Regulation of Macrophage Gene Expression by Mycobacterium tuberculosis: Down-Regulation of Mitochondrial Cytochrome c Oxidase

SILVIA RAGNO, IRIS ESTRADA-GARCIA,† ROBERT BUTLER, AND M. JOSEPH COLSTON*
Division of Mycobacterial Research, National Institute for Medical Research, London NW7 1AA, United Kingdom

Received 18 March 1997/Returned for modification 3 June 1997/Accepted 30 April 1998

We have investigated changes in gene expression in mouse peritoneal macrophages following infection with virulent Mycobacterium tuberculosis. Using differential-display reverse transcription-PCR (RT-PCR), we have identified a gene that was markedly down-regulated within 6 h of infection and remained so for the duration of the experiment (5 days). On sequencing, this gene was found to encode the murine cytochrome c oxidase subunit VIIc (COX VIIc). Down-regulation of COX VIIc during M. tuberculosis infection was confirmed by three independent techniques: limiting-dilution RT-PCR, RNase protection assay, and Northern analysis. Limiting-dilution RT-PCR and Northern analysis were also used to analyze the specificity of this regulation; heat-killed M. tuberculosis, Mycobacterium bovis BCG, and latex beads had no effect on expression of COX VIIc. Down-regulation of this enzyme was also confirmed by using adherent cells isolated from spleens of M. tuberculosis-infected mice. These ex vivo macrophages showed apoptotic features, suggesting a possible involvement of cytochrome c oxidase in the programmed cell death of the host cells.

Pathogenic mycobacteria such as Mycobacterium tuberculosis and Mycobacterium leprae can survive and grow within host cells, particularly macrophages. In addition to these pathogenic species, the genus Mycobacterium includes many nonpathogenic species and also species which are pathogenic under certain circumstances, such as in immunocompromised hosts. The ability to withstand the hostile macrophage environment is crucial to mycobacterial pathogenicity; for example, M. tuberculosis is capable of remaining dormant for many years within alveolar macrophages but may start to divide when conditions are suitable. In addition, the initial response to the macrophage at the site of invasion is likely to play a key role in determining the outcome of the interaction and the overall regulation of the ensuing acquired response.

The ability to survive within macrophages is likely to be multifactorial. The organisms themselves have evolved mechanisms for surviving exposure to antimicrobial agents produced by activated macrophages. For example, the lepromatous bacillus produces a specific phenolic glycolipid capsule which can protect it against reactive oxygen intermediates (8). Mycobacteria have also evolved mechanisms for regulating their own environment within a host cell. For example, the mycobacterial phagosomal environment does not become acidic due to exclusion of the vesicular proton-ATPase (31), and mycobacterial components are able to down-regulate the induction of an immune response by macrophages (21, 23). Thus, the interaction between the host cell and the bacterium represents a balance between antimicrobial activity of the macrophage and evasion mechanisms of the mycobacterium. The demonstration of trafficking of mycobacterial constituents, particularly lipoarabinomannan, a molecule with diverse regulatory activities on host cells, within macrophages (34) emphasizes the cross-talk which can occur between the host cell and the intracellular parasite.

In order to gain additional insights into the mycobacterium-macrophage interaction, we have investigated changes in macrophage gene expression following invasion by and growth of M. tuberculosis. Using differential-display reverse transcription-PCR (DD RT-PCR), we have identified one particular macrophage gene which is rapidly and persistently down-regulated during infection and have demonstrated that this change in expression is specific to infection with M. tuberculosis. Furthermore, expression of this gene was down-regulated in adherent cells isolated from the spleens of M. tuberculosis-infected mice. Such cells showed phenotypic and transcriptional changes which were consistent with programmed cell death, suggesting that down-regulation of the enzyme could be an early event in the commitment to apoptosis.

(Part of this work was published in the proceedings of the 13th European Immunology Meeting [23a].)

