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Induction of Early-Response Genes KC and JE by Mycobacterial Lipoarabinomannans: Regulation of KC Expression in Murine Macrophages by *Lsh/Ity/Bcg* (Candidate *Nramp*)

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The murine chromosome 1 gene *Lsh/Ity/Bcg* (candidate *Nramp*) regulates macrophage activation for antimicrobial activity against *Salmonella typhimurium*, *Leishmania donovani*, and *Mycobacterium* spp. To determine early events in the activation pathway, the ability of mycobacterial lipoarabinomannan (LAM) to induce early gene (KC and JE) expression in macrophages from susceptible (S) C57BL/10ScSn (*Lsh*^{s}) and congenic resistant (R) B10.L-*Lsh*^{r} mice was investigated. Stimulation with 1.8 μg of arabinofuranosyl-terminated LAM (AraLAM) per ml resulted in similar kinetics for KC or JE expression in S and R macrophages. However, whereas JE/glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA ratios remained equivalent, R macrophages consistently showed enhanced KC/GAPDH ratios within 30 to 40 min of stimulation compared with S macrophages. Significant differences in KC/GAPDH ratios were observed throughout the peak period (0.5 to 6 h) of the KC response and with doses of AraLAM ranging from 0.01 to 2.5 μg/ml. Heavily mannosylated LAM from virulent *Mycobacterium tuberculosis* Erdman, in doses of up to 2.5 μg/ml, failed to stimulate KC or JE in S or R macrophages. Gamma interferon alone (25 U/ml) stimulated equivalent JE expression in S and R macrophages and synergized with AraLAM to enhance JE in both. In contrast, AraLAM-induced KC expression was inhibited in the presence of gamma interferon. Agonist/inhibitor studies were undertaken to determine the signal transduction pathways mediating KC expression. The protein kinase C (PKC) inhibitor Calphostin C (200 nM) inhibited AraLAM-induced KC by 34% ± 4% in S macrophages and 43% ± 5% in R macrophages; the cyclic AMP-dependent PKA inhibitor KT5720 (2 μM) inhibited AraLAM-induced KC by 33% ± 4% (S) and 25% ± 5% (R). A role for Ca^{2+} was indicated because ionophore alone stimulated KC expression and synergized with AraLAM to give a dramatically enhanced response. Induction of KC was also inhibited by (i) blocking constitutive nitric oxide (NO) production by preincubation of macrophages with N^G-monomethyl-L-arginine (400 μM) (48% ± 8% [S] and 40% ± 11% [R]) and (ii) incubation of macrophages with the cyclic GMP-dependent kinase inhibitor KT5823 (4 μM) (65% ± 4% [S] and 72% ± 6% [R]). The manner in which these PKC-, PKA-, and Ca^{2+}-dependent, NO-mediated cyclic GMP-dependent kinase signal transduction pathways may relate to function of the candidate *Lsh/Ity/Bcg* gene *Nramp* is discussed.

Functional analysis of the macrophage resistance gene *Lsh/Ity/Bcg* has been the subject of intensive research over a long time period (reviewed in references 5, 7, 41, and 42). Recently Vidal and coworkers (48) isolated a candidate gene, designated *Nramp* (natural resistance-associated macrophage protein), which encodes a polytopic integral membrane protein that has structural features common to prokaryotic and eukaryotic transporters. The presence of a small consensus motif showing sequence identity with nitrate transporters led these workers to hypothesize that *Nramp* might be involved in direct delivery of nitrates to the phagolysosome of the infected macrophage, where the acid environment would mediate conversion via nitrites to toxic nitric oxide (NO). However, this hypothesis fails to take account of the many studies demonstrating that *Lsh/Ity/Bcg* regulates priming and activation. Although this ultimately results in enhanced tumor necrosis factor alpha (TNF-α)-dependent production of antimicrobial NO (38), the gene has been shown to have many pleiotropic effects, including upregulation of major histocompatibility complex class II, TNF-α production, interleukin 1β expression, AcM.1 antigen expression, oxidative burst, and tumoricidal activity, as well as downregulation of 5′-nucleotidase (reviewed in references 5, 7, 8, 9, 13, 41, and 42). Hence, if transgenesis confirms *Nramp* as *Lsh/Ity/Bcg*, it seems likely that its function will be related to early events in macrophage priming and activation.

More recently, our laboratory has isolated and sequenced a series of full-length *Nramp* clones from an activated B10.L-*Lsh*^{r} macrophage cDNA library (3). These clones all differed in the 5′ region from the published (48) pre-B-cell-derived clone sequence and result in the addition of 64 amino acids at the N terminus of the predicted protein. This new domain is rich in proline, serine, and basic amino acids and contains three protein kinase C (PKC) phosphorylation sites (in addition to the two already identified in the published sequence), as well as a putative Src homology 3 (SH3) binding domain. RNAs containing this extra domain are the only form found within the macrophage. SH3 domains occur as related sequences in tyrosine kinases (32) and are believed to mediate protein-protein interactions obligatory for signal transduction (10). Hence, the new sequence provides compelling evidence that if *Nramp* is *Lsh/Ity/Bcg*, its function must relate to signal transduction for macrophage priming and activation rather than the

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direct delivery of toxic products to the phagolysosome as previously suggested (48). Since most of the earlier parameters of macrophage priming and activation have been measured as responses occurring hours to days after stimulation with bacterial lipopolysaccharide (LPS) and/or gamma interferon (IFN-γ), it seemed important to try to identify the earliest possible influence of *Lsh* (*Nramp*) on the macrophage response.

