INFECTION AND IMMUNITY, Mar. 2003, p. 1125–1133 Vol. 71, No. 3
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.3.1125–1133.2003

Microarray Analysis of mRNA Levels from RAW264.7 Macrophages Infected with *Brucella abortus*

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Received 12 June 2002/Returned for modification 5 September 2002/Accepted 12 November 2002

Identification of host responses at the gene transcription level provides a molecular profile of the events that occur following infection. *Brucella abortus* is a facultative intracellular pathogen of macrophages that induces chronic infection in humans and domestic animals. Using microarray technology, the response of macrophages 4 h following *B. abortus* infection was analyzed to identify early intracellular infection events that occur in macrophages. Of the >6,000 genes, we identified over 140 genes that were reproducibly differentially transcribed. First, an increase in the transcription of a number of proinflammatory cytokines and chemokines, such as tumor necrosis factor alpha, interleukin-1β (IL-1β), IL-1α, and members of the SCY family of proteins, that may constitute a general host recruitment of antibacterial defenses was evident. Alternatively, *Brucella* may subvert newly arriving macrophages for additional intracellular infection. Second, transcription of receptors and cytokines associated with antigen presentation, e.g., major histocompatibility complex class II and IL-12p40, were not evident at this 4-h period of infection. Third, *Brucella* inhibited transcription of various host genes involved in apoptosis, cell cycling, and intracellular vesicular trafficking. Identification of macrophage genes whose transcription was inhibited suggests that *Brucella* utilizes specific mechanisms to target certain cell pathways. In conclusion, these data suggest that *B. abortus* can alter macrophage pathways to recruit additional macrophages for future infection while simultaneously inhibiting apoptosis and innate immune mechanisms within the macrophage, permitting intracellular survival of the bacterium. These results provide insights into the pathogenic strategies used by *Brucella* for long-term survival within a hostile environment.

Bacterial infections require significant interaction between host and pathogen, and the consequences of this interaction determine the outcome of infection. Host defense mechanisms often lead to rapid clearance of the pathogen, and the uptake of bacteria into macrophages is usually fatal for the bacteria. Although many bacteria elude destruction by avoiding macrophages altogether, intracellular bacteria are capable of surviving, and often replicating, inside the macrophage. Thus, for intracellular bacteria, survival and replication within phagocytic cells is the key to pathogenesis.

Brucella abortus, a gram-negative facultative intracellular bacterium and zoonotic pathogen, causes hepatitis, arthritis, and endocarditis in humans and spontaneous abortion in cattle (17). Although, the specific mechanisms of intracellular survival by *Brucella* are not clearly understood, bacteria often alter normal host function to avoid immune detection. Successful strategies for intracellular survival include the ability to survive in acidified membrane-bound vesicles (25, 26), alteration of macrophage apoptosis (5, 8, 14, 19), prevention of phagosome-lysosome fusion (1), and utilization of detoxification and repair mechanisms. Defining the interaction between a host cell and *Brucella* is crucial to understanding the infectious process.

The goal of this study was to define the transcript profile of macrophages exposed to *B. abortus* for 4 h, thus evaluating the early host response to this facultative intracellular bacterium. Microarray technology permits identification of the host response at the gene transcription level and can provide a molecular profile of virulence-associated responses, as well as host defense mechanisms, that occur following infection. As a consequence of infection, we identified 148 macrophage genes that were differentially transcribed in response to 4-h infection by *B. abortus.* Up-regulation in the transcription of proinflammatory cytokines and chemokines likely represents an antibacterial response by host cells. However, transcription of genes involved in cell cycling, apoptosis, and intracellular trafficking was decreased. The last group of genes may permit bacterial intracellular survival in macrophages. Therefore, these data provide a comprehensive foundation of early host gene expression to further understand the infectious process of *B. abortus*.

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MATERIALS AND METHODS

Bacteria and cell line. *B.abortus* strain S2308 (National Animal Disease Center, Ames, Iowa) was grown in 12- by 75-mm tubes on a shaker platform in 4 ml of brucella broth (Difco) or on plates of brucella broth containing 1.5% agar. The cultures were grown at 37°C for 3 days. The mouse macrophage cell line RAW264.7 (ATCC TIB71) was maintained at 37°C with 5% CO_2 in supplemented RPMI 1640 (10% fetal bovine serum, 0.2 mM L-glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin/ml) (Sigma, St. Louis, Mo.).

Cell infection. RAW cells were plated in 75-cm^2 flasks in supplemented RPMI 1640 without antibiotics 1 day prior to infection at a concentration of 6×10^6 per flask. The cells were infected for 4 h with 1 ml of 3-day *B. abortus* culture (multiplicity of infection, ~100). Following infection, the cells were washed three times with phosphate-buffered saline to remove extracellular bacteria.

Target preparation for microarray analysis. Target RNA was prepared according to protocols in the Affymetrix Gene Chip Expression Analysis technical manual (Affymetrix, Inc., Santa Clara, Calif.). Total RNA was isolated from RAW cells using the RNeasy Mini Kit (Qiagen, Valencia, Calif.) according to the manufacturer's protocol with the following modifications. Following lysis, the supernatants were centrifuged for 2 min to remove intact bacterial cells. RNA integrity was determined by gel electrophoresis. RNA was converted to double-

