Early Gamma Interferon mRNA Expression Is Associated with Resistance of Mice against *Yersinia enterocolitica*

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T cells in cooperation with macrophages play an important role in resolution of primary *Yersinia enterocolitica* infection in mice. Previous work from this laboratory demonstrated that gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) are essential mediators of these processes. In an attempt to elucidate early mechanisms of resistance, we investigated cytokine mRNA production, including that for interleukin-1β (IL-1β), IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ, after primary as well as secondary *Y. enterocolitica* infection in *Yersinia*-susceptible BALB/c mice and *Yersinia*-resistant C57BL/6 mice. In both strains of mice, proinflammatory cytokines such as IL-1β, IL-6, and TNF-α were expressed rapidly and to comparable degrees, while IFN-γ expression was enhanced two- to eightfold in C57BL/6 mice, as revealed by semiquantitative reverse transcription PCR. Similar results were found in both mouse strains after secondary *Y. enterocolitica* infection. IL-2 mRNA was detected only during secondary infection and disappeared rapidly in BALB/c mice. IL-4 mRNA expression was detectable in C57BL/6 but not BALB/c mice. The levels of cytokine mRNA expression correlated closely with the number of injected bacteria. The findings reported here support the hypothesis that early and enhanced production of IFN-γ may be associated with a state of heightened resistance against *Y. enterocolitica* infection.

Cytokines are important mediators of the host immune response against infectious pathogens. In various experimental infection models, particularly those involving intracellular pathogens such as *listeria*, *leishmania*, and *mycobacteria*, the role of cytokines has been extensively studied (for a review, see references 6, 14, 28, and 29). It was shown that an immune response against these pathogens is associated with increased interleukin-1 (IL-1), IL-2, IL-3, IL-4, tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) mRNA expression (6, 14, 29). Moreover, expression of IFN-γ can be associated with resistance or protection against a microbial pathogen, while expression of IL-4 can be associated with progression of an infectious disease (22, 23).

*Yersinia enterocolitica* is a gram-negative, predominantly extracellularly located pathogen (18, 27, 34). Because yersiniosis in rodents resembles closely *Yersinia* infection in humans, experimental yersiniosis in mice turned out to be a suitable model for investigation of the immunobiology of *Y. enterocolitica* infection (7, 8, 20). Similar to the case of infection by intracellular pathogens, T cells, particularly CD4+ Th1 cells, in cooperation with macrophages are involved in and required for clearance of a primary *Yersinia* infection (1, 1a, 2, 4, 5). These processes are mediated by various cytokines. Thus, neutralization of IFN-γ or TNF-α, respectively, in vivo abrogates resistance to yersiniae and leads to fatal disease progression (1, 3). More recently, we found that administration of anti-IL-4 antibodies prior to infection rendered *Yersinia*-susceptible BALB/c mice resistant to yersiniae while this treatment did not significantly affect yersiniosis in *Yersinia*-resistant C57BL/6 mice (1). Furthermore, T cells isolated from *Yersinia*-resistant C57BL/6 mice produce significant quantities of IFN-γ upon stimulation with heat-killed yersiniae, while T cells from *Yersinia*-susceptible BALB/c mice do not do so (1).

Mechanisms of early resistance can best be studied when mouse strains with different susceptibilities to infection are compared. Resistance of mice to yersiniae is not related to certain gene loci such as *Ity* or *H-2* (16, 17). In the present study we wanted to compare the patterns of cytokine mRNA expression upon infection by *Y. enterocolitica* in the livers of *Yersinia*-susceptible BALB/c mice and of *Yersinia*-resistant C57BL/6 mice.

Female 6- to 8-week-old C57BL/6 and BALB/c mice were purchased from Charles River Wiga (Sulzfeld, Germany). Mice were provided with food and water ad libitum and kept for 1 week in our animal care facility under specific-pathogen-free conditions (positive-pressure cabinets) before being used in the experiments.

Plasmid-bearing *Y. enterocolitica* WA (9, 10, 21) was passed in mice and cultivated as described previously (4, 5). For intravenous infection, an aliquot of a frozen bacterial suspension was thawed and washed. After appropriate dilution in phosphate-buffered saline (PBS), pH 7.4, mice were injected in the tail vein with 0.2 ml of the bacterial suspension. The actual number of bacteria administered was confirmed by plating serial dilutions of this suspension on Luria-Bertani agar and counting CFU after incubation for 2 days at 26°C. For primary infection, mice were challenged with either sublethal or lethal doses (103, 104, 105, and 107 bacteria). For secondary infection, mice were challenged with 106 bacteria 28 days after a sublethal (0.5 50% lethal dose) primary infection.

