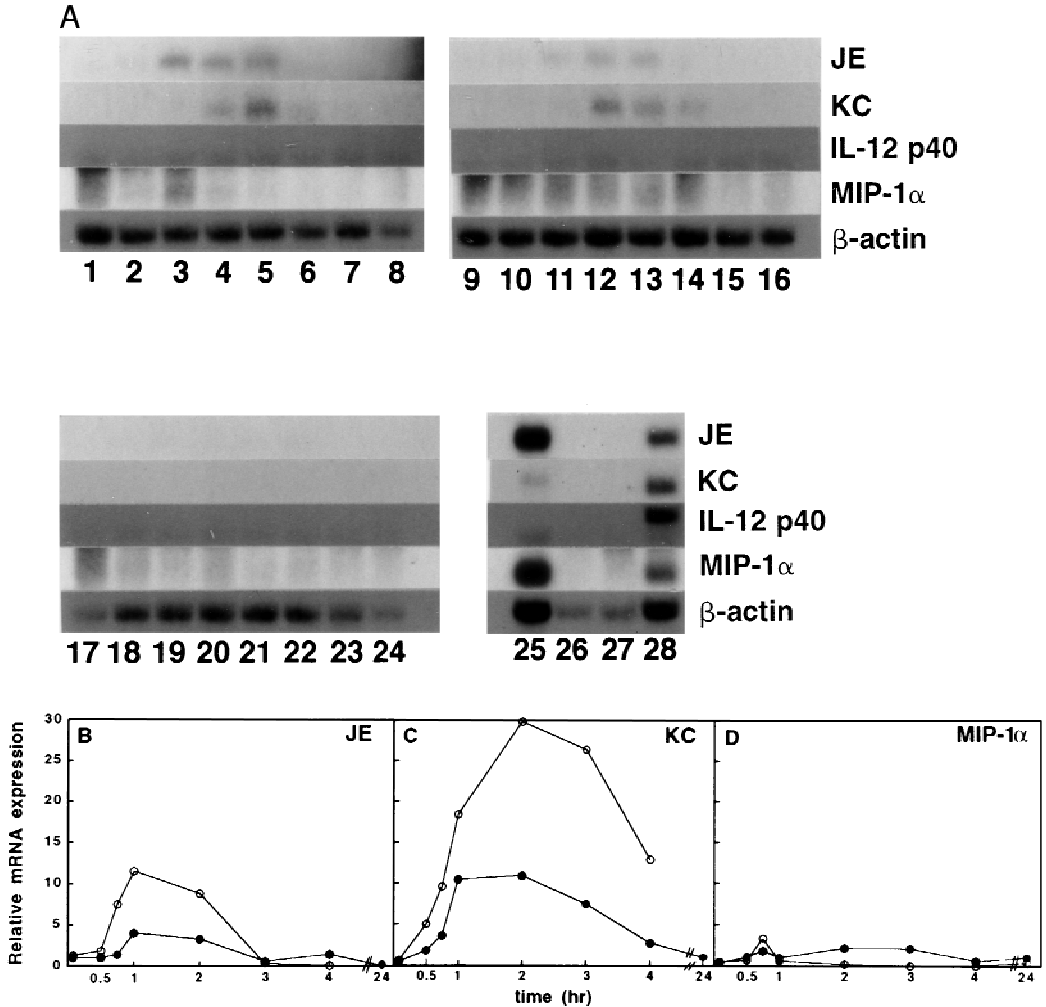


FIG. 1. Macrophages infected with stationary-phase promastigotes transiently induce JE and KC chemokine gene expression. (A) BMM were incubated for 5 min and 0.5, 0.75, 1, 2, 3, 4, and 24 hr with Lc79 promastigotes (lanes 1–8), Lc5 promastigotes (lanes 9–16), or with medium alone (lanes 17–24). Total RNA was isolated and subjected to successive Northern blot analysis with cDNA probes indicated to right of each row; the autoradiogram is shown. Controls for RNA expression were: RAW 264.7 cells + LPS for 6 hr (lane 25); BMM + M-CSF (lane 26); L929 fibroblasts (lane 27); and BMM + LPS + IFN- γ for 6 hr (lane 28). (B–D) Data from the Northern blots shown in A were used to calculate the relative expression of JE (B), KC (C), and MIP-1 α (D) to that of β -actin, for Lc79-infected BMM (open circles) and Lc5-infected BMM (closed circles). These ratios then were normalized to that of uninfected BMM and this value is plotted. A second experiment gave similar results (not shown).