MATERIALS AND METHODS

Bacterial culture. M. tuberculosis H37Rv was grown in Middlebrook medium, and stock cultures of mid-log-phase bacilli were stored in 1-ml aliquots of Dulbecco modified Eagle medium (DMEM) containing 20% heat-inactivated fetal calf serum (FCS) (Advanced Protein Products, Brierly Hill, United Kingdom) in liquid nitrogen. Viable counts of the stock cultures were determined by performing 10-fold serial dilutions in saline and plating onto Middlebrook agar medium. Plates were incubated at 37°C for 3 weeks, and CFU were counted. Mycobacterium bovis BCG was obtained as a lyophilized suspension from Evans Medical Ltd. (Langhurst, England).

Macrophage culture and infection. Peritoneal macrophages were collected from 6- to 8-week-old female BALB/c mice and cultured in DMEM (Flow Laboratories, High Wycombe, United Kingdom) plus 20% FCS without addition of antibiotics. The cells were aliquoted into six-well culture plates (Nunc, Roskilde, Denmark) at a concentration of approximately 10⁶ cells per well. After 2 days, the medium was replaced with medium containing approximately 10⁸ CFU of live M. tuberculosis (strain H37Rv) or equivalent concentrations of heat-killed (85°C for 30 min) M. tuberculosis, live BCG, or 6.4-μm-diameter latex beads (Sigma). Ziehl-Neelsen staining was carried out 24 h later to confirm that the M. tuberculosis had been phagocytosed.

* Corresponding author. Mailing address: Division of Mycobacterial Research, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom. Phone: (0181) 959 3666. Fax: (0181) 913 8528. E-mail: j-colston@nimr.mrc.ac.uk.
† Present address: Instituto Politecnico Nacional, Departamento de Immunologia, Mexico DF 11340, Mexico.
DD RT-PCR. Total RNA was extracted from noninfected and infected macrophages at 6 h and 5 days after addition of *M. tuberculosis*, as described previously (33). To minimize the risk of amplification of contaminating DNA, total RNAs were digested with 10 U of DNase I (Boehringer Mannheim, Lewes, United Kingdom) at 37°C in accordance with the manufacturer's instructions, followed by phenol-chloroform extraction and reprecipitation in 2 volumes of 100% ethanol and 0.5 volume of 3 M sodium acetate (33). cDNAs for DD RT-PCR were synthesized from 300 ng of total RNA by using 1 μM oligo (dT)12 anchored primers (DD1, T12CA; DD2, T12CG; and DD3, T12CC), where V may be A, G, or C, and at 65°C for 10 min. The RT mix, consisting of 1× buffer, 200 μM of Moloney murine leukemia virus reverse transcriptase (Life Technology, Paisley, United Kingdom), 20 μM deoxynucleotidetriphosphates (dNTPs) (Boehringer Mannheim, Hermsdorf, United Kingdom), and 10 μM dithiothreitol (Life Technology), was added, and the reaction mixture was incubated at 37°C for 1 h.

PCR (40 cycles of 94°C for 30 s, 55°C for 2 min, and 72°C for 30 s and 1 cycle at 72°C for 5 min) was carried out in an Omegene thermocycler (Hybaid, Middlesex, United Kingdom) with 1 μM anchored primers (DD1, DD2, and DD3), 0.2 μM random 10-mer primers (OPA 14, TCT GTG CTG G; OPA 18, AGG TGA CCG T; and OPA 20, GTC GTC ATC C), 2 μM dNTP, 1× PCR buffer I (1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl [pH 8.3], 0.001% gelatin), and 1 U of Taq polymerase (AmpliTag, Perkin-Elmer, London, United Kingdom), and 0.37 MBq of α-32P-ATP (Amersham, Buckinghamshire, United Kingdom). DD RT-PCR products were electrophoresed on a denaturing 6% polyacrylamide gel with urea gel methodology (Amer, 1987), and the film exposed overnight.