At various time intervals after the infection, mice were killed and the livers were infused with 10 to 20 ml of PBS, pH 7.4 (4°C); removed; and homogenized in 5 ml of buffer consisting of 4 M guanidine-isothiocyanate (Sigma Chemical Co., Deisenhofen, Germany), 25 mM Na-citrate (Serva, Heidelberg, Germany), 0.5% *N*-lauroylsarcosine (Sigma), and 100 mM 2-mercaptoethanol (Fluka, Buchs, Switzerland). The homogenates were stored at −70°C until further processing. After thawing of
an aliquot, lysates were mixed with 50 μl of 2 M sodium acetate (pH 4.0), 500 μl of water-saturated phenol, and 100 μl of chloroform according to the phenol-chloroform extraction method described elsewhere (11). This mixture was vortexed, incubated for 15 min at 4°C, and centrifuged at 15,000 x g for 15 min. The aqueous phase was recovered and again mixed with phenol and chloroform. The RNA was precipitated with isopropanol for 2 h at -20°C. The precipitates were centrifuged at 4°C for 5 min at 10,000 x g, washed once with 70% ethanol, dried, resuspended in 50 μl of diethylpyrocarbonate-treated distilled water (dH2O), and repelleted. Dried pellets were resuspended in 50 μl of diethylpyrocarbonate-treated dH2O. The purity of extracted RNA was controlled by electrophoresis in a 1.5% formaldehyde agarose gel (33). Reverse transcription (RT) was performed by mixing 20 μg of RNA-dH2O solution with 2 μg of oligo(dT) (United States Biochemical Corp., Cleveland, Ohio). This solution (10 μl) was incubated for 10 min at 65°C. Ten 10 μl of a solution containing 2× reverse transcriptase buffer (100 mM Tris-HCl [pH 8.3], 150 mM KCl, 6 mM MgCl2; Gibco BRL, Life Technologies, Berlim, Germany), 40 U of RNasin (Promega Biotec, Madison, Wis.), 20 mM dithiothreitol (Gibco), Superscript Reverse H reverse transcriptase ( Gibco), and 2 mM deoxynucleoside triphosphate (dNTP) was added, and tubes were incubated for 60 min at 37°C. Finally, tubes were heated to 90°C for 5 min, and 180 μl of dH2O was added to the reaction mixture. Samples were stored at -20°C until further use. This reaction was always performed simultaneously for parallel samples from one experiment.

Primer pairs specific for β-actin, IL-1β, IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α were designed and purchased from Roth (Karlsruhe, Germany). The sequences and the sizes of the amplified fragments are given in Table 1. Five microliters of cDNA extracted as described above was added to 20 μl of a solution consisting of 1 U of Taq DNA polymerase (Amersham, Buckinghamshire, United Kingdom), 200 μM dNTP, 200 to 500 nM 5' and 3' primers, and Taq DNA polymerase buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], and 1.5 mM MgCl2; Amersham). This mixture was overlaid with 25 μl of mineral oil, and PCR was performed in a DNA thermal cycler (Biometra, Göttingen, Germany) with 25 to 35 cycles: 30 s of 94°C denaturation, 45 s of 60°C annealing, and 60 s of 72°C extension. The PCR products were visualized by electrophoresis of 20 μl of the reaction product mixed with 4 μl of loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromphenol blue, 0.4% xylene blue) at 100 V for 60 min in a 2% agarose gel with 0.5× Tris-boric acid-EDTA buffer containing 0.5 μg of ethidium bromide per ml. Marker VI (Boehringer, Mannheim, Germany; providing bands at 2,176, 1,766, 1,230, 653, 517, 453, 394, 298, 234, 220, and 154 bp) was run in parallel to estimate the molecular weights of the amplified fragments. Specificity of the amplified fragments was confirmed by ABI automated DNA sequencing (Applied Biosystems, Weiterstadt, Germany) according to the TaqDyeDideoxy terminator method and 373A DNA sequencer (Applied Biosystems). PCR-assisted mRNA amplification was repeated at least twice for separately prepared cDNA samples. Data shown are representative of at least three experiments including three to five animals per group and time point.