(Fig. 3B). C10 was constitutively expressed in both infected and uninfected BMM (Fig. 3B). These data show that *Leishmania* induced only a subset of chemokine genes.

As noted in the introduction, other investigators have shown that *L. major* infection of mac-

rophages *in vitro* rarely results in induction of macrophage genes (Carrera *et al.* 1996; Reiner *et al.* 1994). We sought to confirm these findings by infecting BMM with different virulent and avirulent *Leishmania* strains for different periods up to 24 hr postinfection and then ana-

TABLE II
Average Increase in JE or KC Expression in Infected BMM 1 hr Postinfection

	<i>L. major</i> strain			
	FA1	FV1	Lc79	Lc5
JE				
Mean \pm standard error ^a	2.1 \pm 0.7	1.8 \pm 0.7	4.0 \pm 1.3	1.9 \pm 0.5
Maximum ^b	5.3	5.0	11.4	4.0
Minimum ^c	0.3	0.5	0.4	0.7
<i>N</i> experiments	6	6	8	7
Avirulent/virulent ^d	4/6		4/7	
KC				
Mean \pm standard error	3.1 \pm 0.6	2.9 \pm 1.3	6.0 \pm 3.1	2.1 \pm 0.6
Maximum	4.3	5.5	18	3.6
Minimum	2.2	1.3	1.2	1.0
<i>N</i> experiments	3	3	5	4
Avirulent/virulent	2/3		3/4	

^a Means were calculated from the cultures infected after 1 hr and normalized to uninfected macrophages incubated similarly in the same experiment.

^b The maximum ratio value of *N* experiments.

^c The minimum ratio value of *N* experiments.

^d The fraction of experiments in which the ratio of gene expression was greater for avirulent strains than for virulent strains. FA1 was compared with FV1, and Lc79 was compared with Lc5.

lyzing the expression of IL-10, IL-12 (p40), TNF- α , TGF- β , and i-NOS. An example of these results is shown in Fig. 2A with Lc79-infected BMM (which showed the greatest induction of the chemokines) and i-NOS, TNF- α , or IL-10 and in Fig. 3A with all four *Leishmania* strains and the TGF- β probe. No significant induction of genes other than the chemokines JE and KC was observed (Figs. 2 and 3; data not shown). In contrast, all genes tested were induced by treatment of uninfected BMM with either LPS + IFN- γ or LPS alone (Fig. 2A, lanes 9 and 10).

Several lines of evidence suggest that the effects on gene expression observed here cannot be attributed to trace contamination by LPS. First, LPS stimulated greater, more sustained JE expression than *L. major* promastigotes (Figs. 2A, lanes 9 and 10). Second, macrophages treated with 100 ng/ml LPS expressed the RANTES (Fig. 3), i-NOS, TNF- α (Fig. 2), and IL-1 β (data not shown) genes at high levels, whereas the *L. major*-infected BMM expressed only TNF- α at a low constitutive level (Fig. 2).

In addition, C10 expression is not induced by LPS (Orlofsky *et al.* 1994), yet this gene was expressed constitutively in uninfected and infected macrophages (Fig. 3). Thus, the macrophage response to *Leishmania* infection differs considerably from its response to LPS.