TABLE 1. Sense and antisense primers for RT-PCR-amplified genes

Gene name	Forward primer	Reverse primer	Product size (bp)
Ptgs2	5′ GAGAGAAGGAAATGGCTGCAGAA	5′ GGCTTCCAGTATTGAGGAGAACAGA	193
Scya4 (*MIP-1*β)	5′ ATTCCTGACCAAAAGAGGCAGACA	5′ TGGGAGAGACGCGTCCTATAAACTA	295
c-*myc*	5′ GGAAGAAATTGATGTGGTGTCTGTG	5′ TCTTGTCGTTTTCCTCCGTGTCT	290
Cish3 (SOCS3)	5′ ATGGTCACCCACAGCAAGTT	5′ AATCCGCTCTCCTGCAGCTT	137
Traf1	5′ GGGAGCCCACAATCCATGCA	5′ TCGCTTCCACAGCTGCCTGA	175
Irg1	5′ CTCTGTGGTGGGGACTCTGGGAAGT	5′ ATAGAAGGCACCGAACCCTGACCC	256
*IL-1*β	5′ GTGTGGATCCCAAGCAATACCCA	5′ CCAGCCCATACTTTAGGAAGACACAGA	221
Fcgr1	5′ TCCCTTTAGAGCTGTTTACCACGC	5′ CCTCACACCAGTAGAATCCAGCATC	234
Rab3d	5′ AAGCGAGATCCCACTGAGATGG	5′ GTCTTGACCTTGAAGTCGATGCC	206
Map3k4	5′ GAACACACCGAGTCAGTCTCCACA	5′ GCTACAGCCGAGACTGAGGTAAGG	232
Rab9	5′ AATAATTCTTCTTGGAGATGGTGGAGTTG	5′ ACTAAATGTAAGCAGGCAACAGTCAGAAC	238
Mnt	5′ AGCGGGAACGTGAGAGAGAGCA	5′ GAGACTGCGGGGAATTGGTCAC	210
caspase 3	5′ CCTGGAGAAATTCAAAGGACGGG	5′ GCATGGACACAATACACGGGATCT	192

stranded cDNA using a synthesis kit (GIBCO BRL, Rockville, Md.), except that T-7-$(dT)_{24}$ oligomer (Genset Corp., La Jolla, Calif.) was used. The cDNA was phenol-chloroform extracted and ethanol precipitated prior to the performance of in vitro transcription and labeling with biotin (Enzo Diagnostics, Inc., Farmingdale, N.Y.). The labeled cRNA was fragmented in 50 mM Tris-acetate, pH 8.1, 100 mM KOAc, and 30 mM MgOAc at 94°C for 35 min.

Probe arrays. Briefly, 16 μg of fragmented labeled cRNA was hybridized to murine U74A gene chips (Affymetrix). Washing and staining with streptavidin-phycoerythrin was done using a GeneChip Fluidics station 400 (Affymetrix). Scanning was performed with an Affymetrix GeneArray. GeneChip expression analysis software (Affymetrix) was used to scan and analyze data. The output was stored in an Excel spreadsheet. Difference calls were assigned the following values: increased, 2; marginally increased, 1; no change, 0; marginally decreased, −1; and decreased, −2. The sum of the six difference calls from the intergroup comparisons of two uninfected and three *Brucella*-infected arrays (2 × 3) was calculated. A sum of ≥8 or ≤−8 was the cutoff value for increase and decrease, respectively. Expressed sequence tag sequences were eliminated from the analysis.

Probes for Northern analysis. Probes for β-actin and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were purchased from Clontech (Palo Alto, Calif.). MIP-2 probe (CTTGGCAGGGTCTTCAGGCATTGACAGCGCAG TTCACTGGCCACAACAGC) was obtained from Oligos, Etc. (Wilsonville, Oreg.). Suppressor of cytokine signaling 3 (SOCS3) and tumor necrosis factor alpha (TNF-α) probes were generated by PCR. The SOCS3 probe was amplified by PCR using the sense primer 5′ ATGGTCACCCACAGCAAGTT and the antisense primer 5′ GCCCCCAGAATAGATGTAGT (Oligos, Etc). Primers for TNF-α were obtained from Maxim Biotech, Inc. (San Francisco, Calif.), and PCR was performed according to the manufacturer's protocol. The 531-bp SOCS3 and 351-bp TNF-α PCR products were gel purified using a Qiaex II agarose gel extraction kit (Qiagen) prior to being labeled. The probes (100 ng) were labeled with horseradish peroxidase (HRP) using a North2South Direct HRP labeling, hybridization, and detection kit (Pierce, Rockford, Ill.).

Northern blot analysis. Detection was performed using a North2South Direct HRP labeling, hybridization, and detection kit according to the manufacturer's directions. Briefly, total RNA was electrophoresed on a 1% denaturing gel, transferred onto a nitrocellulose membrane, and cross-linked by UV light. The membranes were prehybridized for 30 min, and HRP-labeled probe was added at a final concentration of 6 ng per ml. The prehybridization and hybridization temperatures were as follows: TNF-α and MIP-2, 50°C; G3PDH, 52°C; SOCS3, 54°C; and β-actin, 55°C. After a hybridization time of 90 min, the membranes were washed at hybridization temperature three times with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate for 5 min and one time with 1× SSC containing 0.1% sodium dodecyl sulfate for 5 min, followed by three washes with 2× SSC at room temperature for 5 min. Substrate development was performed according to the manufacturer's protocol (Pierce). The membranes were wrapped in plastic and exposed to film until the signal was obtained. Blots were stripped of bound probe by boiling the membrane in 0.1× SSC followed by addition of new probe to the total RNA bound to the membrane.

RT-PCR. Total RNA was isolated using the RNeasy Mini Kit according to the manufacturer's protocol, including DNase treatment. RNA (5.0 μg) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Promega, Madison, Wis.) for 1 hour at 37°C. The reverse-transcription mix was diluted 1:10 in DNase- and RNase-free water (Life Technologies, Rockville, Md.). The single-stranded cDNA was then subjected to FailSafe PCR from Epicenter (Madison, Wis.) under standard reaction conditions. Each PCR included 1× FailSafe buffer F, 0.5 to 1.0 μM sense and antisense primers for the gene of interest, 0.04 to 0.08 μM control β-actin primers (Clontech), and 5 μl of cDNA template. Sense and antisense primers for the tested genes are shown in Table 1. PCR was performed for 25 cycles at 95°C for 30 s, 62°C for 30 s, and 72°C for 60 s, followed by 72°C for 5 min. Negative controls for PCR were performed with RNA without M-MLV RT and confirmed the lack of DNA in each reaction mixture. The resultant PCR products were electrophoresed on a 1.5% agarose gel. The gel images were saved as BMP files (Foto/Analyst Archiver; Fotodyne, Hartland, Wis.) and analyzed with Un-Scan-IT Gel software (Silk Scientific Corp., Orem, Utah). Negative segment analysis was performed for each band. The correction factor was determined by dividing the digitized image of the actin band from uninfected cells by the digitized image of the actin band from infected cells.

