J Microbiol Immunol Infect.
2009;42:27-37

Original Article

Differential transcriptional responses between the interferon-γ–induction and iron-limitation models of persistence for *Chlamydia pneumoniae*

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Received: January 18, 2008 Revised: March 2, 2008 Accepted: March 21, 2008

Background and purpose: *Chlamydia* spp. are important pathogens of humans and animals that cause a wide range of acute and chronic infections. A persistence model has been developed in which *Chlamydia* spp. do not complete their developmental cycle, have significantly reduced infectivity for new host cells, and exhibit abnormal inclusion and reticulate body morphology. This study was performed to compare the interferon-γ (IFN-γ) induction and iron-limitation models of persistence for *Chlamydia* spp. to investigate the common and unique transcriptional pathways involved.

Methods: A quantitative real time-polymerase chain reaction approach was used to compare the IFN-γ induction and iron-limitation models of *Chlamydia pneumoniae* persistence at the transcriptional level by analyzing selected genes in each of 5 distinct, functionally relevant subcategories.

Results: The models showed minimal evidence of a general transcriptional stress response in persistence, with only 1 of the 7 genes analyzed in the IFN-γ induction model (*htrA*) and 4 of the genes in the iron-limitation model (*htrA*, *clpB*, *clpP1*, *ahpC*) showing increased mRNA levels. Both models showed similar responses in relation to the genes associated with lack of reticulate body to elementary body conversion (*ctcB*, *lcrH1*, and *hctB* levels were all unchanged or downregulated). The models also showed similar responses to the key cell wall/envelope genes, *ompA*, *omcB*, and *crpA*, exhibiting lower mRNA levels in both models.

Conclusions: These data show that several key transcriptional pathways (lack of late developmental cycle completion, key cell wall components) respond similarly between the models. However, other pathways appear to differ depending on the persistence-inducing mechanism. This result suggests that *Chlamydia* spp. have evolved more than 1 mechanism to respond to different persistence-inducing conditions, but ultimately the pathways probably converge through a common persistence regulon.

Key words: *Chlamydia*; Etiology; Interferon-gamma

Introduction

Chlamydia (Chlamydophila) pneumoniae is an obligate intracellular pathogen of humans causing both acute (bronchitis, pneumonia) and chronic (chronic obstructive pulmonary disease, linked to atherosclerosis) diseases [1,2]. Similar to other *Chlamydia*, *C. pneumoniae* has a distinct developmental cycle involving an extracellular infectious elementary body (EB) from which it initiates the cycle by entering the host cell by endocytosis. Once inside the intracellular vacuole, called an inclusion, the EB differentiates into a metabolically active reticulate body (RB) and

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replicates by binary fission. The RBs subsequently reorganize into EBs, which are released by host-cell lysis at 48 to 84 h postinfection to initiate new cycles of infection [3]. While the acute or lytic phase has been well characterized, the chronic or persistent phase has only recently been described, and is less well characterized.

Chlamydial persistence has been described as a viable but non-cultivable growth stage, resulting in a long-term relationship with the infected host cell often associated with the formation of typical large aberrant reticulate bodies (aRBs) [4]. In vitro, such factors as antibiotic exposure [5], amino acid deficiency [6], iron limitation [7], interferon-γ (IFN-γ) exposure [8], heat shock [9], monocyte infection [10], and phage infection [11] result in persistence. IFN-γ induction is the best studied persistence model, and it is now known that a major reason for the induction of persistence via this model is that IFN-γ induces the enzyme indoleamine 2,3-dioxygenase (IDO), which depletes host cell tryptophan pools, resulting in a tryptophan-limited environment for *Chlamydia* spp. [12]. Addition of tryptophan back to this model releases the organisms from the persistent state [8]. Similarly, limitation of intracellular iron also induces a persistent state in vitro, resulting in aRBs being generated. This model is characterized by depleting the intracellular pool of iron using ferric chelators such as deferoxamine-mesylate (DAM). Subsequent removal of the iron chelator or supplementation with holotransferrin, likewise releases the organisms from the persistent state, resulting in a completed development cycle with the production of infectious EBs [7,13]. Both the IFN-γ–induction and iron-limitation models are particularly relevant in vivo. A poor immune response means that while some IFN-γ is produced, the levels are not sufficient to kill all the *Chlamydia*, potentially pushing them to an IFN-γ–induced persistence. Likewise, iron is an essential element for bacterial growth, including a wide range of metabolic processes, and all pathogenic bacteria have evolved iron binding and transport systems to overcome conditions of poor iron availability inside their host [14]. Limitation of iron in vivo is, therefore, commonly encountered by pathogens, including *Chlamydia*, and this may also push them into a persistent state.

The persistent phase of *Chlamydia* is characterized by a lack of conversion of chlamydial RBs to EBs, which is evident as reduced infectivity on subculture and morphologically abnormal inclusions filled with small numbers of enlarged, morphologically aRBs. However, understanding of persistence at the molecular level is still relatively rudimentary, with many inconsistencies present in the literature. The initial observations of *Chlamydia trachomatis* by Beatty et al reported upregulation of Hsp60 and downregulation of major outer membrane protein antigen levels [15]. Since this early investigation, more than 15 studies have examined gene transcript levels in the various models of chlamydial persistence, with key studies being reported by Al-Younes et al [13], Hogan et al [16], Belland et al [17], Gérard et al [18], Nicholson and Stephens [19], and Slepenkin et al [20]. Some consistencies are evident in many studies, including partial activation of the general stress response, lack of late gene activation, and continued chromosome replication without associated cell wall septation and RB division. Nevertheless, there are also many inconsistencies, both between chlamydial species and between models [16].