Reamplification of DD RT-PCR bands. Differentially expressed bands were cut out from the dried gels and eluted in water. Samples were boiled for 15 min and ethanol precipitated. One microfiter of isolated cDNAs was PCR reamplified (30 cycles of 94°C for 1 min, 40°C for 2 min, and 72°C for 30 s for 30 cycles and 72°C for 5 min) with the same anchored and random primers used for DD RT-PCR but under the following conditions: 2 μM anchored primers, 0.5 μM random 10-mer primers, 2 μM dNTP, 4 mM MgCl2, 1× PCR buffer II (50 mM KCl, 10 mM Tris-HCl [pH 8.3]), 2.5 μU of Taq polymerase (AmpliTag, Perkin-Elmer), and sterile distilled water to 40 μl.

Cloning of reamplified bands. Fresh PCR products of isolated DNA bands were ligated into pCR II or pCR2.1 and transformed by using One Shot Escherichia coli competent cells (Invitrogen, Carlsbad, California). Plasmid DNA was isolated from single transformants and sequenced with T7 and anchored primer linked to the T7 RNA polymerase binding site (5′-ATG AGG TAG TCT GTC AGG T; expected product, 151 bp) (reverse, 5′-ATG GAT GAC GAT GAT ACC GCT; expected product size, 151 bp). β-actin (forward, 5′-ATG GAC GAT GAC GAT ACC GCT; reverse, 5′-ATG GAT GAC GAT GAT ACC GCT; expected product size, 457 bp) (reverse, 5′-ATG GAT GAC GAT GAT ACC GCT; expected product size, 457 bp) was amplified and sequenced for alignment with the published sequences. Sequencing revealed that band U61 showed a high degree of identity with the murine mitochondrial enzyme subunit COX VIIc. We used an RPA to confirm differential expression of COX VIIc. Since DD RT-PCR is known to produce a high rate of false-positive results (7), it was necessary to confirm differential expression of the putative transcript with riboprobe with 20°C of total RNA at 50°C overnight in hybridization buffer (80% formamide, 40 mM Pipes [piperezine-N,N′-bis[2-ethanesulfonic acid]] [pH 6.7], 0.4 M NaCl, 1 mM EDTA). The hybrid (riboprobe and target total RNA) was digested with a cocktail of 1 μg of RNase A and 20 μl of RNase T1 (Ambion, Austin, United Kingdom) for 30 min at 37°C in digestion buffer (10 μM Tris-HCl [pH 7.5], 5 mM EDTA, 200 mM sodium acetate). The reaction was stopped by adding 400 μl of phenol-chloroform. Samples were resuspended in gel loading buffer (80% deionized formamide, 1 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, 0.1% SDS), electrophoresed with a 6% polyacrylamide gel, and exposed overnight at ~70°C.

RT-PCR. PCR was carried out on cDNAs from 1 μg of total RNA by using specific primers for cytochrome c oxidase subunit VIIc (COX VIIc) (forward, 5′-CGG GTG TTT CGC GGT AAA CC; expected product size, 151 bp), β-actin (forward, 5′-ATG GAT GAC GAT GAT ACC GCT; reverse, 5′-ATG GAT GAC GAT GAT ACC GCT; expected product size, 457 bp), COX I (forward, 5′-TCC GAC TCA TCG TCA CAG CAT CAT CAT; reverse, 5′-GTC GGC ATT GAT TGA GAT; expected product size, 341 bp), and COX II (forward, 5′-GTC GGC ATT GAT TGA GAT; expected product size, 341 bp).
Specificity of COX VIIc down-regulation determined by limiting-dilution RT-PCR and Northern analysis. Because phagocytosis alone can lead to changes in the host cell, we investigated the extent to which the down-regulation of COX VIIc was a specific response to infection with *M. tuberculosis*. RNA from macrophage cultures infected with live, virulent *M. tuberculosis* (5 days) was compared to that from macrophages which had phagocytosed latex beads or heat-killed *M. tuberculosis* or had been infected with live BCG, using limiting-dilution RT-PCR and Northern hybridization.