In order to assess cytokine mRNA expression, we performed a semiquantitative competitive PCR as described recently (12, 32). PCR was performed in parallel using target cDNA serially diluted in the presence of a constant amount of competitor control DNA consisting of 5' and 3' primer sequences in tandem array. During coamplification, target and control DNAs compete for the primers and the amount of PCR product is proportional to the amount of input cDNA. Plasmid pmCQ, which was used for quantification of β-actin, was kindly provided by T. Blankenstein, Berlin, Germany. Plasmid pG2PCR106g4, which was used for quantification of IFN-γ, was kindly provided by I. Berberich, Würzburg, Germany. Before determination of cytokine mRNA expression levels, competitive PCR for parallel samples was first performed for β-actin to make sure that the samples contained identical quantities of cDNA. The dilution at which equally dense bands (for control and target DNAs) were obtained during electrophoresis (indicating equal contents of specific cDNA) was used for determination of the cytokine mRNA expression levels.

**Yersinia**-resistant C57BL/6 and **Yersinia**-susceptible BALB/c mice were intravenously infected with 107 CFU of *Y. enterocolitica*. After 6 and 24 h, mice were killed, RNA was extracted from liver tissue, and cytokine mRNA expression in the liver, including IL-1β, IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ was analyzed as described recently (12, 32). Liver tissue was chosen because in noninfected control mice only minute if any background cytokine mRNA expression levels were found in this organ. The PCR conditions chosen for cDNA amplification of the various cytokine mRNAs revealed no distinct bands in noninfected control animals. The patterns of cytokine mRNA expression 6 and 24 h after primary *Yersinia* infection of both strains are depicted in Fig. 1. The level of mRNA expression of the proinflammatory cytokines IL-1β, IL-6, IL-10, IFN-γ, and TNF-α became detectable in both mouse strains after 6 and 24 h. IL-1β and IL-10 mRNAs were rapidly and most abundantly expressed after 6 h. Both TNF-α and IFN-γ were more strongly expressed after 24 h than after 6 h postinfection (p.i.). By contrast, IL-2 was not significantly expressed and IL-4 mRNA was only weakly and transiently expressed in the early phase. Comparison of the cytokine mRNA expression levels in both strains of mice suggested that IL-10 and IFN-γ mRNA expression levels were higher in C57BL/6 mice than in BALB/c mice. However, although we carefully controlled for levels of input cDNA, the RT-PCR analysis used is purely qualitative and hardly allows any con-
conclusions about and comparisons of the quantities of cytokine mRNA present in the tissues.

For a secondary infection, mice were challenged with $10^7$ bacteria 28 days after primary infection with a sublethal dose of yersinia. At this interval after primary infection, the cytokine mRNA expression levels were no longer different from those observed in noninfected mice. After secondary infection, mRNA expression levels of the proinflammatory cytokines IL-1β, IL-6, TNF-α, IL-10, and IFN-γ became rapidly detectable in both mouse strains (Fig. 2) and the maximal expression levels were found already at 6 h p.i. In contrast to primary infection, IL-2 mRNA was expressed already 6 h after secondary infection in both strains of mice. In contrast, IL-4 mRNA was again only transiently expressed in C57BL/6 mice but was not detected in BALB/c mice. Moreover, the mRNA levels of IL-6 in BALB/c mice were increased at 6 h p.i. but, in contrast to those in C57BL/6 mice, decreased to background levels at 24 h after secondary infection. Likewise, IL-2 mRNA expression was not detectable in BALB/c mice at 24 h p.i. Most strikingly, the levels of IFN-γ and IL-10 mRNA expression appeared again to be significantly lower in BALB/c mice compared with those in C57BL/6 mice. Taken together, cytokine mRNA expression developed more rapidly and appeared to be more pronounced in secondary infection than in primary infection.