Recent studies showed that GRO, the human homolog of the murine KC gene, and MAD-5, a gene showing homology to human MCP-1 and MIP-1 α , were activated in human monocytes following attachment to tissue culture plastic coated with various types of extracellular matrix (Sporn *et al.* 1990). This raised the possibility that chemokine induction observed in this study might reflect the general process of macrophage attachment and phagocytosis, rather than a specific stimulus provided by *Leishmania*. Thus, we investigated if phagocytosis of latex beads similar in size to promastigotes would provide a stimulus for JE and KC expression. BMM were incubated with medium containing 4- μ m-diameter latex beads for 1 or 24 hr, and KC and JE expression was probed by Northern blot analysis (Fig. 2A, lanes 11 and 12; data not

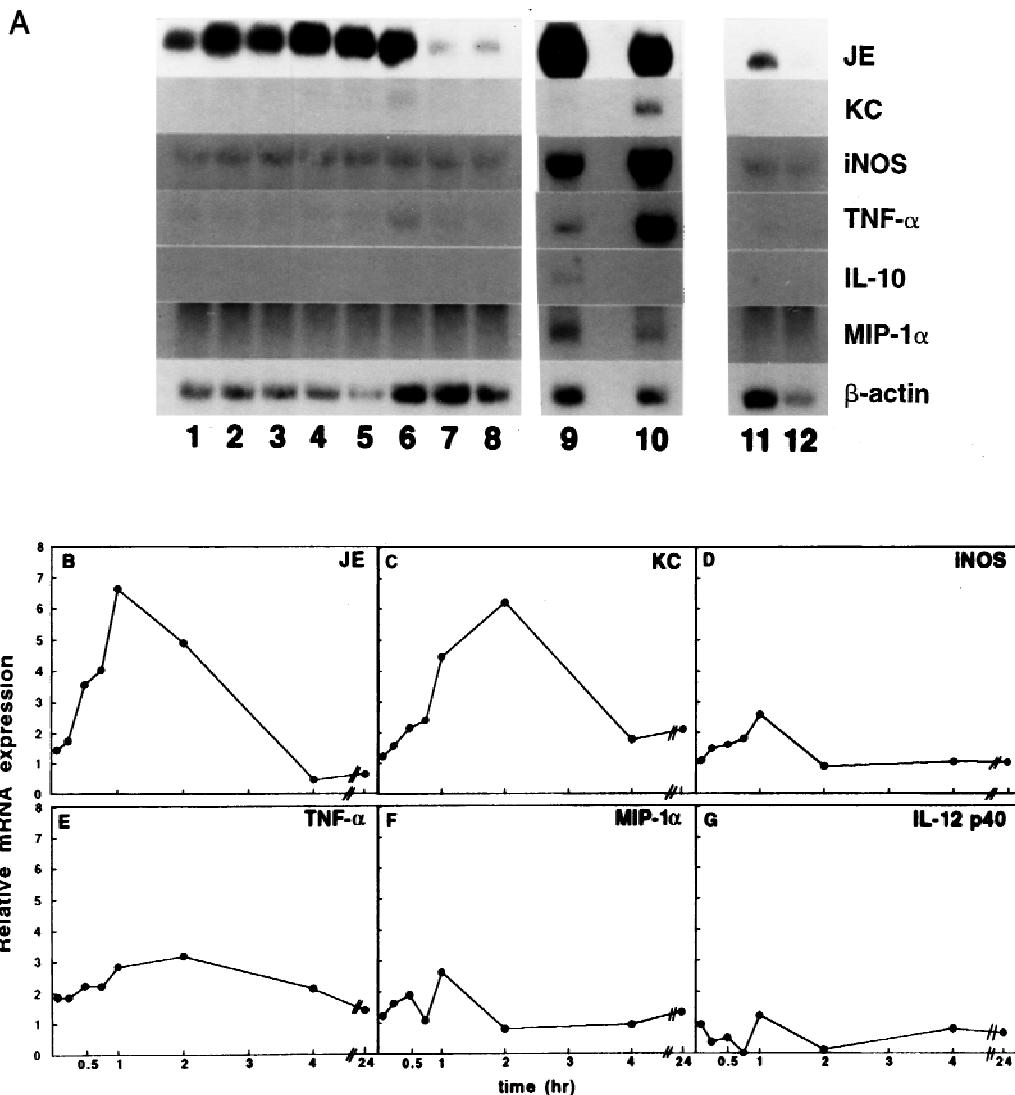


FIG. 2. BMM infected with Lc79 promastigotes induce expression of JE and KC but not other macrophage genes. (A) BMM were incubated in medium alone (not shown) or in medium containing Lc79 promastigotes for 5, 15, 30, and 45 min and 1, 2, 4, and 24 hr (lanes 1–8). Parasites were removed after 2 hr. Additionally, BMM were incubated with medium containing latex beads for 1 or 24 hr (lanes 11 and 12, respectively). Controls for RNA expression were RAW 264.7 cells + LPS for 6 hr (lane 9) and BMM + LPS + IFN- γ for 6 hr (lane 10). Total RNA was isolated and subjected to Northern blot analysis sequentially with the cDNA probes indicated to the right of each row. (B–G) The data from the Northern blots displayed in A (or not shown) were used to calculate gene expression following infection relative to mock-infected macrophages. B: JE; C: KC; D: iNOS; E: TNF- α ; F: MIP-1 α ; G: IL-12 p40 subunit. Data for IL-10 are not shown.

shown). These experiments showed little or no induction of JE and KC expression, relative to infection with Lc79 *Leishmania* performed at the same time (Fig. 2A, lanes 1–8). Nor were