In other studies of macrophage priming and activation, the upregulation of mRNAs for c-*fos*, c-*myc*, KC, and JE was the earliest macrophage response measured following LPS stimulation (27, 28). The proto-oncogenes c-*fos* and c-*myc* are involved in the regulation of gene transcription (17, 18). KC and JE have recently been shown to be members of the new superfamily of peptide cytokines (33, 34, 44) and are specific attractants for neutrophils and monocytes, respectively (49, 52). We recently reported that arabinofuranosyl-terminated (15) mycobacterial lipoarabinomannan (AraLAM), like LPS, induces expression of the macrophage early genes c-*fos*, KC, and JE, and TNF-α, whereas heavily mannosylated LAMs (ManLAMs) from virulent *M. tuberculosis* do not (37). LAM and LPS are major surface molecules of *Mycobacterium bovis* and *M. tuberculosis* and of *Salmonella typhimurium*, respectively, organisms which can come under *Lsh* control (5). Since the induction of KC and JE expression by LPS appears to be under the control of different signal transduction pathways (27, 45, 53), comparison of the LPS- and LAM-induced expression of these two early genes in macrophages from *Lsh* congenic mice might provide key information on the regulatory pathways involved in *Lsh* gene-regulated macrophage priming and activation. This report presents data comparing AraLAM-stimulated early gene responses in bone marrow-derived macrophages from C57BL/10ScSn (*Lsh*^{s}) and congenic B10.L-*Lsh*^{r} mice.

MATERIALS AND METHODS

Mice. C57BL/10ScSn (*Lsh*^{s}) (B10) mice were purchased from Harlan Olac Ltd. (Bicester, Oxfordshire, England) and bred in the animal unit at Cambridge. N10 congenic B10.L-*Lsh*^{r} mice were produced as described previously (9) and bred in the same unit. These strains will be referred to hereafter as susceptible (S) and resistant (R). Mice (7 to 15 weeks old) were matched by age (±2 weeks) and sex within each experiment.

Media and reagents. Dulbecco modified Eagle medium (DMEM) (catalog no. 10-331-26; ICN Flow Laboratories, Irvine, Scotland) with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.05 mM 2-mercaptoethanol was used and supplemented further with 10% fetal calf serum (FCS) (endotoxin and mycoplasma negative; Sigma Chemical Co., Poole, Dorset, England) and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Gibco-BRL, Paisley, Scotland). Murine recombinant IFN-γ was obtained through Gunter Adolf of Boehringer Ingelheim (Ingelheim, Germany). *S. typhimurium* LPS (phenolic extract, tissue culture grade [L6143]), was purchased from Sigma. LAM was generated in the endotoxin-free form by Chatterjee and colleagues as previously described (15). The levels of contaminating endotoxin in the LAM preparations were determined by using a quantitative *Limulus* amoebocyte lysate commercial assay (E-Toxate; Sigma kit no. 210; Sigma, St. Louis, Mo.). AraLAM and ManLAM contained 0.0108 and 0.0268 ng of endotoxin per μg of LAM, respectively. RNase inhibitor vanadyl-ribonucleoside complexes were purchased from Gibco-BRL, dextran sulfate was purchased from Sigma, and Zeta-Probe blotting membranes were purchased from Bio-Rad Laboratories (Hemel Hempsted, England).

L-NMMA (N^G-monomethyl-L-arginine) and its D-NMMA enantiomer were kindly provided by H. Hodson (Wellcome Research Laboratories, Beckenham, Kent, England). A23187 ionophore was purchased from Sigma. Specific protein kinase inhibitors, KT5720 for cyclic AMP-dependent PKA, Calphostin C for PKC, and KT5823 for cyclic GMP (cGMP)-dependent kinase, were obtained from Kamiya Biomedical Co., Thousand Oaks, Calif. Lyophilized inhibitors were dissolved in dimethyl sulfoxide and stored in aliquots at −20°C. In a cell-free system, KT5720 has an inhibition constant (K_i) of 0.056 μM for PKA, compared with >2 μM for PKC or cGMP-dependent protein kinase. KT5823 has a K_i of 0.234 μM for cGMP-dependent kinase, compared with 4 μM and >10 μM for PKC and PKA, respectively. Both are not cytotoxic up to 10 μM. Calphostin C interacts with the regulatory domain of PKC, inhibiting binding of dibutyrate to PKC. It does not compete with Ca^{2+} or phospholipids. The 50% inhibitory concentration for PKC is 0.05 μM, compared with >50 μM for PKA and >25 μM for cGMP-dependent protein kinase. This compound is cytotoxic at 1,000 nM but has been shown to effectively inhibit the effects of epidermal growth factor receptor ligation at 200 nM (12). Doses of inhibitor were chosen to take account of specificity and cytotoxicity, as well as the manufacturer's advice that these inhibitors are ≈10-fold less efficient when used with mammalian cells compared with the cell-free system in which K_i and 50% inhibitory concentration values were determined.

Cell culture and cytoplasmic RNA preparation. Bone marrow-derived macrophages were grown as described previously (37). Briefly, flushed marrows were cultured in 10% FCS–DMEM, supplemented with 15% supernatant from L929 cell cultures, for 6 days. On day 6, half of the medium was removed and replaced with 10% FCS–DMEM. This was repeated on day 7 or day 8, and macrophages were used on day 8 or day 9, respectively. Following the appropriate stimulation, cytoplasmic RNA was harvested as described previously (37). Essentially, macrophages were differentially lysed by using 0.5% Nonidet P-40 in RNA extraction buffer containing 20 mM vanadyl-ribonucleoside complexes. Nuclei and cell debris were removed from the lysates by spinning at 12,000 × *g* for 90 s. Lysate supernatants were then digested with proteinase K (50 μg/ml), for 30 min at 37°C, prior to phenol-chloroform extraction. Cytoplasmic mRNA contained in the aqueous supernatants was then precipitated with an equivalent volume of isopropanol. Approximately 40 μg of RNA was routinely harvested from a 90-mm-diameter petri dish containing about 7.5×10^6 to 1×10^7 macrophages; 8% (approximately 3 μg) of the sample was run on a minigel to check RNA integrity via ethidium bromide staining. In every case, the integrity of RNA was maintained as reported in our previous publication (37) and here in Fig. 4. Slot blots were prepared to allow accurate quantitation of mRNA via densitometry following hybridization of specific probes.