To determine the influence of the number of administered bacteria on the level of IFN-γ, IL-4, and IL-10 mRNA expression, both C57BL/6 and BALB/c mice were infected with $10^3$, $10^4$, and $10^5$ CFU of Y. enterocolitica. On days 1, 3, and 7 p.i., mice were killed, RNA was extracted from liver tissue, and RT-PCR was performed (Fig. 3). After infection with $10^3$ bacteria, C57BL/6 mice expressed significant levels of IFN-γ and IL-10 mRNA during the whole period of observation. In contrast, BALB/c mice showed only minimal IFN-γ mRNA expression levels and moderate IL-10 mRNA expression levels. Expression of IL-4 mRNA was observed in C57BL/6 mice but not in BALB/c mice. After infection with $10^4$ bacteria both IL-10 and IFN-γ mRNA expression levels were higher in C57BL/6 mice. In BALB/c mice, expression levels of these cytokines were increased on day 7 p.i. After infection with $10^5$ bacteria, mice died between days 5 and 7 p.i.
Therefore, data are given for days 1 and 3 p.i. only. This inoculum induced the highest cytokine mRNA expression levels in C57BL/6 mice. Likewise, significant levels of IFN-γ and IL-10 mRNA expression were observed in BALB/c mice on day 3 p.i. However, these levels appeared to be lower than those observed in C57BL/6 mice. In contrast to C57BL/6, expression of IL-4 was not observed in BALB/c mice. Moreover, neither in C57BL/6 mice nor in BALB/c mice did we find detectable amounts of IL-2 mRNA expression (data not shown).

From these data we can conclude that the level of cytokine mRNA expression after Yersinia infection is closely correlated with the number of bacteria administered. Moreover, C57BL/6 mice appeared to develop a faster and more pronounced cytokine mRNA expression in response to higher bacterial loads than BALB/c mice.

In order to compare IFN-γ mRNA expression levels after Yersinia infection between BALB/c and C57BL/6 mice, a semiquantitative competitive RT-PCR (12, 32) was performed. For this purpose, the twofold serially diluted cDNA samples from the livers from mice after Yersinia infection were mixed with a constant amount of a competitor control DNA as described recently (12, 32). Furthermore, we determined that equal amounts of cDNA were obtained from all samples to be compared. Hence, only samples that revealed comparable results in competitive RT-PCR for β-actin were used for determination of IFN-γ mRNA expression levels. The data presented in Fig. 4 and Table 2 indicate that after infection with 10^3, 10^4, and 10^5 CFU of Y. enterocolitica, C57BL/6 mice expressed IFN-γ mRNA levels two- to eightfold higher than did BALB/c mice. Hence, early and strong IFN-γ mRNA expression may be correlated with resistance of mice against Y. enterocolitica.

Infection by the gram-negative bacterial pathogen Y. enterocolitica evokes an acute immune reaction of the host reflecting both inflammatory and protective events, including the involvement of T cells, macrophages, and cytokines (1-5, 20). Primary experimental infection with intracellular Listeria monocytogenes was documented to result in the increased expression of a whole array of cytokines of myelomonocytic origin such as IL-1β, IL-6, monocyte colony-stimulating factor, and granulocyte macrophage colony-stimulating factor, while secondary infection led to an increased expression of TNF-α and IFN-γ, as well as the T-cell-specific cytokines IL-4, IL-2, and IL-3 (12, 29, 31). In an attempt to improve our understanding of the mechanisms involved in the regulation of the protective inflammatory host response to the extracellular pathogen Y. enterocolitica, we characterized by RT-PCR the early cytokine mRNA expression pattern, including that for IL-1β, IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ, upon Yersinia infection in susceptible BALB/c and resistant C57BL/6 mice. For this purpose, we chose liver tissue because (i) the liver is a major target of Yersinia infection (2) and (ii) the cytokine mRNA expression levels in normal uninfected liver

![FIG. 3. Influence of the number of injected yersiniae (10^3, 10^4, or 10^5) on mRNA expression levels of β-actin, IFN-γ, IL-4, and IL-10 in the livers of BALB/c and C57BL/6 mice 1, 3, and 7 days after infection. Lanes M, molecular weight markers.](image)

![FIG. 4. Semiquantitative competitive PCR-assisted amplification of β-actin and IFN-γ mRNA in the livers from C57BL/6 and BALB/c mice 1 day after primary Y. enterocolitica infection with 10^5 CFU. C.F., control fragment (competitor control DNA). Arrows indicate the dilution step at which equally dense bands were obtained.](image)
TABLE 2. Semiquantitative determination of IFN-$\gamma$ mRNA expression after Y. enterocolitica infection in BALB/c and C57BL/6 mice