other cytokines induced in response to phagocytosis of latex beads (Fig. 2A, lanes 11 and 12). Thus, expression of KC and JE is a specific response to infection by *Leishmania*.

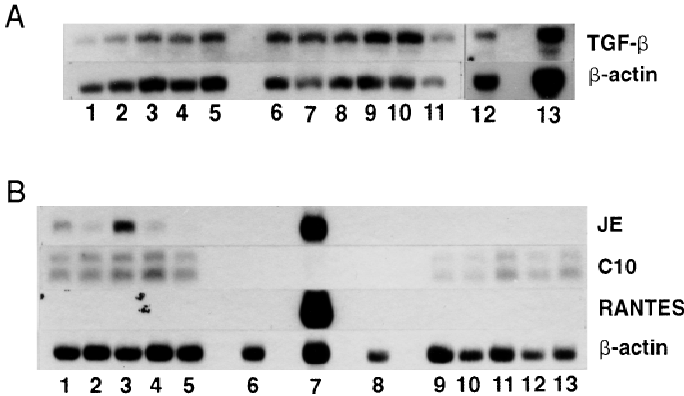


FIG. 3. *L. major*-infected BMM induce JE but not TGF- β 1 or other β -chemokine gene expression. (A) Northern blot analysis of total BMM RNA after incubation for 1 hr (lanes 1–5) or 24 hr (lanes 6–10) with stationary-phase promastigotes of strains FA1 (lanes 1, 6); FV1 (lanes 2, 7); Lc5 (lanes 3, 8); Lc79 (lanes 4, 9), or with no promastigotes (lanes 5, 10). Controls for RNA expression are: BMM + M-CSF (lane 11); RAW 264.7 cells + LPS for 1 hr (lane 12) or for 6 hr (lane 13). The blot was sequentially hybridized with rat TGF- β 1 and β -actin cDNA probes. (B) Northern blot of total RNA from BMM incubated for 1 hr (lanes 1–5) or 24 hr (lanes 9–13) with strain FA1 (lanes 1, 9); FV1 (lanes 2, 10); Lc79 (lanes 3, 11); Lc5 (lanes 4, 12), or with no promastigotes (lanes 5, 13). Controls for RNA expression are: RAW 264.7 cells + LPS for 1 hr (lane 6); RAW 264.7 cells + LPS for 6 hr (lane 7) and L929 fibroblasts (lane 8). The blot was sequentially hybridized with the cDNA probes indicated to the right of each row.

DISCUSSION

In this study we examined the effects of *L. major* infection on gene expression in BMM during the 24-hr period following infection, when virulent organisms must successfully execute steps required for intracellular parasitism. The results showed that BMM infected with *L. major* stationary-phase promastigotes selectively activated the KC and JE genes, members of the α and β chemokine families, respectively. We showed that the induction of JE and KC was rapid and transitory, peaking between 1 and 2 hr and disappearing within 3–4 hr. Significantly, JE and KC were induced specifically in response to parasite invasion, as ingestion of inert latex beads did not result in induction of any gene examined.