For limiting-dilution RT-PCR analysis, specific primers based on the published sequence for COX VIIc were used; these primers gave an expected product size of 151 bp. cDNAs from infected macrophages and control cells were diluted in serial fourfold steps prior to the PCR. The results (Fig. 4a) show that at a dilution of 1:256, the message for COX VIIc disappears from the *M. tuberculosis*-infected macrophages but is still detectable in uninfected and BCG infected cells and in cells which have phagocytosed latex particles or heat-killed *M. tuberculosis*. Similar experiments were carried out with primers for β-actin in order to confirm that the amounts of cDNAs used were similar and that the signal disappeared at the same dilution in each group (Fig. 4b).

Further confirmation of the specificity of the down-regulation of COX VIIc was obtained by Northern analysis. The hybridization probe was a 32P-labelled COX VIIc cDNA product giving a transcript of approximately 0.4 kb. Figure 5a (lane 5) shows that COX VIIc was down-regulated only in live *M. tuberculosis*-infected macrophages. The radioactivity of the bands was integrated over selected areas of the scanned image (ImageQuant software). The histograms show quantitative differences between the amounts of hybridization products: *M. tuberculosis*-infected macrophages (Fig. 5a, lane 5) gave a value of 2,809 U, while killed *M. tuberculosis*-treated macrophages 3954 RAGNO ET AL. INFECT. IMMUN.

FIG. 1. (a) Example of DD RT-PCR polyacrylamide gel analysis after overnight exposure. The arrow indicates band U61, which is down-regulated in *M. tuberculosis*-infected cells 5 days after infection. The primer combination in this case was DD2-OPA 18. Lanes: I, infected macrophages; U, uninfected macrophages. (b) Reamplification of U61. The left panel shows a 197-bp PCR product obtained following elution of the band and amplification with primers DD2 and OPA 18, run in duplicate on a 2% agarose gel. The right panel shows the same PCR product amplified with primers T7-DD2 and OPA 18; this product was used for the RPA.

FIG. 2. Sequence alignment showing sequence identity (95%) between U61 and the murine COX VIIc (accession no. X52940).
(lane 4) (4,450 U), BCG-infected macrophages (lane 3) (4,809 U), and macrophages treated with latex beads (lane 2) (4,249 U) showed no significant difference from the uninfected control cells (lane 1) (4,615 U). For loading controls, filters were stripped and reprobed with a labelled β-actin probe; Fig. 5b shows that the amounts of RNAs in all five lanes were equal. Thus, the 50% reduction in COX VIIc RNA demonstrated by the RPA was confirmed by Northern analysis, and this down-regulation appeared to be specific for macrophages infected with M. tuberculosis.

Down-regulation of COX VIIc also occurs in splenic macrophages of mice infected with M. tuberculosis and is associated with apoptosis. In order to investigate whether down-regulation of COX VIIc is a common feature of M. tuberculosis-infected cells, we used an ex vivo model of infection in which splenic macrophages from infected mice are cultured in vitro for 3 days. Such cells are 90% positive for the macrophage marker F4/80 by immunohistochemical staining (data not shown). RNA was extracted from these cells, and RT-PCR was carried out to detect COX VIIc mRNA. As can be seen in Fig. 6a, there was once again a clear down-regulation of COX VIIc mRNA in infected compared to uninfected cells. Because COX VIIc is a subunit of the holoenzyme COX, we also probed for changes in expression of COX I, which forms the core of the whole oxidase complex; again there was a clear down-regulation of mRNA (Fig. 6a).