In this study, IFN-γ–induction and iron-limitation models of persistence were compared to look for the common and unique transcriptional pathways involved. Five persistence-relevant subcategories were selected and a subset of genes from each category were analyzed as indicators of the chlamydial transcriptional response.

Methods

Cell culture and growth of *Chlamydiae pneumoniae*

HEp2 cells (ATCC CCL-23) were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and maintained in Iscove's Maintenance Medium (IMM; Cellgro, Washington, DC, USA) supplemented with 10% (v/v) fetal bovine serum, L-glutamine 2 mM, 1% (v/v) non-essential amino acids, HEPES buffer 10 mM, gentamycin 10 μg/mL, and vancomycin 25 μg/mL. Cells were grown in 75-cm^2 flasks (Costar, Cambridge, MA, USA) at 37°C in 5% carbon dioxide (CO_2) for 48 h to achieve confluency of the monolayer, and harvested with trypsin-ethylenediaminetetraacetic acid.

C. pneumoniae A-03 (ATCC VR-1452) was originally isolated from an atheroma of a patient with coronary artery disease [21] and was propagated in HEp2-cell monolayers in Iscove's Growth Medium (IGM; IMM with 8.8% [v/v] glucose and cyclohexamide 1.0 μg/mL) centrifuged at 676 × g (Sorvall RT 6000D Centrifuge; Kl Scientific Pty Ltd, Lane Cove, Australia) for 30 min at 10°C and incubated at 37°C in 5% CO_2. EBs were harvested and purified, as previously described

[22], by disruption of HEp2-cell monolayers with a cell scraper, sonication, and centrifugation over a renograffin density gradient. EB suspensions were stored in sucrose-phosphate-glutamic acid buffer at –80°C, and titration of viable EBs was determined following storage.

Induction of persistence

Induction of IFN-γ–mediated persistence was similar to that previously reported [8]. HEp2 cells were seeded in 6-well plates at 1.0×10^6 cells per well in IMM and incubated overnight in 5% CO_2 at 37°C. Cells were subsequently inoculated with *C. pneumoniae* in IGM 2 mL with or without human recombinant IFN-γ 50 U/mL, centrifuged at 676 × g for 1 h at 10°C, and incubated at 37°C in 5% CO_2 for 48 h.

Establishment of the iron-limitation model of persistence involved culturing uninfected HEp2 monolayers in IGM 2 mL with or without exposure to DAM 50 μM (Sigma, St Louis, MO, USA) for 24 h prior to inoculating with *C. pneumoniae* in IGM 2 mL, with or without DAM 50 μM. The cells were then centrifuged at 676 × g for 1 h at 10°C, and incubated at 37°C in 5% CO_2 for 48 h. The monolayers of infected cells from both persistence models were washed twice with phosphate buffered saline 2 mL (1X), lysed in TRIzol reagent 2 mL (Invitrogen, Grand Island, NY, USA) for 10 min and stored at –80°C.

Each cell culture condition (normal, IFN-γ–induction, and iron-limitation) was performed in triplicate. Subsequently, the cultures were harvested, RNA was extracted, and the transcription was assayed (also in triplicate). The data are presented as the mean of these replicates.

Infectivity studies

Parallel monolayers were set up to conduct infectivity studies for each model. After the treatments described above, *C. pneumoniae*–infected HEp2 cells were harvested at 48 h and titrated for viable inclusion forming units (IFUs)/mL. Recovery of infectivity from the inducing conditions was determined by the addition of tryptophan 500 μg/mL (Sigma-Aldrich, St Louis, MO, USA) or holotransferrin 8.0 mg/mL (Calbiochem, San Diego, CA, USA) to the IFN and interleukin models, respectively, which were added immediately after infection.

Transmission electron microscopy analysis of persistence

Transmission electron microscopy (TEM) analysis was used to visualize the level of persistence by analyzing parallel cell cultures as follows. The entire contents of 1 well of a 6-well plate (normal, IFN-γ– or DAM-treated cultures) were fixed with 3% glutaraldehyde (ProSciTech, San Antonio, TX, USA) fixative (in phosphate buffer 0.1 M; pH 7.4) for 1 h at room temperature. The cells were subsequently scraped off, washed, post-fixed in 1% osmium tetroxide, and embedded in Spurr epoxy resin according to standard procedures [23]. Ultrathin sections (50 to 100 nm) were cut and stained with uranyl acetate and lead citrate stains, then examined and photographed with a JEOL 1200 EX TEM (Jeol Pty Ltd, Tokyo, Japan).

Isolation of *Chlamydia pneumoniae* cDNA

Total RNA was extracted from *C. pneumoniae*–infected HEp2 cells using the standard protocol as supplied by the manufacturer (TRIzol reagent). For each treatment (normal, IFN-γ–induction, and iron-limitation), 3 independent triplicate cultures were done and combined prior to RNA extraction. Complete removal of DNA was assured by treating the RNA with RNase-free DNase and RNase inhibitor 40 U (Roche, Castle Hill, Australia) at 37°C for 1 h. The presence of contaminating genomic DNA was excluded by performing polymerase chain reaction (PCR) on RNA samples using 16S rRNA primers as previously described [16]. The RNA was purified through an ammonium acetate/ethanol precipitation followed by 3 × 70% ethanol washes. The final pellet was dried and resuspended in diethylpyrocarbonate-treated double-distilled water 50 μL. cDNA was generated by incubating 1 μg of each total RNA and 1 μg of random hexamers (Roche) at 65°C for 10 min. RNase inhibitor 12 U and a final concentration of 1 × Expand buffer, dithiothreitol 1 mM, deoxynucleosides 1 mM, and Expand reverse transcriptase 50 U (Roche) in a final volume of 20 μL, was added to the RNA-hexamer mix. The reaction mix was incubated at 30°C for 10 min followed by 42°C for 45 min. RNA was removed by incubation with DNase-free RNase 200 ng (Roche) at 37°C for 30 min. The final cDNA was purified using QIAquick columns (Qiagen, Hilden, Germany) as per the manufacturer's specifications and stored at –20°C.