Given that the induction of the chemokines was rapid and transitory, we examined the induction of a number of cytokines at time periods immediately following *Leishmania* infection. We found that *L. major* promastigotes did not significantly activate genes encoding TGF- β , IL-10, IL-12, TNF- α , and i-NOS at any period up to 24 hr postinfection. Our findings were in

agreement with previous reports using PCR-based methods (Carrera *et al.* 1996); (Reiner *et al.* 1994), although Carrera *et al.* found that in some of their experiments, metacyclic *L. major* promastigotes did induce considerable BMM expression of IL-10 (Carrera *et al.* 1996). It is notable that, in the latter study, metacyclic promastigote induction of JE expression and slight induction of TNF- α expression were correlated with a lack of IL-10 induction (Carrera *et al.* 1996), which was also seen in the present study. Like Reiner *et al.*, we found that promastigotes did not induce BMM IL-12 expression. However, Schariton-Kersten *et al.* found that 24 hr after injection of *L. major* promastigotes into Balb/C mice, IL-12 was produced within popliteal lymph nodes (Schariton-Kersten *et al.* 1995). Their result indicates that other immune cells present at the site of *L. major* infection may play a role in subsequent cytokine gene induction and protein synthesis. We surmise that exposure of BMM to exogenous cytokines is not required for *in vitro* expression of the JE and KC chemokines. Induction of chemokine genes thus represents the first known transcrip-

tional response of the macrophage to *L. major* infection. Of course, there may be other genes not analyzed here that may be induced similarly, and differential screening methods (Palazzolo *et al.* 1989) could be employed in future studies to identify these.

In agreement with the results presented here, Carrera *et al.* (1996) used a semiquantitative PCR-based assay to show that *L. major* infection of murine BMM induced transient induction of JE. In contrast, Badolato *et al.* (1996) showed that *L. major* infection of human peripheral blood monocytes resulted in a sustained induction of both MCAF (the human homolog of JE) and IL-8, which continued to increase throughout the 12-hr period postinfection. Presently we do not know whether the differences in transient vs sustained induction arise from the use of different infecting parasite strains, constant exposure to parasites, or the type or species of host macrophage employed. Our data now show that chemokine induction is selective, in that the β -chemokines C10, RANTES, and MIP-1 α were not induced at any point following *L. major* infection of murine BMM. Thus, chemokine induction is restricted to JE/MCP-1/MCAF, KC/GRO, and IL-8.

Although we did not perform assays to confirm the secretion of active chemokine protein following JE and KC gene induction, several recent studies have shown a correlation between mRNA expression of JE and KC homologues and protein secretion. For example, cytokine-induced neutrophil chemoattractant (CINC), the rat homolog of KC, was secreted by stimulated normal rat kidney cells as early as 3 hr after IL-1 induction (Watanabe *et al.* 1989). LPS-stimulated human monocytes secreted active MCP-1 following gene induction at 4 hr of treatment (Colotta *et al.* 1992). Finally, Badolato *et al.* (1996) showed that active IL-8 and MCAF protein were produced in response to infection of human monocytes with *L. major*. Because JE and KC gene expression were sustained for at least 3 hours in our assays (Figs. 1 and 2), we feel that it is likely that the chemokines were secreted in our assays.