RESULTS

Effect of *B. abortus* infection on RAW cell gene expression. Differential gene transcription was determined using RAW264.7 cells infected with *B. abortus* strain 2308. Three independent experiments were performed, and the RNA transcription levels for over 6,000 genes were determined. Transcripts for ~40% of the probe sets were detected. Of the RAW cells infected with *B. abortus*, ~3% of the expressed transcripts showed an increase in gene transcription. An equal percentage demonstrated a reduction in transcription.

Table 2 lists the genes up-regulated 4 h after *Brucella* infection, an early time following macrophage infection. Up-regulation of transcription occurred reproducibly in 69 genes. The majority of genes up-regulated during infection were associated with inflammation or apoptosis. The proinflammatory genes, *IL-1*α, *Scya2* (*MCP-1*), and *Scyb2* (*MIP-2*), were strongly induced following *B. abortus* infection, with average increases of >100-fold. Additionally, *IL-1*α,*TNF*-α, and glucocortoid-regulated inflammatory prostaglandin (*Ptgs2*) genes were also up-regulated, with increases of 42- and 67-fold, respectively. The up-regulated apoptotic genes included those for apoptosis inhibitors, such as *Naf1*, *Gadd45b*, *A1-b*, and the zinc finger protein A20, as well as the proapoptotic genes *Fas*, *LT*, *Cash*, and *Tnfrsf1b*. Changes for these genes ranged from 1.9- to >17-fold (Table 2). Other genes that were greatly increased in expression were associated with the cell membrane, e.g., the gene for a macrophage C-type lectin, which was increased >25-fold. Extracellular matrix protein gene transcription was increased, e.g., that for MMP9, a metalloproteinase (17-fold). Regulatory gene transcription was increased, e.g.,

TABLE 2. Macrophage gene expression induced by *B. abortus* infection[a]

Probe set	Accession no.	Fold increase[b]	Gene symbol	Protein or gene
Inflammation and chemokines				
103486	M15131	188.58	*Il-1*β	IL-1β
94755	M14639	42.97	*Il-1*α	IL-1α
104647	M88242	67.35	*Ptgs2*	Glucocorticoid-regulated inflammatory prostaglandin
101160	X53798	102.88	*Scyb2* (*MIP-2*)	Macrophage inflammatory protein 2
94142	M13926	20.55	*Csf3*	Granulocyte colony-stimulating factor
102736	M19681	126.23	*Scya2*	Platelet-derived growth factor-inducible protein
94761	X70058	8.07	*Scya7*	Small inducible cytokine A7
93858	M33266	6.42	*Scyb10*	Macrophage interferon-inducible protein 10 (IP-10)
94146	X62502	7.95	*Scya4* (*MIP-1*β)	Macrophage inflammatory protein 1β
102629	D84196	57.98	*TNF*-α	TNF-α
93871	L32838	8.35	*Il-1rn*	IL-1 receptor antagonist IL-1rn
95344	U65747	6.97	*IL-13ra2*	IL-13 receptor alpha 2
102712	X03505	32.30	*Saa3*	Serum amyloid A protein (SAA)
Cell membrane				
92217	U05265	3.73	*Gp49b*	Glycoprotein 49B
100325	M65027	2.2	*Gp49a*	Glycoprotein 49A
104469	M73748	5.83	*Gp38*	Glycoprotein 38
99434	AF001036	2.72	*Cd83*	CD83 antigen
92415	L15435	3.38	*Tnfsf9*	TNF (ligand) superfamily, member 9
92962	M83312	5.52	*Tnfrsf5*	TNF receptor superfamily, member 5
92730	L07264	4.22	*Hegfl*	Heparin binding epidermal growth factor-like growth factor
96551	AB024717	25.70	*Clecsf9*	Macrophage C-type lectin
Apoptosis				
104755	AJ242778	3.65	*Naf1*	A20-binding inhibitor of NF-κB activation
102921	M83649	5.02	*Fas*	Fas antigen
102779	X54149	17.15	*Gadd45b*	Myeloid differentiation primary-response gene
102940	U16985	6.78	*Ltb*	Lymphotoxin beta
103217	Y14041	1.62	*Cash*	CASH alpha protein
99392	U19463	5.87	*Tnfaip3* (A20)	TNF-induced protein 3
94928	X87128	8.05	*Tnfrsf1b*	TNF receptor superfamily, member 1b
102914	U23778	1.88	*A1-b*	Hematopoietic-specific early-response A1-b protein
93869	U23781	2.12	*A1-d*	Hematopoietic-specific early-response A1-d protein
Adhesion				
103005	X66084	2.95	*Cd44*	CD44
96752	M90551	3.77	*ICAM1*	Intracellular adhesion molecule 1
102280	AB006758	2.40	*Pcdh7*	Pcdh7 mRNA for BH-protocadherin-a
Cell cycle and proliferation				
94246	J04103	3.33	*Ets2*	Avian leukemia oncogene 2
102292	U00937	16.33	*Gadd45a*	GADD45 protein
103349	M57696	1.67	*Lyn*	Yamaguchi sarcoma viral (v-yes-1) oncogene homologue
92472	AF099973	4.15	*Slfn2*	Schlafen 2
96285	AJ001616	2.55	*Myadm*	Mycloid-associated differentiation protein
Extracellular matrix				
94147	M33960	5.55	*Serpine1*	Plasminogen activator protein PAI-1
92978	X16490	5.53	*Serpine2*	Plasminogen activator protein PAI-2
101561	K02236	5.38	*Mt2*	Metallothionein II (MT-II)
99957	X72795	17.55	*MMP9*	Matrix metalloproteinase
Transcription				
98427	M57999	3.68	*Nfkb1*	NF-κB transcription factor
92925	M61007	2.08	*Cebpb*	CCAAT/enhancer binding protein (C/EBP) beta
102362	U20735	4.35	*JunB*	JunB
94189	AB011665	3.45	*Bazf*	BcL6-associated zinc finger protein
104712	L00039	7.88	*c-myc*	Myelocytomatosis oncogene
92855	Z50159	1.75	*Suil-rsl*	Suppressor of initiator codon mutations-related sequence Sui1
98007	AJ131021	3.83	*Rsk3*	pp90 ribosomal protein S6 kinase 3
Stress				
101995	U40930	3.58	*Sqstml*	Oxidative stress-induced protein
96042	L35528	2.43	*MnSOD*	Manganese superoxide dismutase (MnSOD) gene
95722	AB013137	3.95	*GLRX*	Glutaredoxin
Transport				
103065	M73696	4.70	*Slc20aI*	Solute carrier family 20
102198	AF042487	1.90	*Kcnn4*	Intermediate conductance potassium channel mIK1