COX is associated with the membranes of mitochondria (10). Since perturbation of mitochondrial membranes is an early event in apoptosis (14, 16, 35) and since apoptosis of mycobacterium-infected cells has been reported (15, 20), we investigated whether changes in COX VIIc and COX I mRNAs could be correlated with changes in the expression of genes associated with apoptosis. We therefore compared expression of the gene encoding the prosurvival molecule Bcl-2 (16, 22, 30, 32, 35) with that of the gene encoding the proapoptotic protein Bax (22). As is evident from Fig. 6, there is a striking down-regulation of bcl-2 and a corresponding up-regulation of bax in the infected ex vivo cells. The fact that these ex vivo cells were apoptotic was confirmed by using a TUNEL assay to demonstrate the presence of cells undergoing programmed cell death (Fig. 6b).

**DISCUSSION**

In order to be able to survive and grow within macrophages, pathogenic bacteria, such as mycobacteria, have to avoid or resist exposure to the microbicidal potential of those cells. In order to investigate mechanisms by which this might be achieved, we have used DD RT-PCR to investigate the influence of phagocytosed M. tuberculosis on macrophage gene expression. DD RT-PCR allows identification of mRNAs that are uniquely expressed by a cell or tissue type, by cells at various stages of development, or by cells responding to different stimuli (2, 18, 19). The value of DD RT-PCR lies in the possibility of isolating and identifying new genes involved in a particular cellular response rather than monitoring expression of known genes. The nine combinations of arbitrary and anchored primers which were used in this study allow analysis of only a small fraction of the macrophage mRNA population to be investigated. Nevertheless, several differentially expressed mRNAs were identified. We were particularly interested in one of these, designated U61, because it was rapidly down-regulated following infection and remained so throughout the course of the infection. Sequencing of this band revealed it to encode COX VIIc; subsequent analysis of expression of the gene encoding COX VIIc, carried out by using probes and primers based on the COX VIIc sequence (rather than the U61 sequence), confirmed that this gene is down-regulated.

**FIG. 3.** RPA to confirm down-regulation of U61. The product obtained from the M. tuberculosis-infected macrophages (lane I) shows a reduction in intensity of approximately 50% in comparison with that from uninfected cells (lane U). Lane −, negative control consisting of the probe plus an irrelevant RNA.

**FIG. 4.** Limiting-dilution RT-PCR analysis of cDNAs from uninfected, live M. tuberculosis (Mtb)-infected, and BCG-infected macrophages and from macrophages treated with latex beads and heat-killed M. tuberculosis 5 days after infection or phagocytosis. Specific primers for murine COX VIIc (a) or for beta-actin (as a loading control) (b) were used. PCR products were electrophoresed on a 2% agarose gel.
Three techniques, i.e., RPA, limiting-dilution RT-PCR, and Northern analysis, were used to confirm and quantify the degree of down-regulation of the COX VIIc gene and to investigate the specificity of the response. These three techniques were all used with independently generated material, and hence the findings were confirmed by using the three different techniques. The results indicate that there is approximately a 50% reduction in COX VIIc mRNA in macrophages infected with live *M. tuberculosis* compared to macrophages infected with BCG, untreated macrophages, or macrophages which had phagocytosed latex particles or heat-killed *M. tuberculosis*. Since *M. tuberculosis* and BCG are closely related organisms, this implies that down-regulation of COX VIIc is a very specific response and hence may be important in the infection process. The fact that both COX VIIc and COX I are significantly down-regulated in ex vivo cells cultured from *M. tuberculosis*-infected mice (Fig. 6a) suggests that this response is genuinely associated with the infection process.