Preparation of slot blots, probes, and hybridization conditions. RNA was denatured with formamide-formaldehyde and loaded onto Zeta-Probe membranes cut to fit the slot blot apparatus (Schleicher & Schuell, Dassel, Germany). Loaded samples were rinsed twice with TE solution (10 mM Tris-Cl [pH 8], 1 mM EDTA [pH 8]). Membranes were then baked at 80°C for 1 h. Plasmid pcJE.1, 0.6-kb *Eco*RI fragment (JE; no. 37590; American Type Culture Collection, Rockville, Md.), plasmid pBC-KC, 0.8-kb *Pst*I fragment (KC; American Type Culture Collection no. 37591), and plasmid pGPDN5, 1.3-kb *Eco*RI-*Hin*dIII fragment (rat glyceraldehyde 3'-phosphate de-

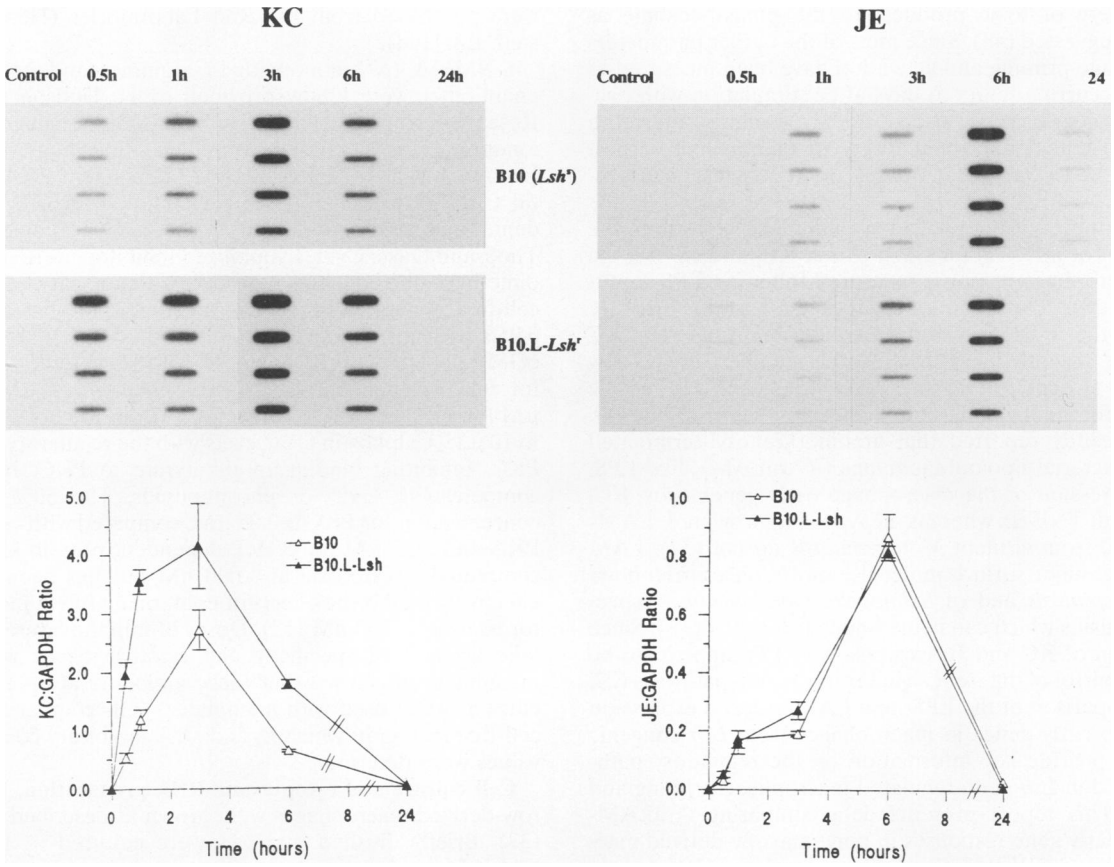

FIG. 1. Kinetics of KC and JE induction in bone marrow-derived macrophages from Lsh^s and Lsh^r mice stimulated with 1.8 μg of AraLAM per ml. Slot blots were loaded vertically with doubling dilutions of RNA starting with 5 μg per slot and probed with KC or JE. The same slots were later reprobed with GAPDH. All autoradiographs were exposed overnight. Graphs show KC/GAPDH and JE/GAPDH ratios (mean ± SD for the four dilutions). Results of three such time course experiments using AraLAM are shown in Table 1, together with results of statistical analyses.

hydrogenase [GAPDH], kindly supplied by P. P. Fort, Laboratoire de Biologie Moleculaire, Montpellier, France), were random primed by using [α-^{32}P]dATP (ICN Flow) to a specific activity of approximately 1×10^8 to 2×10^8 cpm/μg of DNA. Membranes were prehybridized, hybridized, and washed as previously described (38). Washing was down to a final stringency of 0.015 M NaCl–0.0015 M sodium citrate–1% sodium dodecyl sulfate at 65°C. Membranes were exposed to Fuji RX film at −70°C for the times indicated in figure legends. The specificities of all probes used were checked by hybridization to Northern (RNA) blots of control and IFN-γ/LPS-activated macrophage RNA. As reported previously (37), transcripts of 1,200 and 990 bp for KC and JE, respectively, and 1,600 bp for GAPDH were observed. Autoradiographs were scanned with a Joyce Loebl Chromosan 3 densitometer (Applied Imaging, Sunderland, England). Scan peak integrals were used to quantitate relative levels of mRNA.

Statistical analyses. Within-experiment variation in KC/GAPDH or JE/GAPDH ratios is indicated as standard deviations (SD) for triplicate or quadruplicate readings for each time point, dose of AraLAM, or macrophage population. Since absolute values for ratios are not comparable between experiments, a nonparametric Wilcoxon paired comparison test was applied to determine whether differences between macrophage populations were significant over multiple experiments, time points, or doses of AraLAM stimulation.