Continued on following page

TABLE 2—*Continued*

Probe set	Accession no.	Fold increase[b]	Gene symbol	Protein or gene
Signal transduction				
101457	L16956	2.13	*Jak2*	Janus kinase 2
93680	D89728	3.03	*Stk10*	Serine-threonine kinase 10
94378	U94828	3.83	*Rgs16*	Retinally abundant regulator of G-protein signaling
Regulatory				
99109	M59821	2.65	*ler2*	Growth factor-inducible protein (pip92)
102957	U20159	2.37	*SLP-76*	76-kDa tyrosine phosphoprotein SLP-76
92232	U88328	55.72	*Cish3*/*SOCS3*	Suppressor of cytokine signaling 3
94186	L35302	36.30	*Traf1*	TNF receptor-associated factor 1
Other				
100515	X54056	1.93	*PC3*	Proprotein convertase subtilisin-kexin type 3
102313	L09737	2.30	*Gch*	GTP cyclohydrolase 1
94085	M34603	2.30	*Prg*	Proteoglycan core protein
102663	X62700	9.70	*Plaur*	Urokinase plasminogen activator
98774	L38281	54.33	*Irg1*	Immunoresponsive gene 1
94384	X67644	9.70	*ler3*	Growth factor-inducible immediate-early gene gly96
101554	U57524	6.12	*Nfkbia*	I κBα
100981	U43084	10.97	*Ifit1*	Interferon-induced protein with tetratricopeptide repeats 1

[a] Difference calls were assigned the following values: increased, 2; marginally increased, 1; no change, 0; marginally decreased, −1; and decreased, −2. The sum of the six difference calls from the intergroup comparisons of two uninfected and three *Brucella*-infected arrays (2 × 3) was calculated. A sum of ≥8 was the cutoff value for increase determination.

[b] Average of six (2 × 3) increase values.

that of *SOCS3* increased 56-fold. Similarly, the TNF receptor-associated factor 1 gene (*TRAF1*) was increased 36-fold.

Genes down-regulated during infection are shown in Table 3. The majority of genes down-regulated during infection were associated with cell cycle and proliferation or intracellular trafficking. The transcription of 22 genes involved in cell cycle proliferation or differentiation was down-regulated in the presence of *B. abortus*. For example, *Cdc6* and *Lyl1* were decreased >4-fold, and *BRCA1* was decreased 6-fold. Additionally, a decrease in the transcription of nine genes involved in intracellular trafficking was observed. Members of the Rab family (Rab9 and Rab3d) and kinesin motor proteins (Kif1 and Kif4) were down-regulated, as well as genes (*Gsn* and *Pip5k*). Six genes involved in apoptosis also showed decreased transcription; one of these genes, *caspase 3*, had a >5-fold reduction in expression. The transcription of the other apoptotic genes, *Nix*, *Bip3*, *Bad*, *Birc5*, and *Siva*, was decreased ~2-fold (Table 3). *MEK kinase 4b* was decreased fivefold, while transcription of other genes, e.g., that for a nuclear protein (acidic nuclear phosphoprotein 32), was decreased 9-fold.

Confirmation of array data. To further validate the gene array results, Northern blot analysis or RT-PCR was performed on selected genes. For Northern blot analysis, total RNA from RAW cells infected for 4 h with *B. abortus* was isolated, and 5, 10, and 15 μg were loaded onto a 1% formaldehyde gel. Transcription of the *MIP-2*, *TNF*-α, and *SOCS3* genes was assessed. With uninfected RAW cells, no *MIP-2*, *TNF*-α, or *SOCS3* transcription was detected. However, in the presence of *B. abortus*, all three transcripts were present (Fig. 1). The eukaryotic housekeeping genes, the β-actin gene and *G3PDH*, were used as references for comparison of gel loadings.

RT-PCR was performed for 13 genes. All reactions were done with and without RT (data not shown) and with actin primers. No bands were observed in reactions without RT, confirming the lack of DNA in RNA samples. Transcription of β-actin gene was used as a positive control and to ensure equal amounts of cDNA in each reaction and that the PCR products were equally loaded onto the gel. RT-PCRs were performed at least twice for each gene. Representative gels are shown in Fig. 2. Increased transcription of *IL-1*α, *Traf1*, and *Ptgs2* (Fig. 2A, lane 2) and decreased transcription of *Mnt*, *Fcgr1*, and *Rab9* (Fig. 2B, lane 2) was observed, as well as equal concentrations of the β-actin product. Gel images were digitized, and the percent change in transcription between infected and uninfected macrophage RNA was determined. As shown in Table 4, *Traf*, *SOCS3*, *Ptgs2*, *Irg*, *MIP-1*α, c-*myc*, and *IL-1*α were up-regulated during infection. The percent change ranged from 17 to >200%. Six genes, *Map3k4*, *Rab3d*, *Fcgr1*, *Rab9*, *Mnt*, and *caspase 3*, exhibited decreased transcription with *B. abortus* infection. These results confirm the gene array data.