Quantitative real-time polymerase chain reaction

Primer sets were designed for all target genes using the PrimerExpress 2.0 software (Applied Biosystems, Foster City, CA, USA) [Table 1]. Amplicon standards were generated for each primer pair by amplifying *C. pneumoniae* cDNA using the ABI Prism 7000 Sequence Detection System (Applied Biosystems).

Table 1. Primer sequences for each gene studied with real-time polymerase chain reaction

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
16S rDNA	GGGTAAAGAAGCACCGGCTAA	AACGCTAGCACCCTCCGTATT
Ndk	GAGCGTCCTTTTTTCCAAGAAC	TTTGCCCCTTCCAATACTAAAACT
trxA	CGTTCTCGTTGATTTCTTTGCA	CGCAGCAAGATTTTCTAAGATAGGA
Adk	GCAAAGCCAAGCCTGCTCTA	AAGATGCGCCTGATCTAAGGTT
pyrH	CGGCGGTGGCAATATTTTAA	TTTGATCTGCCGATACACGATT
birA	AAGAAGCTCCCTGGTAGTCTCAAG	TGTCGGACAACCTGCAACAT
crpA	GGAGTGTCACCGCTGGTCTAC	TTTTGTCTTTGCCCATCCTATGA
ompA	AAAAAACTCTTAAAGTCGGCGTTATT	GGCTTGTAAGGAGCCAACAGA
omcB	AGGCGATCGATTTTCCAGACT	CTGAATTCGTAAGCAGTGATCCA
ahpC	ACACTCTCCCTAGTTGGAAAGGAA	CACGTTTCGCCATTAACAACA
gspD	GAACCCTACGGATAAAACAGTATTTTG	GAAAGCAATGCCGCCAACT
Tal	TGGCCCAAGAACCCAAATT	TCACCGTTCTGTCGGATTCC
Pyk	TCTTCAGGTGTCCCCCTATCTCT	TGGCGTCTGCCCTTTCTG
groES	AACGACCCTCCGAATTAAACC	CCACGAGCAGTGGCTTCTTC
dnaK	CGCATTGCAGGTCTAGATGTAAA	TTTATCGATTCCGTAGGCAAGAG
grpE	TTCTTCAATAGGCCAAAAGTTTAACC	CCCCTCAGGAACTTCAGAAGTCT
htrA	TCCCGCTGTTGTGTACATAGAAA	CACGGCGTCCAGGAGAAG
clpP1	CCAGGAGCTGCACATAAACTCA	CACCCGTCACTACTGTTGTGACA
clpB	GGTAGGACAGCCTTTTGCAGTCT	GACGTTGAGGATCATTTAAACCTACA
greA	TCCAAACCGTCGTGAAAGG	TTTCGAGACGGCATTTAAAAGTT
ftsK	AAGCAATGCCTCCGCAAAG	GGGTCTGTAGGAACCGCTCTAATTTTT
murA	GACAACCGCGGCCATTC	AGACCACACCTTCCTTCCAGATAA
glmU	GATTTCAGTACAGTGACCACAACA	GCCTATCTGCGAGGCAATGT
Euo	CCCAAGCGGTCCCTTAC	GCAGAAGGTCTACTATGCAACTCGTA
ctcB	AAAAATATCGCAGAACTTGGGAAA	AACGATTCCACTTAGCGGATTG
hctB	CAACTAGATGTATGCTTATGGTTTTTGTG	CTACAGTGGCTAAAGGTTCTCCTAAGA
lcrH1	GGCTTCGCGTTTTTTTATTGA	GAGCAAGCCCTCTCCTCGTA
tyrP	ACTCCCTACTGATTGCCTATTTCTG	TTTTGTTCCCGCCATAATGATA

The standard reaction mix contained cDNA 1.0 µL in a final concentration of 1 × ABI SYBR green master mix (Applied Biosystems), each primer 10 µM in a final volume of 25 µL. Cycling parameters for all reactions were as follows: denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 sec and annealing and extension at 60°C for 1 min; and melting curve analysis from 60°C to 95°C with acquisition every 1°C. Amplicon standards were purified using the QIAquick columns (Qiagen) as per the manufacturer's specifications. Each standard was diluted to 10^3, 10^5, 10^7, and 10^9 for use as a standard curve with the respective PCR. The ABI Prism 7000 Sequence Detection System (Applied Biosystems) was used to quantify *C. pneumoniae* cDNA for 48 h with all target genes, plus 1 internal reference gene, 16S rRNA. Each PCR was carried out for the gene of interest on the cDNA generated from each treatment condition along with the relevant amplicon standard and a negative control (negative). All reactions were performed in triplicate using the standard reaction mix and cycling conditions as described for the generation of amplicon standards. For each real time-PCR (RT-PCR) analysis, a new batch of sample RNA was prepared and analyzed by PCR. ABI Prism 7000 SDS software V1.0 (Applied Biosystems) was used to analyze amplification traces for each gene. A standard curve was generated by the software and the transcript copy numbers of the gene of interest for each cDNA sample was calculated on the given copy number of the amplicon standards. Grubb's test was used to identify outlying copy numbers, which were subsequently excluded from further calculations. Data are presented as the mean cDNA copy number obtained for each gene, divided by the corresponding mean 16S rRNA values to standardize for the number of chlamydial bodies present in each sample [16].