COX is the terminal enzyme of the electron transport chain (10). It catalyzes the reduction of oxygen to water. The electron donor is cytochrome c, and the reaction results in translocation of protons across the organelle membrane (6). COX is located in the mitochondrial membrane, and some of its subunits are encoded in mitochondrial DNA, while others are encoded in the nucleus. The subunit COX VIIc is encoded in the nucleus (11, 27), and the protein is translocated to the mitochondria, where it is incorporated into the membrane-bound COX complex. Several possible physiological effects of down-regulation of COX VIIc by *M. tuberculosis* might be envisaged. First, it could reflect a general interference with

---

**FIG. 5.** Northern analysis of RNAs from uninfected (lanes 1), live *M. tuberculosis*-infected (lanes 5), and BCG-infected (lanes 3) macrophages and from macrophages treated with latex beads (lanes 2) and heat-killed *M. tuberculosis* (lanes 4) 5 days after infection or phagocytosis. (a) Expression of COX VIIc; (b) expression of beta-actin. In each case the top panel shows an autoradiogram of the hybridization membrane, and the bottom panel shows quantification of the hybridization signal by phosphorimaging analysis.

---

**FIG. 6.** (a) RT-PCR analysis of cDNAs from adherent spleen cells of *M. tuberculosis*-infected mice and uninfected control mice. Specific primers for murine COX VIIc, COX I, Bcl2, and Bax or for beta-actin (as a loading control) were used. PCR products were electrophoresed on a 2% agarose gel. (b) TUNEL staining of adherent spleen cells from *M. tuberculosis*-infected mice. The arrow indicates apoptotic cells in green.
oxidative metabolism and in particular the generation of reactive oxygen species. Reactive oxygen species are known to be important components of the antimicrobial defense system of macrophages, and *M. tuberculosis* is thought to inhibit the macrophage oxidative burst (5, 24, 28) as a survival mechanism; this could be achieved by transcriptional regulation of host cell genes involved in oxidative metabolism. Alternatively, the response could reflect the importance of apoptosis in the macrophage-mycobacterium interaction. A number of intracellular pathogens have been shown to induce apoptosis in infected host cells (1, 9, 20, 38), and this has been shown to be the case for macrophages infected with mycobacteria (15, 20). Perturbation of the mitochondrial outer membrane is a key feature of apoptotic death (14, 17, 35, 36). Since COX is a mitochondrial membrane-embedded protein, down-regulation of COX could represent either an early apoptotic signaling event or a downstream consequence of apoptotic signaling.

In order to investigate the association between changes in expression of COX-encoding genes and genes known to be involved in apoptosis, we used the ex vivo model to look at changes in expression of *bcl-2* and *bax*. The *bcl-2* oncogene promotes hemopoietic survival and cooperates with c-myc to immortalize cells (32); thus, up-regulation of *bcl-2* is associated with cell survival (35). Bax, on the other hand is a proapoptotic protein; up-regulation of *bax* is associated with accelerated programmed cell death (22). In our ex vivo model system the down-regulation of *bcl-2* and up-regulation of *bax* (Fig. 6a) indicate that the host cells were committed to apoptosis, a fact confirmed by TUNEL staining. Proapoptotic changes in expression of *bcl-2* and *bax*, along with the down-regulation of the genes encoding COX I and COX VIIc, were also shown to occur within 6 h of infection of peritoneal macrophages in vitro, before apoptotic cells could be detected by TUNEL staining (data not shown); this suggests that changes in COX gene expression are part of an early commitment to programmed cell death.

The regulation of cytokine-encoding genes in macrophages infected with mycobacteria has been widely investigated (3, 4, 12, 13, 24, 25, 29, 37). In this study we demonstrate that mycobacterium-macrophage interactions are likely to involve the cross-regulation of many genes other than those encoding cytokines. Of course, many of these changes are likely to be the result of phagocytosis or nonspecific interactions between intracellular bacteria and macrophages. However, the demonstration of at least one gene which is transcriptionally regulated in a manner which is relatively specific to infection with *M. tuberculosis* emphasizes that both the pathogen and the host have evolved specific mechanisms for coping with the interaction. Understanding of these mechanisms should shed new light on the pathogenesis of tuberculosis.

**ACKNOWLEDGMENT**

I.E.-G. was supported by a fellowship from the EDD/COFFA/SNI.

**REFERENCES**