RESULTS

Kinetics of AraLAM-induced KC and JE in S and R macrophages. Stimulation of macrophages with 1.8 μg of AraLAM per ml induced both KC and JE expression in S and R macrophages (Fig. 1). The overall kinetics of the responses for either KC (peaking at ≈3 h) or JE (peaking at ≈6 h) were similar in the two strains of mice. However, an enhanced level of KC expression was induced in R macrophages within 30 min of stimulation with AraLAM and remained consistently higher throughout the peak period of KC induction (Table 1; $P < 0.005$, Wilcoxon paired comparison test for multiple time points over three experiments). In contrast, no significant differences in the JE/GAPDH mRNA expression ratios were observed for R versus S macrophages (Table 1).

Dose response for KC and JE following stimulation with AraLAM, ManLAM, or LPS. Figure 2 demonstrates that the early (40 min) enhanced expression of KC in R macrophages was maintained over the dose range 10 to 1,000 ng of AraLAM per ml as well as with 1 ng of LPS per ml. This was reproducible at different time points over multiple experiments (Table 2; $P < 0.005$, Wilcoxon paired comparison test). Increasing the dose of AraLAM to 2,500 ng/ml still failed to induce a response as great in S macrophages as that observed in macrophages from R mice (Table 2, experiment 3). No significant differences between S and R macrophage JE re-

TABLE 1. Results of three separate experiments showing kinetics of KC and JE induction in bone marrow-derived macrophages from S (*Lsh*^{s}) versus R (*Lsh*^{r}) mice following stimulation with 1,000 ng of AraLAM per ml

Expt[a]	Time (h)	Ratio[b]			
		KC/GAPDH		JE/GAPDH	
		Lsh^s	*Lsh*^{r}	*Lsh*^{s}	*Lsh*^{r}
1	0	0.05 ± 0.09	0.05 ± 0.09	0.02 ± 0.01	0
	0.5	0.10 ± 0.12	1.62 ± 0.16	0.30 ± 0.04	0.08 ± 0.04
	1	1.66 ± 0.06	12.34 ± 2.02	0.65 ± 0.02	0.32 ± 0.07
	2	6.13 ± 2.70	14.48 ± 3.50	ND	ND
	4	2.58 ± 0.37	11.27 ± 1.08	1.25 ± 0.03	0.96 ± 0.06
	6	2.85 ± 0.99	2.55 ± 0.42	2.77 ± 0.15	2.91 ± 0.28
	8	0.62 ± 0.54	0.71 ± 1.23	ND	ND
	24	ND	ND	0.31 ± 0.06	0.43 ± 0.11
2	0	0	0	0	0
	0.5	0.53 ± 0.09	1.95 ± 0.22	0.03 ± 0.01	0.06 ± 0.01
	1	1.18 ± 0.17	3.55 ± 0.21	0.22 ± 0.02	0.21 ± 0.03
	3	2.72 ± 0.34	4.16 ± 0.73	0.25 ± 0.01	0.38 ± 0.03
	6	0.63 ± 0.02	1.77 ± 0.08	1.08 ± 0.03	1.07 ± 0.04
	24	0.01	0.02	0.03 ± 0.01	0.03 ± 0.04
3	0	0	0	0	0
	0.5	0.57 ± 0.06	5.28 ± 0.59	0.19 ± 0.02	0.16 ± 0.04
	2	0.43 ± 0.04	6.32 ± 1.47	0.18 ± 0.03	0.21 ± 0.04
	6	ND	ND	ND	ND

[a] Experiment 1: KC, R > S ($P < 0.025$); JE, R ≈ S (not significant). Experiment 2: KC, R > S ($P < 0.005$); JE, R ≈ S (not significant). Experiments 1 to 3: KC, R > S ($P < 0.005$); JE, R ≈ S (not significant).

[b] Mean ± SD ($n = 1$) for triplicate or quadruplicate values for each time point. ND, not done. The nonparametric Wilcoxon paired comparison test was used to determine whether the response of R mice was significantly higher than that of S mice throughout the peak period (0.5 to 6 h) of expression and over all three experiments.

sponses were observed over the same range of doses and time points tested for KC (Table 2). Erdman ManLAM, in which the Ara terminals are extensively capped with mannose residues (20), failed to elicit KC or JE responses in either S or R macrophages at doses of 1,000 ng/ml (KC; Fig. 2) or 2,500 ng/ml (KC and JE, zero values compared with parallel AraLAM response in experiment 3, Table 2). Indeed, Erdman ManLAM in doses up to 2,500 ng/ml fails to induce any early gene (c-*fos*, KC, JE, and TNF-α) responses in either S (37) or R macrophages (data for c-*fos* and TNF-α not shown). The fact that Erdman LAM fails to elicit any responses negates the possibility that results obtained with AraLAMs could be due to the low level of LPS contamination. Erdman LAM contained ≈2-fold more contaminating LPS than AraLAM, making the highest possible level of LPS contamination 0.067 ng/ml when 2,500 ng of Erdman LAM per ml was used. Clearly this did not elicit a response.

IFN-γ priming for KC and JE expression. IFN-γ alone (25 U/ml) failed to induce KC expression at 30 min or 2 h poststimulation in S or R macrophages (Fig. 3). In the same experiment, JE expression (Fig. 4) was induced by IFN-γ alone, which synergized with AraLAM (1,000 ng/ml). In contrast, IFN-γ given as a costimulus with AraLAM inhibited KC induction in both S and R macrophages (Fig. 3). As with previous experiments, AraLAM-induced enhanced KC expression in R macrophages was maintained, while JE expression remained equivalent even in the presence of IFN-γ. ManLAM (1,000 ng/ml) again failed to induce either KC or JE expression (2 h poststimulation) in either S or R macrophages (Fig. 3 and 4). This lack of responsiveness to ManLAM does not result from slower kinetics for induction of KC or JE compared with AraLAM (49; unpublished observations). ManLAM (1,000 ng/ml) showed minimal synergy with IFN-γ for JE expression, but KC expression remained negative. These data reinforce previous observations (27, 53) that different signal transduction pathways are involved in induction of KC and JE, with IFN-γ acting as an antagonist for one and an agonist for the other.