Results

Transmission electron microscopy analysis of persistence

Comparison of the morphological features of the normal, IFN-γ, and iron-limitation *C. pneumoniae*

cultures is important for the interpretation of the gene expression profiles. Fig. 1 shows the morphologies observed for normal (Fig. 1A), IFN-γ–treated (Fig. 1B), and iron-limitation–treated (Fig. 1C) cultures using TEM. Table 2 shows a summary of the populations of normal and aberrant chlamydial particles and inclusions observed in hundreds of cells using TEM. For the IFN-γ–induction model, at 48 h postinfection, untreated cultures (without IFN-γ) showed primarily normal inclusion morphology. By comparison, 90% of the IFN-γ–treated *C. pneumoniae* infected cells at 48 h postinfection contained inclusions that were morphologically abnormal in some way. The inclusion size in these IFN-γ–treated cultures was smaller (3 μm in diameter) than for the untreated cultures (5 μm) and contained an average of 5 enlarged morphologically aRBs per inclusion (Fig. 1B). The iron-limitation cultures (treated with DAM 50 U/mL) at 48 h postinfection identified that approximately 30% of inclusions (22 of 72 inclusions examined) were morphologically abnormal (Table 2, Fig. 1C). Again, the size of these inclusions was smaller (3 μm in diameter), and contained morphologically aberrant, often enlarged, giant aRBs.

Infectivity studies

To validate the models used in this study, infectivity studies were conducted with *C. pneumoniae*–infected HEp2 cells 48 h postinfection. Fig. 2A shows the reduction in viable *C. pneumoniae* IFUs by 3 orders of magnitude due to treatment with IFN-γ. Replenishing tryptophan 500 μg/mL led to the recovery of infectivity by 2 to 4 logs. Similarly, Fig. 2B shows loss of infectivity by 2.5 logs in the presence of DAM 50 μM. Addition of iron-saturated holotransferrin 8 mg/mL to the monolayers led to an increase in IFUs.

Transcriptional response to interferon-γ–induction and iron-limitation models of persistence

The relative levels of mRNA for 25 genes during normal, IFN-γ– and DAM-treated *C. pneumoniae* infections were determined by RT-PCR on cDNA generated by random priming of total RNA isolated from the infected cells. The complete set of data obtained for IFN-γ induction and iron limitation resulting from chelation with 50 μM DAM at 48 h postinfection are shown in Fig. 3. For each of the genes analyzed, the mRNA data are normalized to the 16S rRNA copy number, represented as log10 copy number × 10^8 per 16S rRNA copy number. Fifteen of the 25 genes

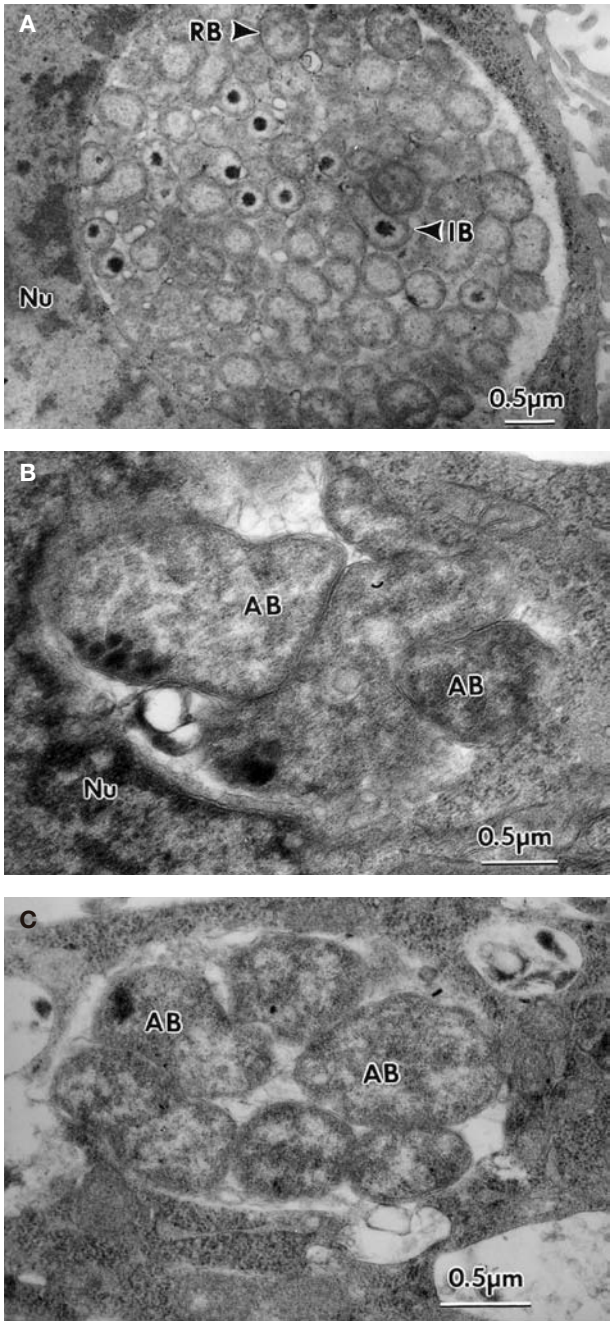

Fig. 1. Ultrastructural analysis by transmission electron microscopy of the morphological features of normal, interferon-γ–, and iron-limitation–treated cultures of *Chlamydia pneumoniae*–infected HEp2 cells at 48 h postinfection. (A) Untreated culture showing characteristic reticulate bodies (RBs) within a characteristic chlamydial inclusion (average diameter, 5 μm). (B) Interferon-γ–treated culture showing smaller inclusions (average diameter, 3 μm) with enlarged and morphologically abnormal RBs. (C) Iron-limitation–treated culture showing smaller inclusions (average diameter, 3 μm) with enlarged and morphologically abnormal RBs.
Abbreviations: AB = aberrant body; IB = intermediate body; Nu = nucleus.