DISCUSSION

Although the entry of pathogens into macrophages is usually fatal, *Brucella* cells not only survive but replicate within these hostile cells (16). Macrophages respond to pathogens by producing cytokines, eliciting an inflammatory response, and inducing the death of the bacteria (4, 8, 28). Pathogens like *Brucella* have developed sophisticated evasion strategies, often utilizing normal host cell functions to avoid destruction (6, 20, 25). To better understand the complex interaction between the host cell and *B. abortus*, we have analyzed the differential transcription of >6,000 murine genes following a 4-h *B. abortus* infection of RAW264.7 macrophages. At 4 h, transcription of host genes would likely be activated as an early response to the intracellular infection, and likewise, the bacteria might influence the transcription of host genes at this early period of infection to help ensure their intracellular survival.

Inflammation is a powerful protective mechanism coordinated and controlled by cytokines and chemokines. Increases in macrophage cytokines have been detected in gene array

TABLE 3. Macrophage gene expression decreased by *B. abortus* infection[a]

Probe set	Accession no.	Fold decrease[b]	Gene symbol	Protein or gene
Inflammatory cytokines and chemokines				
102794	Z80112	5.0	*Cmkar4*	*lcr-1* gene
Cell membrane				
101793	X70980	10.2	*Fcgr1*	Fc receptor; immunoglobulin G; high-affinity I
94425	AB007599	1.6	*Ly86*	MD-1
Apoptosis				
93836	AF041054	2.4	*Bnip3*	E1B 19K/Bcl-2-binding protein homologue (Nip3)
99670	L37296	2.6	*Bad*	BAD protein
97828	AF033115	2.7	*Siva*	Proapoptotic protein (Siva) gene
96255	AF067395	2.4	*Nix*	NIX (Nix) mRNA; nuclear-gene-encoding mitochondrial protein
101521	AB013819	2.1	*Birc5*	TIAP mouse homologue of inhibitor of apoptosis
98436	U54803	5.4	*caspase 3*	Caspase 3; apoptosis-related cysteine protease
Intracellular trafficking				
97415	M89777	4.3	*Rab3d*	GTP-binding protein (Rab3D) mRNA
95516	AB027290	3.0	*Rab9*	SID 99 mRNA for small GTP-binding protein
102221	AJ002306	2.7	*Syngr1*	Synaptogyrin 1b
93750	J04953	1.7	*Gsn*	Gelsolin
102318	X86000	2.9	*Siat8d*	*N*-Glycan alpha 2,8-sialyltransferase
104644	D12646	1.8	*Kif4*	Kinesin heavy-chain, member 4
99541	AJ223293	2.2	*Kif11*	Kinesin-related mitotic motor protein
101109	U43512	3.3	*Dag1*	Dystroglycan 1
98428	AJ246002	2.0	*Spg4*	Spastin protein orthologue (Spast gene)
101865	AB009615	2.5	*Pip5k2a*	Type II phosphatidylinositolphosphate kinase alpha
Adhesion				
99577	M57647	4.1	*KitI*	Mouse mast cell growth factor (MGF)
95016	D50086	1.9	*Nrp*	Neuropilin
Cell cycle, differentiation, proliferation				
92481	AF086905	2.9	*Chk2*	Protein kinase Chk2 (Chk2)
103821	AJ223087	4.4	*Cdc6*	Cdc6-related protein
93666	M64360	2.5	*Lmo2*	LIM only
100467	X57687	4.7	*Lyl1*	LYL gene
103001	U43836	1.9	*Vegfb*	VEGF-related factor mvrf186 precursor mRNA
93319	U20238	3.8	*Rasa3*	GTPase-activating protein GAPIII
101027	AF069051	1.7	*PTTG*	Pituitary tumor transforming gene protein
101484	U73039	2.0	*Nbr1*	Next to Brca 1
102976	U32446	6.1	*BRCA1*	Breast cancer 1
104476	U27177	3.4	*Rb11*	Retinoblastoma-like 1 (p107)
99076	U09504	2.9	*Thra*	Thyroid hormone receptor alpha
93356	D26091	1.6	*Mcmd7*	mCDC47
92262	AF012923	2.3	*Wig1*	p53-inducible zinc finger protein (Wig-1)
99564	D87908	1.9	*Np95*	Nuclear protein np95
97963	D11374	3.2	*Spa1*	Signal-induced proliferation-associated gene 1
99632	U83902	1.8	*Mad211*	Mitotic checkpoint component Mad2
93099	U01063	2.4	*Plk*	Polo-like kinase homologue
99532	D78382	2.1	*Tob1*	Tob family
97468	AB025409	1.7	*CksI*	sid1334p
92210	AF004326	2.5	*Agpt2*	Angiopoietin 2
100427	U37465	3.0	*Ptpro*	Protein tyrosine phosphatase; receptor type; O
103057	AF024570	2.8	*Pold1*	DNA polymerase delta 1; catalytic domain
Transcription				
94698	X59421	2.4	*FliI*	Friend leukemia integration 1
94296	AF043220	2.5	*Gtf2i*	TFII-I protein short-form mRNA; alternatively spliced
98122	AF074600	2.5	*Lmo4*	LIM domain transcription factor LMO4
92300	Y07609	5.7	*Mnt*	Max-binding protein
104591	L13171	3.1	*Mefc2*	Myocyte enhancer factor 2C
99602	AF064088	2.1	*Tieg*	Transcription factor GIF mRNA
102963	L21973	3.7	*E2F-1*	E2F transcription factor 1
Stress				
94897	D87896	1.6	*Gpx4*	Glutathione peroxidase 4
102792	U55040	1.8	*Ung*	Uracil-DNA glycosylase (*ung*) gene; exon 1
98398	U22262	1.8	*Apobec1*	Apolipoprotein B editing complex 1
98071	X77731	2.5	*Dck*	Deoxycytidine kinase
97327	L26320	1.9	*Fen1*	Flap structure-specific endonuclease 1