Signal transduction pathways in AraLAM induction of KC expression. Since AraLAM stimulation of macrophages led to such dramatic differences in KC expression in S and R macrophages, further inhibitor/agonist experiments were performed to determine which of the major signal transduction pathways are involved (Fig. 5). The PKC-specific inhibitor (Calphostin C) reduced KC expression by ≈30 to 40% in both S and R macrophages, while the PKA-specific inhibitor (KT5720) reduced expression by ≈20 to 30% (Fig. 5a). Neither completely ablated the response. The importance of Ca^{2+} in the signal transduction pathway for KC was demonstrated by the observation that ionophore alone induced KC expression and synergized with AraLAM to give a dramatically enhanced response (Fig. 5b). A role for NO and cGMP-dependent kinase activity was also demonstrated by (i) inhibition (≈65 to 75%) of AraLAM-induced KC expression by the cGMP kinase-specific inhibitor KT5823 and (ii) ≈40 to 50% inhibition when constitutive NO production was blocked by pretreating (3 h) macrophages with 400 μM L-NMMA (Fig. 5a). Adding L-NMMA simultaneously with the stimulus did not inhibit KC induction (data not shown), stressing a role for constitutively expressed Ca^{2+}-calmodulin-dependent NO synthase (cNOS) in the generation of NO mediating this response. Cell morphology and adherence appeared unaltered throughout all experiments, indicating that none of these effects was due to cytotoxicity of the inhibitors. Interestingly, no consistent differences in percentage inhibition were observed between S and R strains. Hence, although PKC, PKA, and Ca^{2+}-dependent, NO-mediated cGMP-dependent kinase pathways are involved in signal transduction for KC expression, amplification of the

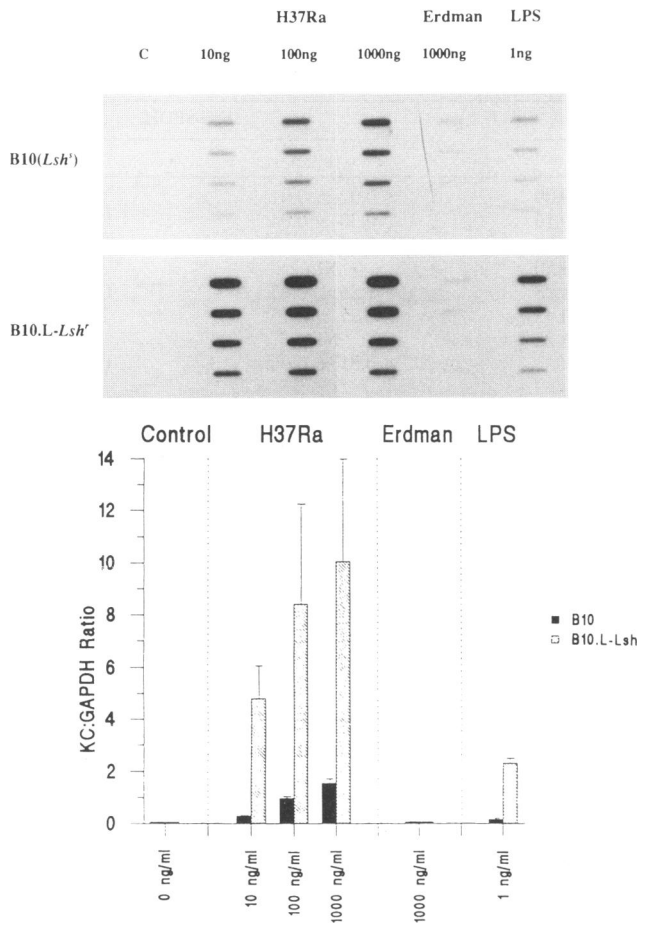

FIG. 2. Dose response of macrophages at 40 min poststimulation with an attenuated H37Ra AraLAM, virulent *M. tuberculosis* Erdman ManLAM, and *S. typhimurium* LPS. Slot blots showing the induction of KC mRNA were loaded as described in the legend to Fig. 1. Autoradiographs were exposed overnight. KC/GAPDH ratios (mean ± SD) are shown below. Results of three such dose-response experiments using AraLAM are shown in Table 2, together with results of statistical analyses. C, control.

response in R macrophages might precede and/or diverge from the input of these kinases into the signal transduction pathway.