Table 2. Numbers of inclusion types and different chlamydial particles observed by transmission electron microscopy analysis at 48 h postinfection of normal, and interferon-γ– and deferoxamine-mesylate–treated *Chlamydia pneumoniae* infections.

Culture conditions	Type of inclusion No. (%)	Inclusions examined	Chlamydial particles observed			
			Reticulate body	Intermediate body[a]	Elementary body	Aberrant body[b]
Untreated	Normal	102 (95.3)	4420 (82.0)	788 (14.5)	200 (3.5)	0 (0)
	Abnormal	5 (4.7)	150 (81.0)	9 (9.0)	5 (3.0)	13 (7.0)
Interferon-γ	Normal	6 (10.0)	114 (84.0)	22 (16.0)	0 (0)	0 (0)
	Abnormal	54 (90.0)	184 (60.0)	32 (10.0)	0 (0)	92 (30.0)
Deferoxamine-mesylate	Normal	50 (69.4)	1138 (97.0)	30 (3.0)	0 (0)	0 (0)
	Abnormal	22 (30.6)	138 (58.0)	6 (2.0)	0 (0)	94 (40.0)

[a]An intermediate body is one in which the chlamydial particle is bigger than an elementary body, but with electron-dense DNA.
[b]An aberrant body is a morphologically aberrant chlamydial particle bearing little similarity to a reticulate body, intermediate body, or elementary body.

analyzed showed similar mRNA patterns between the 2 persistence models, although the level of response differed between the various subcategories. Table 3 summarizes the responses measured by quantitative RT-PCR, where the ratios indicate the fold change for each persistence model at 48 h postinfection when compared with the untreated infection.

Stress response

Seven genes were analyzed in the stress response category, *groES*, *dnaK*, *grpE*, *htrA*, *clpB*, *clpP1*, and *ahpC* (Table 3). In the IFN-γ–induction model, there was a general reduction in mRNA levels for all of the stress response genes analyzed, except for *htrA*. A consistent response with both models was the marked increase in mRNA levels for the predicted DO serine protease gene, *htrA* (28.06-fold increases in the IFN-γ–induction model and 21.14-fold increases in the iron-limitation model). The 2 *clp* protease genes analyzed, *clpB* and *clpP1*, showed opposite responses between the 2 persistence models. Both genes had reduced mRNA levels in the IFN-γ–induction model, whereas the same genes had significantly increased mRNA levels in the iron-limitation model. The alkyl hydroperoxide reductase gene, *ahpC*, was also analyzed as a marker of response to peroxide/peroxidase levels in the host cell, and the 2 persistence models also showed opposite responses for this gene, with the IFN-γ–induction model showing significantly decreased mRNA levels (5.00-fold decreased levels), whereas the iron-limitation model showed significantly increased mRNA levels (8.56-fold increased levels).

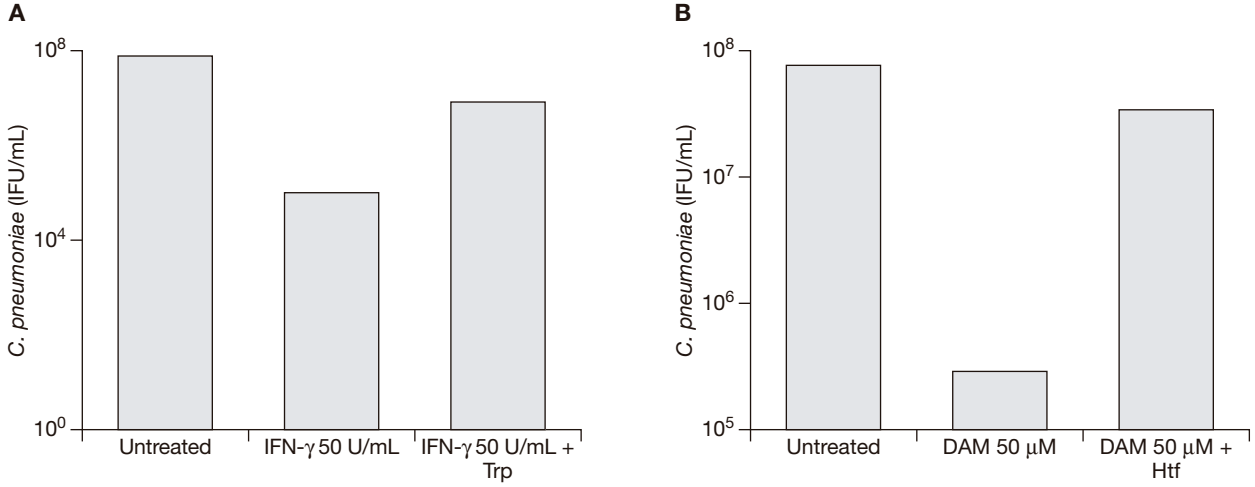

Fig. 2. In vitro infectivity studies showing loss of viable *Chlamydia pneumoniae* in interferon-γ (IFN-γ)–induction (A) and iron-limitation (B) models of persistence. Restoration of infectivity is also shown following either (A) addition of L-tryptophan (Trp) 500 μg/mL (A) or addition of holotransferrin (Htf) 8.0 μg/mL (B).
Abbreviations: IFU = inclusion forming units; DAM = deferoxamine-mesylate.