<table>
<thead>
<tr>
<th>No. of bacteria administered</th>
<th>Day(s) p.i.</th>
<th>Ratio of IFN-$\gamma$ to $\beta$-actin&lt;sup&gt;a&lt;/sup&gt; in:</th>
<th>C57BL/6: BALB/c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BALB/c mice</td>
<td>C57BL/6: BALB/c</td>
</tr>
<tr>
<td>$10^9$</td>
<td>1</td>
<td>&lt;0.0015 0.003 2:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>&lt;0.0015 0.006 4:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>&lt;0.0015 0.012 8:1</td>
<td></td>
</tr>
<tr>
<td>$10^4$</td>
<td>1</td>
<td>&lt;0.0015 0.003 2:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.003 0.006 2:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.006 0.012 2:1</td>
<td></td>
</tr>
<tr>
<td>$10^3$</td>
<td>1</td>
<td>0.003 0.012 4:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.003 0.003-0.006 1:1-2:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>ND ND</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Serially diluted target cDNA was amplified by PCR in the presence of constant amounts of competitor control DNA (480 pg of pmcQO per ml, 22 PCR cycles; 12 pg of pG3PCR106g4 per ml, 30 PCR cycles). The dilution at which equally dense bands (for control and target DNA) were obtained was used for determination of cytokine mRNA expression levels.

<sup>b</sup> The ratio of IFN-$\gamma$ to $\beta$-actin was calculated as follows: [concentration of IFN-$\gamma$ control fragment $\times$ dilution factor of target DNA (IFN-$\gamma$)]/[concentration of $\beta$-actin control fragment $\times$ dilution factor of target DNA ($\beta$-actin)].

These conflicting data do not necessarily argue against the hypothesis that increased IL-4 production in BALB/c mice might be related to their increased susceptibility to yersiniae. Moreover, both C57BL/6 and BALB/c mice showed comparable IL-2 expression levels. Similar contradictory results were found in murine listeriosis. Thus, anti-IL-4 treatment rendered susceptible mice resistant against listeriae, but there were no differences found for resistant and susceptible mouse strains in terms of IL-2 and IL-4 mRNA expression (15, 26). Therefore, other mechanisms, e.g., the presence of soluble IL-4 receptors, may account for these differences (6).

The role for a seemingly increased expression of IL-10 in C57BL/6 mice is at present unknown. It is provocative in that IL-10 has been shown to downmodulate macrophage activation and would thus outbalance the protective effect afforded by IFN-$\gamma$ (13, 24). Comparison of the cytokine expression patterns of primary and secondary infections showed qualitative and quantitative differences. After secondary infection, cytokine expression developed more rapidly. While IL-2 mRNA was not detected after primary infection, we observed IL-2 mRNA expression 6 h after secondary infection in both C57BL/6 and BALB/c mice, suggesting acquired immune mechanisms, including the involvement of T cells already in the early phase of secondary infection. Comparable results were found in murine listeriosis (12, 25).

During both primary and secondary infections, the cytokines IL-1$\beta$, TNF-$\alpha$, and IL-10, which are primarily produced by myelomonocytic cells, appeared to be most abundantly expressed. However, these signals were expressed earlier and more strongly in C57BL/6 mice than in BALB/c mice. This observation is consistent with the finding that in BALB/c mice the recruitment or influx of inflammatory cells into Yersinia-induced liver lesions appears to be delayed (1a).

The fact that the increase of the cytokine mRNA expression is closely correlated with the number of administered bacteria should be kept in mind when cytokine expression data from different studies or experiments are to be compared. Nevertheless, because the cytokine mRNA expression observed in this study resembled that observed by various groups after infection with other bacterial pathogens, e.g., L. monocytogenes (12), we feel that this cytokine gene expression profile reflects the uniform mechanism(s) of (non)specific resistance operative in a wide variety of microorganisms that tends to be rather stereotypic regardless of whether the insulting pathogen resides intra- or extracellularly. We are currently investigating to what extent the T-cell-specific response during secondary infection differs in terms of its cytokine pattern and how this component of the immune response may account for the different tissue pathology observed. Finally, studies that focus on host response mechanisms, including cytokine production after intestinal infection, are required.

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