DISCUSSION

LAMs are major surface molecules (15, 47) of *M. bovis* and *M. tuberculosis* which come under the control of the macrophage resistance gene *Lsh/Ity/Bcg* (*Nramp*). In this report, two important observations were made: (i) that arabinofuranosyl-terminated (15) mycobacterial AraLAM induced differential levels of KC (a neutrophil chemoattractant), but not JE (a monocyte chemoattractant), expression in macrophages from S and R mice; and (ii) that in addition to their inability to induce TNF-α production (15, 37), heavily mannosylated ManLAMs from virulent *M. tuberculosis* Erdman also failed to induce expression of either KC or JE in S or R macrophages. The latter observation is interesting in relation to earlier studies (35) demonstrating that highly virulent mycobacterial strains do not come under *Lsh* gene regulation in vivo. This is consistent with the hypothesis that *Lsh* regulates an early event in macrophage priming and activation which must be triggered by molecules on the surface of the infecting microbe before the resistance mechanism, the TNF-α-dependent production of reactive nitrogen intermediates (38), is expressed. The former may also reflect earlier biological observations on infection in vivo. An influx of fresh monocytes to the site of infection has never been demonstrated to be important in *Lsh* resistance (19). The work of Appelberg and coworkers (2) has, however, demonstrated higher neutrophilia in the peritoneal granulocytosis of congenic C.D2-*Idh-1-Pep3* (*Lsh*^{r}) mice following intraperitoneal infection with *M. avium* compared with BALB/c (*Lsh*^{s}) mice. The rapid influx of neutrophils, which can be observed early in infection with either *Leishmania donovani* (50) or mycobacteria (22, 43), may play a direct cidal role in the initial knockdown of extracellular parasites. In addition, activated neutrophils are also able to secrete TNF-α (20, 21) and NO (51), which might influence the early macrophage response. So too would the ability of neutrophils to synthesize the lipid integrin-modulating factor 1, which controls the avidity of CR3 and LFA-1 (26). The β_2 integrins, principally CR3, play a major role in the uptake of *L. donovani* (6, 18) and *M. tuberculosis* (39, 40), and we have recently demonstrated (24) that engagement of β_1 and β_2 integrins by plating macrophages onto fibronectin or fibrinogen directly stimulates enhanced TNF-α release in *Lsh*^{r} compared with *Lsh*^{s} mice. Of interest too is the recent report by Schlesinger (39) that the attenuated H37Ra and the virulent H37Rv and Erdman strains of *M. tuberculosis* all use CR3 for entry into macrophages, but only the virulent strains also use the mannose-fucose receptor (MFR). Engagement of the MFR by plating macrophages onto mannan does not elicit a TNF-α response in either *Lsh*^{s} or *Lsh*^{r} macrophages (24). In other studies, AraLAM stimulation of TNF-α and interleukin-1β secretion by macrophages was inhibitable with anti-LAM or anti-CD14 monoclonal antibodies (55), demonstrating that in addition to direct ligation of macrophage receptors, LAM may also interact directly or indirectly with CD14. This differential engagement of macrophage surface receptors (CR3, MFR, CD14) during infection may determine the distinctive triggering of interacting signal transduction pathways and ultimately the pattern of gene expression observed. Whether the enhanced expression of KC does offer an advantage to *Lsh*^{r} mice during initial infection with *L. donovani*, *Mycobacterium* spp., or *S. typhimurium* requires further investigation.

The immediate importance of the observation that AraLAM induces differential expression of KC in macrophages from *Lsh*^{r} and *Lsh*^{s} mice lies in the potential this provides for studies aimed at determining the precise mode of action of the gene, especially now that a candidate gene (*Nramp*) has been cloned and sequenced (3, 48). All of the earlier functional analysis pointed to an important role for the *Lsh/Ity/Bcg* gene in regulating the priming and activation of macrophages (reviewed in references 5, 7 to 9, 13, 41, and 42). Because of the many pleiotropic effects of the gene, it was earlier hypothesized (8) that *Lsh* operated early in the pathway to macrophage activation, at the level of signal transduction and/or DNA binding activity. The observation that enhanced KC expression in *Lsh*^{r} macrophages occurred within 30 min of exposure to AraLAM or LPS supports this contention. Our observation that specific inhibition of PKC reduced AraLAM-induced KC expression is consistent with previous studies in which *Porphyromonas gingivalis* fimbriae were used as the stimulus for induction (25) and may relate to the multiple PKC binding sites now identified on the candidate *Nramp* molecule (3, 48). A role for the PKA pathway has not previously been demonstrated (45). Our result may reflect the different stimulus (AraLAM versus LPS) used and/or the fact that we were

TABLE 2. Results of three separate experiments showing KC and JE induction in bone marrow-derived macrophages from S (*Lsh*^{s}) versus R (*Lsh*^{r}) mice following stimulation with a range of doses of AraLAM

Expt[a]	Dose (ng/ml)	Ratio[b]			
		KC/GAPDH		JE/GAPDH	
		Lsh^s	*Lsh*^{r}	*Lsh*^{s}	*Lsh*^{r}
1 (at 40 min)	Control	0	0	0	0
	10	0.28 ± 0.04	4.79 ± 1.28	0.23 ± 0.01	0.26 ± 0.05
	100	0.94 ± 0.09	8.41 ± 3.86	0.45 ± 0.06	0.45 ± 0.06
	1,000	1.53 ± 0.19	10.05 ± 3.96	0.66 ± 0.05	0.63 ± 0.04
	1 (LPS)	0.15 ± 0.05	2.30 ± 0.20	0.08 ± 0.03	0.08 ± 0.01
2 (at 2.5 h)	Control	0	0	0	0
	10	0.03 ± 0.01	0.02 ± 0.01	0.06 ± 0.02	0.04 ± 0.02
	100	0.71 ± 0.03	0.57 ± 0.08	0.38 ± 0.03	0.32 ± 0.07
	1,000	1.76 ± 0.21	2.73 ± 0.69	0.61 ± 0.01	0.64 ± 0.19
	2,500	2.98 ± 0.04	3.68 ± 0.78	2.91 ± 0.02	2.88 ± 0.64
3 (at 3 h)	Control	0	0	0.38 ± 0.26	0.09 ± 0.12
	100	0.47 ± 0.09	1.89 ± 0.12	2.75 ± 0.25	1.26 ± 0.31
	1,000	0.69 ± 0.27	2.63 ± 0.47	1.66 ± 0.12	1.70 ± 0.59
	2,500	1.49 ± 0.20	5.53 ± 0.86	3.66 ± 1.04	3.48 ± 0.64

[a] Experiments 1 to 3: KC, R > S ($P < 0.005$); JE, R ≈ S (not significant).
[b] Mean ± SD ($n = 1$) for triplicate or quadruplicate values for each dose of AraLAM. The nonparametric Wilcoxon paired comparison test was used to determine whether the response of R mice was significantly higher than that of S mice for all doses and time points over the three experiments. Results obtained with LPS (1 ng/ml) are shown for comparison.