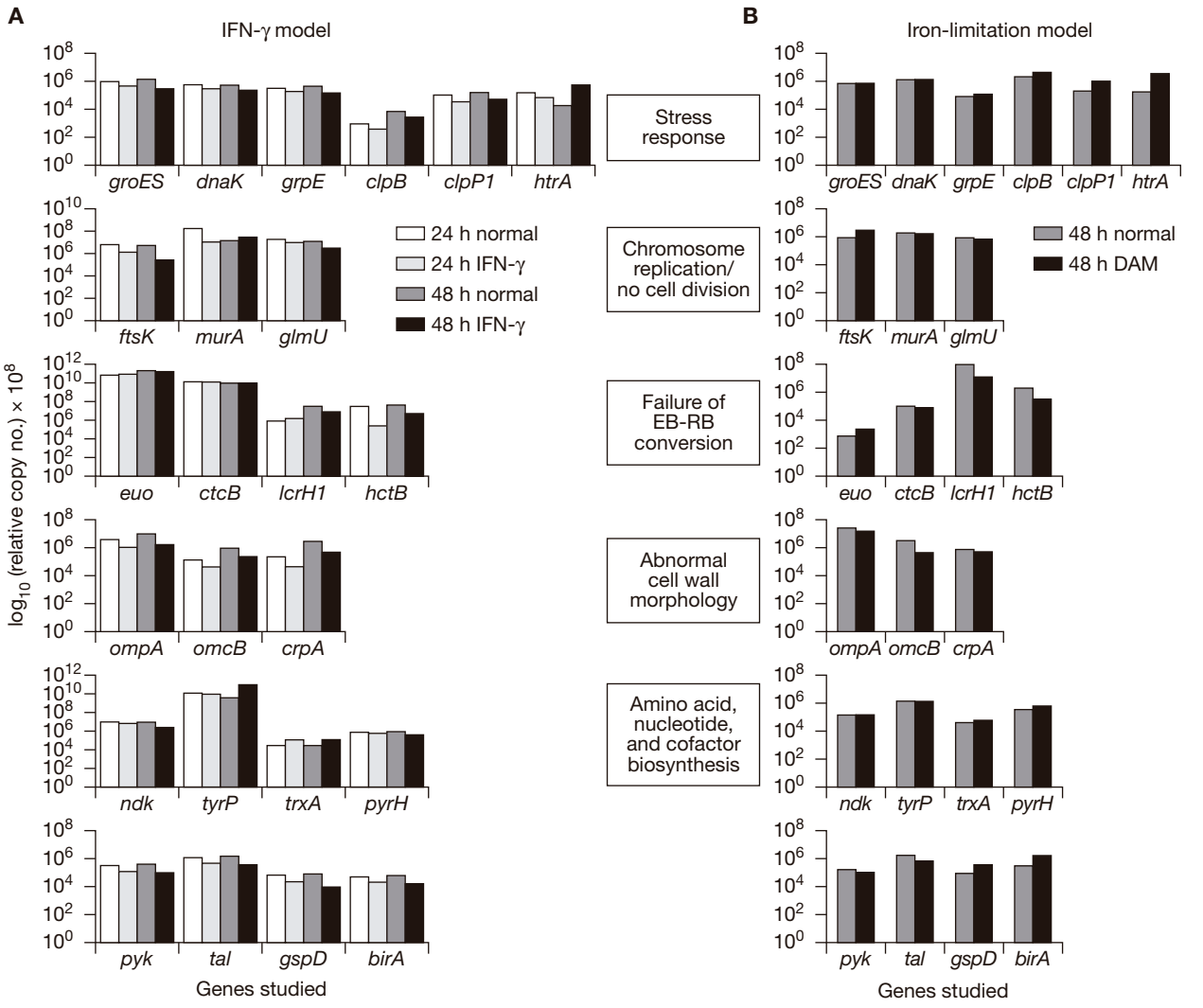

Fig. 3. Relative expression of specific genes during both interferon-γ (IFN-γ)– and iron-limitation–induced persistence. Relative mRNA levels were generated by standardizing the cDNA copy numbers of the gene of interest to the rRNA 16S levels using data obtained by real time-polymerase chain reaction with random-primed RNA isolated from *Chlamydia pneumoniae* infected HEp2 cells treated with either IFN-γ 50 U/mL (A) or deferoxamine-mesylate (DAM) 50 μM (B) at 24 h and 48 h postinfection. The data represent the summary of repeat experiments performed in duplicate (the error bars are mostly eclipsed by the log scale). Abbreviations: EB = elementary body; RB = reticulate body.

Chromosome and cell division response

Three genes were analyzed in this category: *ftsK* as a measure of chromosome replication, and *murA* and *glmU* as measures of cell division. Both models showed similar transcriptional response trends for all 3 genes, although the responses for the IFN-γ–induction model were greater than those for the iron-limitation model. FtsK transcript levels were significantly reduced in the IFN-γ–induction model (0.05) but less reduced in the iron-limitation model (0.59). MurA transcript levels remained unchanged in the iron-limitation model (0.97) but were marginally increased in the IFN-γ–induction model (1.87). Finally, *glmU* levels showed downward trends in both models, again with the IFN-γ–induction model showing the greatest change.

Failure of reticulate bodies to convert to elementary bodies

As a measure of the lack of late trigger observed previously in chlamydial persistence, 4 genes, *euo*, *ctcB*, *lcrH1*, and *hctB* were analyzed. There was a general similarity of transcript levels for 3 of the genes between both models (*ctcB*, *lcrH1*, *hctB*). The early gene, *euo*, showed different responses between the models, with a significant downregulation in the

Table 3. Summary of the mRNA responses of *Chlamydia pneumoniae* in the interferon-γ–induction and iron-limitation models of persistence.