Continued on following page

TABLE 3—*Continued*

Probe set	Accession no.	Fold decrease[b]	Gene symbol	Protein or gene
Transport				
102892	U65592	2.3	*Kcnab2*	K^+ channel beta 2 subunit mRNA
Signal transduction				
97411	L11316	2.0	*Ect2*	Ect2 oncogene
103070	AB018194	3.1	*Ptpns1*	BIT
104272	U85608	5.4	*Map3k4*	MEK kinase 4b (MEKK4b)
Regulatory				
101966	AF037206	1.9	*Rnf13*	RING zinc finger protein (Rzf)
94061	M13018	1.8	*Crip*	Cysteine-rich intestinal protein
92975	L14543	2.4	*Sh3bp2*	SH3 binding protein 3BP2
Other				
93320	AF017175	8.4	*Cpt1a*	Carnitine palmitoyltransferase 1; liver
92608	D88793	1.8	*Csrp*	Cysteine-rich protein
93582	AF080580	1.7	*Coq7*	CLK-1 (*clk-1*)
94324	U49878	1.8	*Hmgcl*	hydroxy-3-methylglutaryl-coenzyme A lyase
96310	L07508	1.7	*Mbp*	Golli-mpb
96887	Y08702	1.7	*Np15.6*	Neuronal protein 15.6
98989	AF057368	2.5	*Dhcr7*	7-Dehydrocholesterol reductase
96110	U3196	3.7	*Cbr1*	6:Carbonyl reductase
93908	X16670	2.1	*Ccr4*	Type IIB intracisternal A-particle (IAP) element-encoding integrase
93372	U73478	9.1	*Anp32*	Acidic nuclear phosphoprotein 32
100323	Z23077	1.9	*Samdc*	*S*-Adenosylmethionine decarboxylase 3
100596	M32032	4.7	*Selenbp1*	Selenium binding protein 1
101104	AB001990	2.2	*dcra*	Dcra
100978	U62105	1.9	*FSHD*	FSHD region gene 1
103032	AF038008	1.5	*TPST-1*	Tyrosylprotein sulfotransferase-1
94815	X13586	3.3	*Bpgm*	2,3-Bisphosphoglycerate mutase
96081	X60980	6.1	*TK*	TK gene encoding thymidine kinase

[a] Difference calls were assigned the following values: increased, 2; marginally increased, 1; no change, 0; marginally decreased, −1; and decreased, −2. The sum of the six difference calls from the intergroup comparisons of two uninfected and three *Brucella*-infected arrays (2 × 3) was calculated. A sum of ≤−8 was the cutoff value for decrease determination.

[b] Average of six (2 × 3) decrease values.

experiments using other gram-negative intracellular bacteria, such as *Salmonella enterica* serovar Typhimurium (27) and *Listeria monocytogenes* (2). Similar up-regulation of inflammation-associated genes was observed during *B. abortus* infection. Up-regulation of transcription in *B. abortus*-infected cells was verified by RT-PCR with five genes, *Ptgs2*, *MIP2*, *IL-1*β, *MIP-1*α, and *TNF*-α. Additionally, our results confirm reports that *B. abortus* up-regulates *TNF*-α in mouse macrophages (10). Thus, *B. abortus*-infected macrophages mount a powerful inflammatory response in an effort to clear this pathogen. Alternatively, the influx of inflammatory cells to the site of infection may provide additional host cells for *B. abortus* to infect.

Sustained or excessive production of inflammatory cytokines can have damaging consequences. A strong inflammatory response can enhance the invasiveness of some bacteria by increasing tissue destruction, permitting bacterial dissemination (29). To counterbalance inflammatory cytokines, anti-inflammatory cytokines and/or inhibitors of signal transduction are produced. The balance between pro- and anti-inflammatory signals may influence the outcome of disease (34). Anti-inflammatory cytokines include interleukin 10 (IL-10), transforming growth factor β, and IL-1 receptor antagonist (IL-1ra) (12, 23). Transcription of *IL-1ra* was increased in the presence of *B. abortus*. During *Brucella* infection, anti-inflammatory signals may decrease the potentially damaging effects of proinflammatory cytokines on host tissue.

Alterations in cell surface and adhesion molecules may

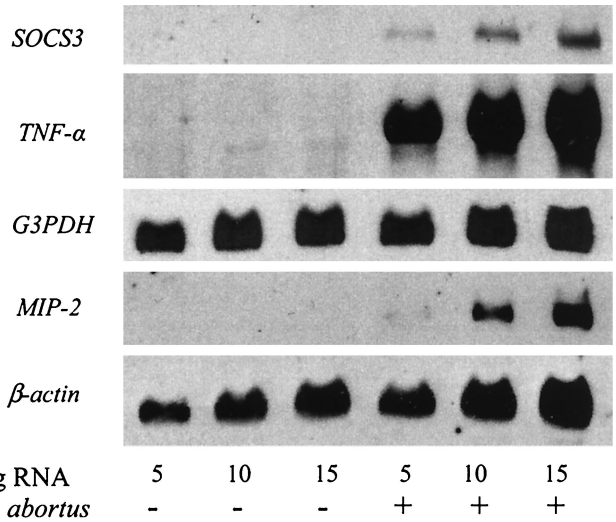

FIG. 1. Northern blot analysis of *SOCS3*, *TNF*-α, and *MIP-2* transcription in RAW264.7 macrophages infected with *B. abortus* or uninfected. Total RNA was isolated from RAW cells infected for 4 h with *B. abortus* and compared to RNA from uninfected cells. RNA (5, 10, or 15 μg) was separated by denaturing gel electrophoresis and transferred to a nitrocellulose membrane. The blots were hybridized with probes to *SOCS3*, *TNF*-α, and *MIP-2*. Probes to the housekeeping genes *G3PDH* and β-*actin* were used as controls.