obliged to use KT5720 at concentrations bordering on the K_i at which this inhibitor loses its specificity for PKA in a cell-free system. Other studies have demonstrated, however, that PKA-dependent activation of CREB can lead to enhanced induction of early gene (c-*fos*) expression (36). Stimulation of KC expression by the calcium ionophore A23187, as well as the dramatic synergism observed between the ionophore and AraLAM, demonstrated the importance of Ca^{2+} in the signal transduction pathway. The ability of ionophore to stimulate gene expression has been linked to signalling via pathways involving the initial hydrolysis of phosphatidylinositol-4,5-biphosphate (1). The products of this hydrolysis, diacylglycerol and inositol-1,4,5-triphosphate, are direct and indirect activators of PKC, respectively (1, 29). Inositol-1,4,5-triphosphate releases Ca^{2+} ions from intracellular stores (1), and certain isoforms of PKC (31), in addition to calmodulin-dependent kinases (16), require Ca^{2+} for their activation. Interestingly, the constitutive generation of NO, which our L-NMMA inhibition studies suggest is important in KC induction, also relies on the Ca^{2+}-calmodulin-dependent activity of cNOS (11, 14). NO generated by cNOS can directly activate guanylate cyclase and hence feeds into the cGMP-dependent kinase pathway (4, 46), which our studies have demonstrated is also important in induction of KC. NO can also act as an amplifier of Ca^{2+} signals (36). In particular, PKA-dependent activation of CREB for enhanced c-*fos* expression (36) involved this NO amplification of calcium activity, perhaps providing further explanation for the role of PKA in the KC signal transduction pathway.

Our interest in examining the potential role of NO in signal transduction stemmed from the observation that *Nramp* has structural features common to prokaryotic and eukaryotic transporters and that it contains a small (20-amino-acid) motif showing sequence identity with nitrate transporters (48). Although Vidal and coworkers (48) hypothesized that this might be important in direct delivery of nitrates for conversion via nitrites to toxic NO in the acidic phagolysosome, we considered an alternative hypothesis that nitrate transport function might be involved in the generation of NO for amplification of

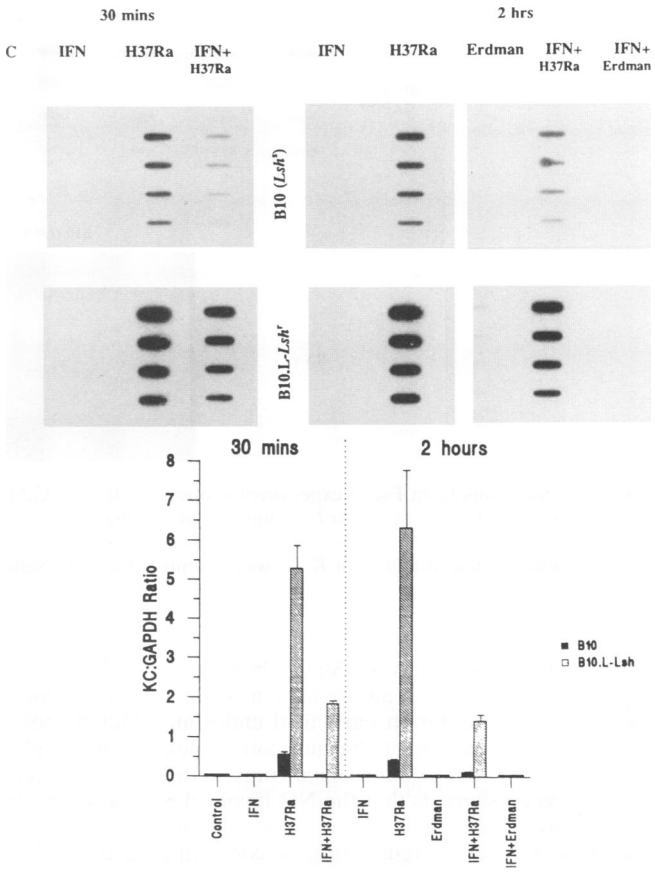

FIG. 3. Macrophages were stimulated with IFN-γ (25 U/ml), AraLAM (1,000 ng/ml), or ManLAM (1,000 ng/ml), either alone or in combination. Slots were loaded as described in the legend to Fig. 1. Autoradiographs were exposed overnight. Histograms show KC/GAPDH ratios (mean ± SD). Similar results have been obtained in three independent experiments. C, control.