Subcategory of persistence	Target gene	Interferon-γ–induction model[a]	Iron-limitation model[a]
Stress response	*groES*	0.21[b]	1.02
	dnaK	0.43[b]	1.04
	grpE	0.35[b]	1.38
	htrA	28.06[c]	21.14[c]
	clpB	0.39[b]	4.98
	clpP1	0.32	4.81
	ahpC	0.20	8.56
Continued chromosome replication, no cell division	*ftsK*	0.05	0.59
	murA	1.87[c]	0.97[c]
	glmU	0.23	0.81
Failure of reticulate bodies to convert to elementary bodies (lack of late trigger)	*euo*	0.38	2.94
	ctcB	1.01[b]	0.81
	lcrH1	0.26[b,c]	0.14[c]
	hctB	0.12[b,c]	0.15[c]
Abnormal cell wall morphology	*ompA*	0.21[b,c]	0.69[c]
	omcB	0.27[b,c]	0.15[c]
	crpA	0.17[b,c]	0.78[c]
Amino acid, nucleotide, cofactor biosynthesis, and energy metabolism	*trxA*	4.37[b,c]	1.47[c]
	ndk	0.28[b]	1.00
	pyrH	0.56	1.62
	pyk	0.27[c]	0.64[c]
	tal	0.25[c]	0.37[c]
	tyrP	27.87[b]	1.02
	gspD	0.12[b]	4.31
	birA	0.29	6.08

[a]Values show the ratio of mRNA changes in the persistence condition at 48 h postinfection compared with untreated controls.
[b]Similar response to interferon-γ–induction model between *Chlamydia trachomatis* (Belland et al[17]) and *Chlamydia pneumoniae* (present study).
[c]Similar trend in response between the interferon-γ–induction and iron-limitation models.

IFN-γ–induction model (0.38) compared with an upregulation in the iron-limitation model (2.94). All 3 genes analyzed at the 48-h time point, *ctcB*, *lcrH1*, and *hctB*, showed decreased mRNA levels in both the IFN-γ–induction and iron-limitation models.

Cell wall morphology

Three cell wall/membrane protein genes, *ompA*, *omcB*, and *crpA*, were analyzed as measures of chlamydial RB morphology and development. The response for all 3 genes was highly consistent between the 2 models, with all 3 genes showing decreased mRNA levels under persistence conditions. Larger mRNA decreases were seen with the IFN-γ–induction model (3.6- to 5.5-fold reduction in mRNA levels) compared with the iron-limitation model (1.2- to 5.0-fold reduction in mRNA levels).

Amino acid, nucleotide, and cofactor biosynthesis responses

Comparable transcript levels for 8 genes were determined to represent the amino acid, nucleotide, and cofactor biosynthesis responses, *trxA*, *pyrH*, *pyk*, *tal*, *ndk*, *tyrP*, *gspD*, and *birA*. Only 3 of these target genes showed the same trends between IFN-γ–induction and iron-limitation models, 1 with increased mRNA levels (*trxA*) and 2 with decreased mRNA levels (*pyk* and *tal*). The transcript levels for *ndk*, *pyrH*, *gspD*, and *birA* were decreased in IFN-γ–induced persistence but remained unchanged (*ndk*) or slightly increased (*pyrH*, *gspD*, and *birA*) under iron-limitation persistence. *tyrP* transcript levels were unchanged in the iron-limitation model but showed markedly increased mRNA levels (27.87) under IFN-γ–induced persistence conditions.

Discussion

Chlamydial persistence can be induced by a wide range of factors in vitro, including IFN-γ treatment, amino acid depletion (in particular, tryptophan depletion), heat shock, antibiotics, limitation of free iron, and phage infection. Some of these persistence-inducing conditions are better characterized than others (IFN-γ treatment, tryptophan depletion). As more is understood about *Chlamydia* spp., it is possible to select categories of functions by which to analyze chlamydial persistence: general stress response; failure to complete the developmental cycle as evidenced by a lack of conversion of RBs to EBs (which results in reduced infectivity in cell culture assays); continued chromosome replication without matching RB septation and cell division; alterations to RB morphology with resultant large and morphologically aRBs; and other more direct responses to the particular persistence-inducing factor. A subset of genes from each of these categories was chosen to analyze the persistence response in the IFN-γ–induction and iron-limitation models and to compare the 2 models for both common and model-specific hallmarks of persistence. Several examples of common persistence responses between the 2 models were found, but important evidence of model-specific differences were also observed.

The results obtained from the quantitative RT-PCR analysis for IFN-γ–induced persistence in *C. pneumoniae* were compared with the microarray results reported by Belland et al for IFN-γ–induced persistence with *C. trachomatis* [17]. Fourteen of the 25 genes showed the same trends between the 2 studies, with only 4 (*htrA*, *ahpC*, *ftsK*, and *euo*) having a dramatically different expression profile. These differences could be explained by the fact that the 2 chlamydial species might be expected to have some different responses to persistence and because the 2 studies used different timepoints postinfection for analysis, which would reflect the different stages of the developmental cycle analyzed in each study.

One of the first persistence responses reported was the upregulation of the Hsp60 protein in the IFN-γ–induction model [15]. In this study, 7 genes were selected as markers of a stress response and, contrary to some previous reports [15,16,18], the majority of stress response genes (*groES*, *dnaK*, *grpE*, *clpB*, *clpP1,* and *ahpC*) showed decreased mRNA levels in response to IFN-γ 50 U/mL. Apart from *clpP1*, these observations are consistent with the microarray data published by Belland et al for IFN-γ–induced persistence in *C. trachomatis* [17]. These results suggest that, at least at the transcriptional level, a global stress response is not necessarily a consistent outcome of chlamydial persistence.