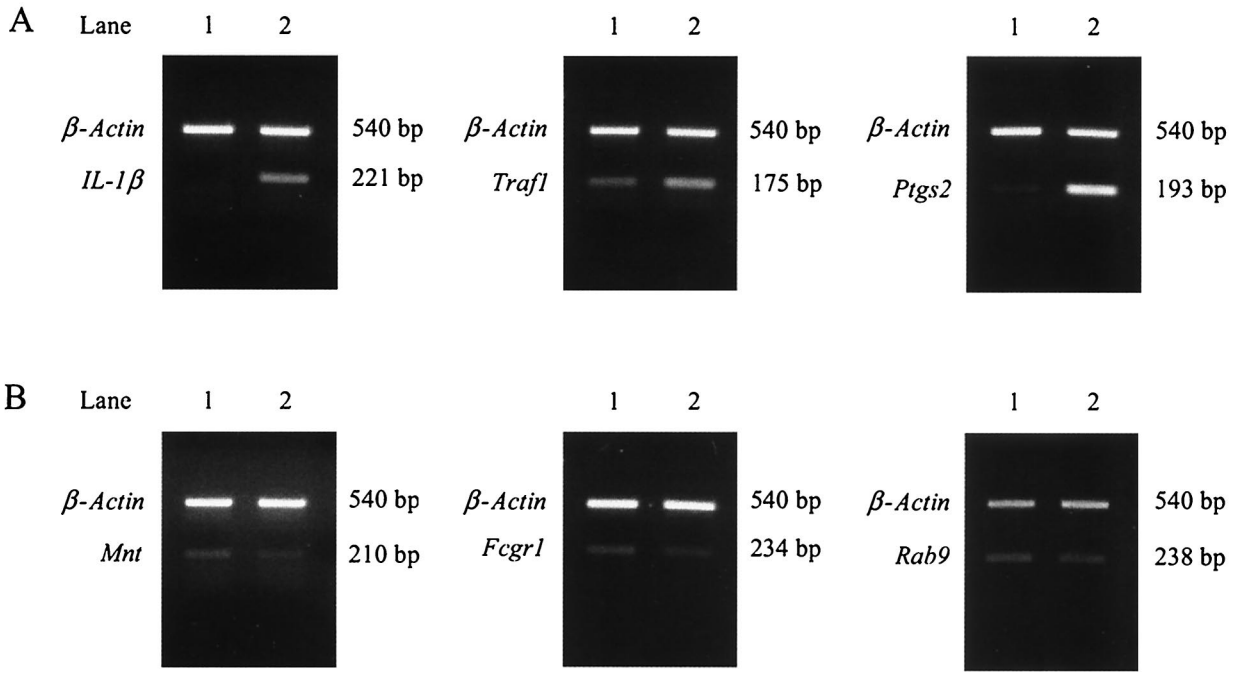

FIG. 2. RT-PCR analysis of selected gene transcription in RAW264.7 macrophages infected with *B. abortus* or uninfected. Total RAW cell RNA from uninfected (lanes 1) cells or 4 h postinfection with *B. abortus* (lanes 2) was reverse transcribed with M-MLV RT, and PCR was performed. Transcription of up-regulated (A) and down-regulated (B) genes is shown. β-Actin (540 bp) was included in all reactions to verify equal cDNA concentrations in the PCR and on the gel.

facilitate bacterial clearance or macrophage infection. For example, up-regulation of the metalloproteinase MMP9 may facilitate macrophage-bacterium contact through extracellular matrix digestion, while expression of macrophage C-type lectin on the macrophage surface may serve to enhance the uptake of bacteria (31).

Cell function depends on multiple signaling pathways that control the decision to proliferate, differentiate, or initiate apoptosis (33). Disruption of these pathways by pathogens leads to alterations in both proliferation and cell death. Central to the control of cell proliferation are the retinoblastoma (*Rb*) and *p53* genes. p53 inhibits the proliferation of damaged cells, thus inhibiting cell cycle progression and inducing apoptosis. Also involved in the p53 signaling pathway are Chk2 kinase, which triggers the p53 pathway, and Wig-1, which is induced in human cells following DNA damage (13). Transcription factor E2F1 activates the transcription of genes involved in cell cycle progression, as well as DNA synthesis. However, the association of E2F1 with Rb prevents cell proliferation. Furthermore, this E2F/Rb pathway interacts with pathways that control apoptosis. As with the E2F/Rb and p53 pathways, c-myc influences both proliferation and apoptosis pathways. Down-regulation of cell cycle genes during *B. abortus* infection may permit cell cycle progression and inhibition of apoptosis.

Stress also affects cell cycle progression. Specifically, Lyn and GADD45a are induced in response to DNA damage and function to prevent proliferation of damaged cells (9, 30). JunB represses cyclin D transcription (24), thereby arresting cell cycle progression. An increase in mRNA levels by stress-associated genes may block macrophage proliferation yet allow intracellular *Brucella* organisms to multiply. Findings with these genes appear contradictory to those with *Rb*, *Wig*, *Chk2*, and c-*myc*. However, one set of genes may be the result of the host response rather than the influence of the pathogen. Additional experiments will be necessary to identify the contributions of host and pathogen.