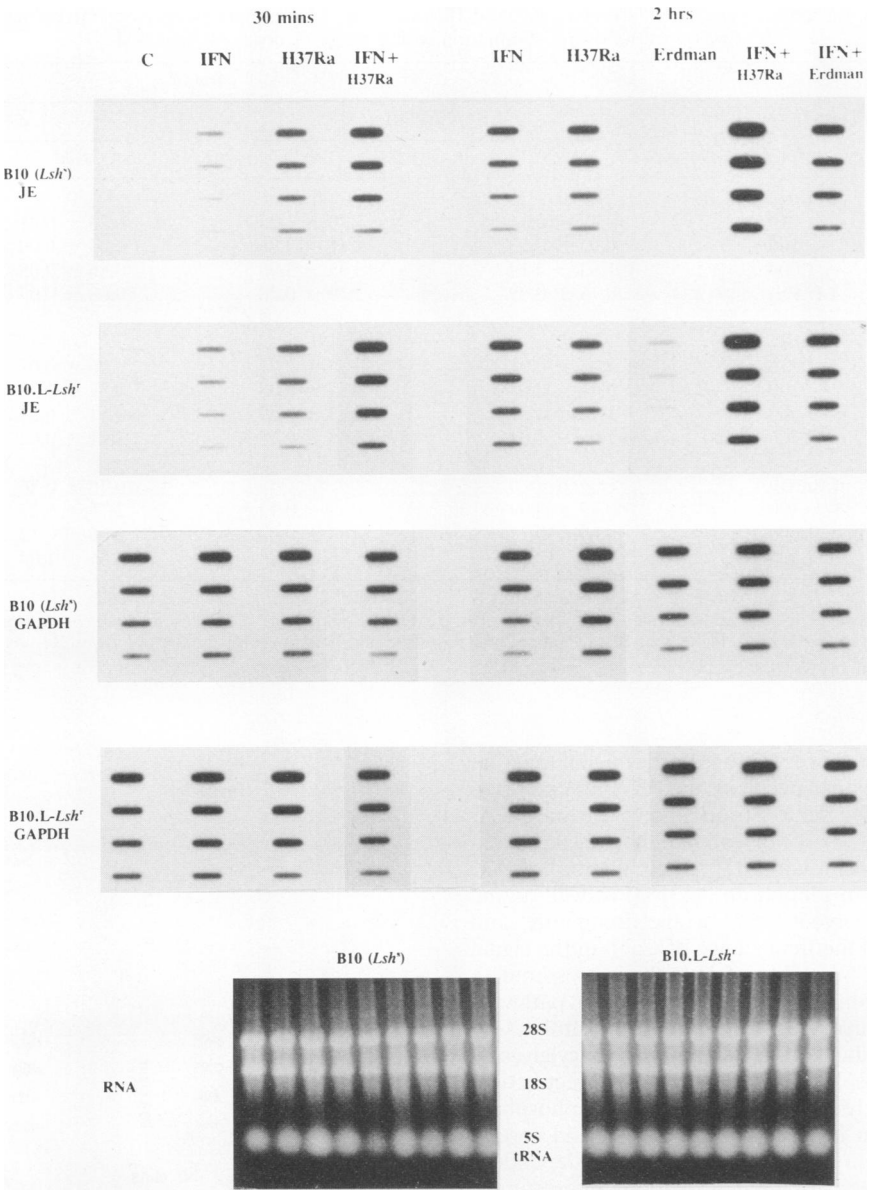

FIG. 4. Slot blots from Fig. 3 experiment probed for JE and GAPDH. Autoradiographs were exposed for 6.5 h and overnight, respectively. JE/GAPDH peak integral ratios for Lsh^s and Lsh^r macrophages were equivalent (see Table 2) and hence are not graphed. Similar results have been obtained in three independent experiments. Integrity of total RNA is demonstrated by ethidium bromide-stained minigels from the nine treatments for each mouse strain. Integrity of RNA was examined for every sample in all experiments. C, control.

signal transduction pathways. Such a hypothesis would also be dependent on delivery of nitrate to an acid compartment which could, in this case, be an early acid endosome. Our demonstration that pretreatment (but not cotreatment) of macrophages with L-NMMA inhibits signal transduction for enhanced KC expression suggests that the NO involved is dependent on the activity of cNOS in the resting macrophage. Whether *Nramp* is involved in redirecting or concentrating the stable nitrate end product generated from cNOS to the site of signal transduction remains to be determined.

The recent demonstration by our laboratory that macrophage-expressed *Nramp* contains a putative SH3 binding domain (3) suggests a mechanism by which *Nramp* regulates early events in transmembrane signalling in resistant macrophages by binding tyrosine kinases. This would be consistent with other studies demonstrating that (i) integrin-dependent signalling is important in macrophage immediate-early gene expression (54) as well as enhanced TNF-α production in Lsh^r versus Lsh^s macrophages (24); (ii) integrin-dependent phosphorylation occurs via tyrosine kinases (30); (iii) the myeloid-specific tyrosine kinase Hck is upregulated in activated macrophages (56); and (iv) Hck is involved in signal transduction for TNF-α release in murine macrophages (23). By binding tyrosine kinases, *Nramp* might provide a primary focus for protein-protein interactions important in signal transduction, and/or phosphorylation of *Nramp* on tyrosines may regulate its transport function in providing additional substrate for the generation of NO involved in signal transduction. Our further

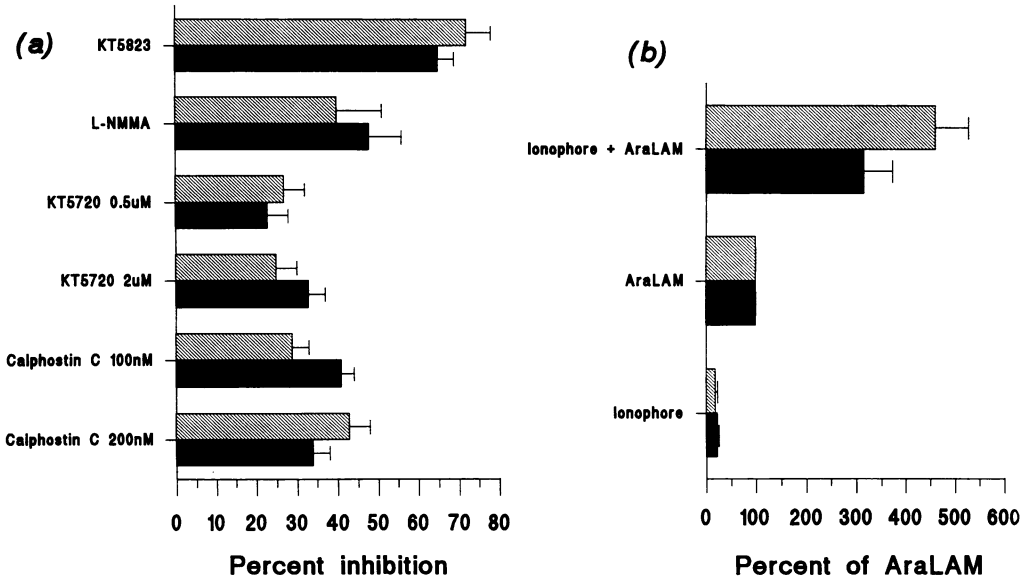

FIG. 5. (a) Inhibition of the KC response (at 1.5 h poststimulation with 1,000 ng of AraLAM per ml) with different doses of the specific kinase inhibitors Calphostin C (PKC), KT5720 (PKA), and KT5823 (cGMP kinase), as well as by pretreating macrophages for 3 h with L-NMMA; (b) stimulation of KC expression by ionophore, AraLAM, and ionophore plus AraLAM (expressed as a percentage of the control AraLAM response).

research is designed to determine whether amplification of transmembrane signalling in resistant mice involves binding of tyrosine kinases to *Nramp* early in the cascade of signalling events which lead to the multiple pleiotropic effects important in the action of this resistance gene.

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