However, several stress response genes did show markedly increased mRNA levels under iron-limitation conditions: *clpB*, *clpP1*, and *htrA*. These are all proteases and, in other bacteria, have been shown to be responsible for the proteolysis of degraded or damaged proteins, often in response to harsh external stimuli such as thermal or oxidative stress [24,25]. HtrA transcript levels, but not *clpB* or *clpP1*, were also increased under IFN-γ–induced persistence in this study. This confirms that chlamydial persistence per se does not trigger a broad stress response, and certainly does not upregulate all stress response genes. However, because of subsequent blockage of several biosynthetic pathways, proteases such as HtrA are increased to remove any subsequent excess or misfolded proteins.

A consistent feature of all persistence models is the reduction of infectious chlamydial progeny due to the failure of RBs to convert to EBs, presumably because of a key block that prevents this final trigger event. As a result of this, late genes do not get switched on and their relative transcriptional response appears as downregulated. This characteristic was common to both the models in this study, with the late genes *lcrH1* and *hctB* showing significantly reduced mRNA levels. Apart from the *euo* response, all the other gene responses in this category were consistent with Belland et al's *C. trachomatis* IFN-γ–induction model microarray findings [17].

Another commonly observed feature of chlamydial persistence is that inclusion and RB cell wall morphology is abnormal, usually visible by TEM as enlarged and aberrant-shaped RBs. One explanation for this observation is that the structural cell wall components, such as OmpA, CrpA, and OmpB, are disregulated during persistence. Data from the literature are inconsistent in this respect [16]. The transcription data from this study show that the 2 models agreed strongly in their responses, with these cell wall/membrane genes showing decreased mRNA levels for all 3 genes under persistence conditions. The decrease in mRNA levels was much more marked for the IFN-γ–induction model compared with the iron-limitation model. This downregulation is not surprising and might indicate that one or more key intermediates in the pathways to the synthesis of these cell membrane

proteins is depleted during persistence, thereby reducing the ability of the organism to correctly synthesize its membrane proteins.

While the gene transcript levels for stress response, lack of late trigger, and disregulated cell wall/membrane proteins showed general consistency between the 2 persistence models, genes in the other categories investigated either showed potentially important differences or a mixed response to the 2 different persistence inducers of IFN-γ and iron limitation. This probably indicates the different incoming signals to the *Chlamydiae*, prior to entering a common persistence pathway.

IFN-γ and iron limitation both have several important direct effects on the host cell, which subsequently affect intracellular pathogens such as *Chlamydia* spp. The best studied effect is the upregulation of the enzyme IDO by IFN-γ, which depletes the host cell tryptophan pool [12]. Unlike *C. trachomatis*, *C. pneumoniae* does not possess any enzymes in the tryptophan biosynthesis pathway and therefore is unable to rescue tryptophan from the host cell and presumably must get sufficient levels of tryptophan by some other means. One means might be to increase the transport of aromatic amino acids such as tryptophan from the host cell into the chlamydial inclusion, via chlamydial transporters such as TyrP. In support of this hypothesis, this study found *tyrP* mRNA levels to be significantly upregulated (27.87-fold increase) under IFN-γ conditions. By comparison, *tyrP* levels remained unchanged in the iron-limitation model, which reflects the different mechanisms, since the IDO response is not induced in this model.

While there have been several studies analyzing the transcriptional response of *Chlamydia* spp. to persistence [16-20], none have yet analyzed 1 model in both *C. trachomatis* and *C. pneumoniae* in the same experiment or 2 different persistence models in the same experiment. These authors attempted to use the data obtained from this study of 2 models with *C. pneumoniae*, combined with the data previously published [6,17-20] to propose some transcriptional hallmarks of persistence. Of the genes that have been analyzed in at least 2 studies, only 5 show consistent changes in all *Chlamydia* spp. and across all persistence models; *hctB*, *omcB*, *ompA*, and *crpA* are all downregulated, while *htrA* is usually upregulated. The upregulation of the stress response gene *htrA* is not unexpected, but of all the stress response genes analyzed, the *htrA* response appears to be consistent with a greater increase in transcript levels than the others. The downregulation of *hctB* is also predictable, being a late gene involved in DNA condensation, but it appears to be one of the better late gene markers of persistence. The consistent downregulation of the cell wall/envelope genes, *omcB*, *pmpA*, and *orpA* is interesting and could possibly suggest that *Chlamydia* spp. has evolved a mechanism to dampen its immune induction by limiting the expression of its antigenic wall components. The IFN-γ–induction model has been the best studied persistence model, and in addition to the 4 model-wide hallmarks mentioned above, 5 other genes appear to give consistent changes in both chlamydial species in the IFN-γ–induction model: *groES*, *dnaK*, *grpE*, and *ndk* are all downregulated, while *tyrP* is upregulated. The *tyrP* response is not unexpected as, for both species, tryptophan becomes limiting, hence the upregulation of aromatic amino acid transporters such as TyrP might assist the bacterium to obtain as much tryptophan from the host as possible. The downregulation of the 3 stress response genes is less obvious, and either suggests that this component of the stress response is deleterious to parasite survival, or else that transcriptional control of these genes is not as important as translational control mechanisms. Comparison of persistence markers between the chlamydial species is less straightforward, as many studies have not compared the same genes. However, it seems obvious that there are numerous examples of species differences in the transcriptional response to persistence and this might suggest important differences in the evolution of the 2 species in their response to host-induced persistence.

Acknowledgments

We would like to thank Erin Sullivan for technical support of culturing the *Chlamydia pneumoniae*. This work was supported financially by grants from the National Health and Medical Research Council (NHMRC), Canberra, Australia, and the National Instutes of Health, Bethesda, MD, USA (HL-68874 and A1-51255, respectively).

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