Apoptosis plays a significant role in regulating the pathogenesis of infection. To survive, intracellular pathogens may induce and/or block apoptosis. The main components of the apoptotic process include the surface receptors (death domain

TABLE 4. Changes in gene expression detected by RT-PCR

Gene	Digitized counts		% Change[b]
	Without *B. abortus*	With *B. abortus*[a]	
*IL-1*β	25,787	59,434	130
Ptgs2	51,368	155,445	203
*MIP-1*β/*Scya4*	222,869	308,561	38
c-*myc*	115,055	215,706	88
Cish3/*SOCS3*	75,038	168,048	124
Traf1	448,160	522,370	17
Irg1	135,260	239,045	77
Fcgr1	51,011	41,805	−18
caspase 3	32,502	29,505	−9
Rab3d	16,429	11,814	−28
Rab9	42,724	37,378	−13
Mnt	59,939	51,396	−14
Map3k4	6,092	4,772	−22

[a] Digitized image of band from infected macrophages was normalized to uninfected band by calculating the correction factor. Correction factors ranged from 0.95 to 1.03.

[b] Percent change was calculated using the formula percent change = (value for *B. abortus* treated − value for not treated/value for not treated) × 100.

receptors, FAS, and TNF receptors), cysteine proteases (caspases), and the Bcl-2-like family of proteins. The family of death receptors includes Fas cell surface receptor, FADD, TNFR1, and TRAIL. The binding of Fas ligand to Fas antigen induces apoptosis. Although an increase in expression of *Fas* was seen in our experiments, no change in transcription was observed with the Fas-associated (FADD-like) protein or TRAIL gene. Furthermore, although the transcription of *TNFR2*, a death domain gene that can trigger apoptosis, was increased, no increase was observed in its ligand, *TRADD*.

Several pathogenic organisms alter the caspase pathway, which is important in apoptosis. *Shigella* (15) and *Salmonella* (14) utilize similar mechanisms to induce apoptosis via the caspase-1 pathway. *Legionella*-induced apoptosis of macrophages utilizes caspase 3 (7), as does *Mycobacterium* (3). *Chlamydia* protects infected cells against apoptosis early in infection but induces apoptosis during late stages of infection (5, 22). We determined that *caspase-3* was down-regulated in *B. abortus* infection at 4 h. Prevention of apoptosis during infection would permit the survival and replication of intracellular *Brucella* organisms. Studies are under way to examine the roles of caspase proteins during *Brucella* infection in greater detail.

The Bcl-2 family is composed of prosurvival (Bcl-2 and A1) and proapoptotic (Bad, Bax, Bak, and Bid) proteins. Our results indicate that the *A1* gene is up-regulated in *B. abortus* infection, similar to a report about *Brucella suis* in which up-regulation of the *A1* gene blocked apoptosis (11). Additionally, genes encoding two proapoptotic Bcl-2 proteins, *Bnip-3* and *Bnip-31*, were down-regulated, as well as *Bad*. Thus, many of the genes that function in proapoptotic pathways are down-regulated in *B. abortus* infection of macrophages, while the prosurvival gene *A1* is up-regulated. This alteration of apoptosis may result in the increased survival of *Brucella* within the macrophage.

A key defense mechanism of the host cell during infection is the production of reactive oxygen. The gene encoding the oxidative stress-induced protein, *Sqstml*, was up-regulated, suggesting the generation of reactive oxygen species. Manganese superoxide dismutase, a reactive-oxygen scavenger, was up-regulated, as well as the metallothionein gene (*MT2*), which plays a role in the detoxification of heavy metals and scavenging of free radicals. Metallothionein may alter the induction of apoptosis due to oxidative stress (18).

Transcription was down-regulated in nine genes whose proteins are involved in intracellular trafficking. With *Salmonella*, interference in intracellular membrane trafficking leads to the inhibition of phagosome-lysosome fusion (32). The down-regulation of two intracellular-trafficking genes, those for *Rab3d* and *Rab9*, was verified by RT-PCR. The Rab proteins are key regulators of membrane trafficking and function in tethering and vesicle movement. Specifically, Rab9 appears to mediate vesicle transport from endosomes to the trans-Golgi. The modulation of genes involved in intracellular trafficking may serve to increase survival of *B. abortus* within macrophages by redirecting vesicular movement and preventing phagosome-lysosome fusion.

In summary, determining the macrophage genes that are transcribed during the early stages of *Brucella* infection can establish new hypotheses regarding the molecular pathogenesis of brucellosis. Using the RAW264.7 macrophage cell line, common host defense mechanisms, as well as pathogen-specific manipulations of those defenses, were identified. First, the increase in a number of proinflammatory cytokines was evident, similar to findings with human macrophages activated by several gram-negative and -positive bacteria (21). These proinflammatory components may constitute a general host recruitment of antibacterial defenses. However, *Brucella* may subvert newly arriving macrophages for additional intracellular infection. Second, mRNA levels of receptors and cytokines associated with antigen presentation, e.g., IL-12p40 and major histocompatibility complex class II, were not evident at this 4-h period of infection. Third, *Brucella* infection inhibited the mRNA levels of a number of host genes involved in apoptosis, cell-cycling, and intracellular-trafficking mechanisms among cytoplasmic compartments. Decreased mRNAs of specific genes suggests that *Brucella* utilizes specific mechanisms to alter the expression of certain genes. Our results provide a unique opportunity to select particular host pathways to determine how intracellular *Brucella* organisms can alter such pathways to ensure a bacterial advantage for intracellular survival. Pathogen-specific manipulations of host pathways have practical applications in designing vaccines and therapies that engage the innate immune system in a targeted fashion. The DNA microarray expression data in the present study provide a foundation for further understanding the long-term survival of *Brucella* in an immunocompetent host.

ACKNOWLEDGMENTS

This work was supported by grants RO1 AI48490 from the National Institutes of Health and US.-2968-98C from the Binational Agricultural Research and Development (BARD) Fund.

We thank Bruce Jarvis and Gireesh Rajashekara for valuable discussions and critical review of the manuscript. Appreciation is expressed to Sandra Splinter for probe array hybridization and scanning.

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Editor: B. B. Finlay