The ability of *L. major* promastigotes to se-

lectively induce expression of JE and KC chemokines *in vitro* represents the earliest host response to macrophage invasion and could play a role in early events following infection *in vivo*. MCP-1/JE not only attracts monocytes and macrophages (Miller and Krangel 1992), but stimulates these cell types to release granule enzymes and reactive oxygen intermediates (Zachariae *et al.* 1990). In addition, MCP-1/JE has also been shown to augment monocyte-mediated killing of several tumor cell lines *in vitro* (Matsushima *et al.* 1989). KC attracts murine neutrophils (Bozic *et al.* 1995), which are also capable of exerting oxidative burst activities. Because of these properties, JE and KC could attract activated infiltrating macrophages and neutrophils and repress the expansion of a *Leishmania* infection. This theory was supported in a recent study comparing resident mononuclear cells and expression of chemokines in healing lesions of localized cutaneous leishmaniasis with those of nonhealing lesions of diffuse cutaneous leishmaniasis in patients infected with *L. mexicana mexicana* (Ritter, *et al.* 1996). These investigators found high levels of MCP-1/JE expression and moderate levels of MIP-1 α in healing lesions and, in contrast, low levels of MCP-1/JE and high levels of MIP-1 α in the nonhealing, diffuse lesions. In addition, the high levels of MCP-1/JE in the healing lesions were associated with a low frequency of infected macrophages, while the low level of MCP-1 and high level of MIP-1 α in nonhealing lesions were associated with highly parasitized macrophages. These findings suggest that MCP-1/JE and MIP-1 α might attract differentially activated macrophages to sites of infection or have the ability to modulate macrophage activity. In contrast, the other chemokines examined in the present study, RANTES and MIP-1 α , attract memory CD4⁺ T cells (Schall *et al.* 1990), and monocytes (Fahey *et al.* 1992), respectively, but MIP-1 α was not found to trigger a macrophage oxidative burst (Fahey *et al.* 1992). At present, it is not known what cell type responds to C10, although its expression is induced by the cytokine IL-4, known to exacerbate *Leishmania* infection. Thus, it appears that

the induction of JE and KC favors the development of an innate, cell-mediated immune response at sites of infection. Our finding that avirulent strains of *L. major* induce greater expression of JE and KC than virulent strains implies that avirulent strains would induce a healing response in the host, whereas virulent strains would be less likely to do so.

Ingestion of *Leishmania*, rather than phagocytosis itself, was responsible for the induction of high levels of JE and KC expression in cultured BMM. This raises the question as to what parasite molecule(s) provides this specific stimulus. JE and KC gene induction have been shown to occur in macrophages treated with purified lipoarabinomannan from an avirulent but not virulent strain of *Mycobacterium tuberculosis* (Roach *et al.* 1993), suggesting that mycobacterial virulence may be dependent on the ability to avoid stimulation of expression of macrophage chemokines. The implication of glycolipid molecules in differential induction of host chemokine expression is interesting because the avirulent Lc79 and virulent Lc5 strains used in the present study exhibit differences in expression of the major surface molecule lipophosphoglycan (LPG) (Marchand *et al.* 1987; Shankar *et al.* 1993) which binds to macrophage surface receptors (Talamas-Rohana *et al.* 1990). Lc79 *L. major* exhibits lower levels of LPG than Lc5 and does not synthesize the elongated, modified metacyclic form of LPG (Shankar *et al.* 1993). This suggests a possible involvement of the parasite LPG in modulating the signal for chemokine induction and an explanation for the differential induction of JE and KC expression seen by the strains used in this study. For example, the metacyclic form of LPG expressed by FV1 and Lc5 may somehow act to repress chemokine expression in infected macrophages; it is known that purified LPG inhibits protein kinase C-mediated monocyte responses (Descoteaux and Turco 1993). Alternatively, defective forms of LPG, such as those expressed on the FA1 and Lc79 strains used in this study, may fail to repress or stimulate macrophage chemokine expression. Now that methods for the creation of isogenic

LPG-deficient and reconstituted mutant lines are available (Ryan *et al.* 1993), it will be possible to probe the role of LPG on host gene expression *in situ*. Infective *Leishmania* promastigotes also express on their surface gp63 protease (Chang and Chaudhuri 1990), gp46/PSA-2 (Lohman *et al.* 1990; Murray *et al.* 1989) and an abundant class of small glycolipids termed GIPLs (McConville and Ferguson 1993). Any of these parasite molecules, singly or in combination, could provide the signal required for chemokine induction.

In summary, current data show that the *Leishmania* parasite is able to actively manipulate the host cell gene expression program. For some genes this involves avoiding or even repressing expression, while for others this involves activation. This selectivity is exemplified most clearly by the chemokines, as only a subset of this large gene family is induced. How and why the parasite chooses to differentially manipulate chemokine and other host gene expression is obviously a matter of great interest and active research.

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