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Reduced interferon-γ release in patients recovered from Legionnaires’ disease

K D Lettinga, S Weijer, P Speelman, J M Prins, T van der Poll, A Verbon

Background: Legionella pneumophila, a Gram negative facultative intracellular pathogen, is the causative agent of Legionnaires’ disease (LD). Interferon (IFN)-γ is important for host defence against L pneumophila so reduced IFN-γ production capacity and/or responsiveness might render humans more susceptible to infection with L pneumophila.

Methods: Seventy seven patients who suffered from LD after a point source outbreak one year earlier participated in the study. Whole blood was incubated with non-specific stimuli (lipopolysaccharide (LPS) or interleukin (IL)-12) or specific stimuli (viable or heat killed L pneumophila) to evaluate IFN-γ production, and with IFN-γ to evaluate IFN-γ responsiveness. Expression of complement receptor 3 on monocytes was determined by flow cytometry. Thirty seven companions who were also exposed but had not developed LD served as controls.

Results: Patients released less IFN-γ than controls in response to stimulation with LPS (mean (SE) 393 (58) pg/ml v 914 (178) pg/ml; p=0.001) and IL-12 (96 (14) pg/ml v 177 (41) pg/ml; p=0.058). IFN-γ responsiveness, measured by release of IFN-γ inducible protein (IP)-10, tumour necrosis factor α, IL-12 production capacity, and monocyte expression of complement receptor 3, did not differ between patients and controls. IFN-γ release after stimulation with LPS and IP-10 release after stimulation with IFN-γ were weakly associated with severity of LD in the former patient group (ρ=–0.3, p=0.011 and ρ=–0.3, p=0.037, respectively).

Conclusion: These results suggest that impaired IFN-γ production may contribute to susceptibility to L pneumophila infection.
Legionnaires’ disease (LD) in study patients (n=77)  

<table>
<thead>
<tr>
<th>Positive diagnostic test for LD</th>
<th>No of patients</th>
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<tbody>
<tr>
<td>Sputum culture</td>
<td>9</td>
</tr>
<tr>
<td>Fourfold rise in titre/seroconversion</td>
<td>33</td>
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<tr>
<td>Urinary antigen test</td>
<td>50</td>
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<tr>
<td>Polymerase chain reaction</td>
<td>3</td>
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<td>Single high titre ≥ 1:256</td>
<td>6</td>
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</table>

Statistical analysis
Values are given as mean (SE) unless otherwise indicated. Differences between patients and controls were analysed by the χ² test, Student’s t test, or the Mann-Whitney U test. Spearman’s ρ was used to determine correlation coefficients. A p value of <0.05 was considered to be statistically significant.

RESULTS
Patient characteristics
A total of 77 subjects were studied 1 year after their episode of LD. The median age was 66 years (range 46–88) and 62% were men. The diagnosis of LD was based on epidemiological, clinical, and diagnostic features (table 1). Twenty patients (26%) suffered from severe LD (severity score ≥2), of whom eight had required mechanical ventilation. Underlying diseases such as COPD, cardiovascular disease, and diabetes mellitus were present in 10%, 30%, and 9%, respectively, of the patients. Two patients used immunosuppressive medication at the time of this study and 44% were active smokers. None of the patients had a history of recurrent infections. The median age of the control subjects was 64 years (range 47–78) and 32% were men. All control subjects denied underlying diseases except one who had diabetes mellitus.

Cytokine release
The number of leucocytes, lymphocytes, and CD4+ and CD8+ T lymphocytes did not differ between former patients and control subjects (t test, all p>0.05, data not shown). Whole blood stimulation with LPS and IL-12 resulted in a lower IFN-γ release in patients than in controls (393 (58) pg/ml in patients v 914 (178) pg/ml in controls (p=0.001) after stimulation with LPS and 96 (14) pg/ml v 177 (41) pg/ml (p=0.058) after stimulation with IL-12, fig 1). In contrast, no difference in IFN-γ response was found between patients and controls after stimulation with live bacteria (204 (81) pg/ml and 107 (19) pg/ml, respectively, p=0.65) or with heat killed bacteria (104 (21) pg/ml and 66 (8) pg/ml, respectively, p=0.73).

Since former patients and control subjects were not completely comparable with regard to sex, smoking habit, and underlying diseases, we determined whether IFN-γ release was influenced by these factors. A comparison was made of IFN-γ release in patients and controls with or without underlying conditions (Mann-Whitney U test; data not shown). None of the factors significantly affected IFN-γ release. The only exception was that active smokers had a higher IFN-γ release than non-smokers after stimulation with dead bacteria but not after stimulation with live bacteria, LPS, or IL-12. Differences in IFN-γ release between former LD patients and control subjects are therefore not likely to have resulted from differences between the groups with respect to sex, smoking habit, or underlying diseases.

The production of IP-10 by various cell types is strongly dependent on IFN-γ and has been suggested to play a role in innate immunity. To examine IFN-γ responsiveness, IP-10 levels were measured after stimulation with IFN-γ. IP-10 release
was similar in patients (581 (83) pg/ml) and control subjects (541 (104) pg/ml, *p*=0.41), indicating that the response to IFN-γ was normal in both groups.

IFN-γ is predominantly produced by lymphocytes. To determine whether cytokine production by mononuclear cells is also decreased in former LD patients, TNF-α and IL-12 levels were measured. Stimulation with IFN-γ and IL-12 did not increase TNF-α release compared with incubation with RPMI alone (fig 2A). Stimulation with LPS and dead *L. pneumophila* increased TNF-α levels in patients and control subjects to a similar extent (LPS: 1940 (156) pg/ml and 1434 (107) pg/ml, respectively (*p*=0.23); dead bacteria: 884 (95) pg/ml and 936 (96) pg/ml, respectively (*p*=0.14)). After stimulation with live *L. pneumophila*, TNF-α production was significantly higher in patients than in controls (1463 (151) pg/ml and 962 (163) pg/ml, respectively, *p*=0.028).

IL-12p70, the active form of IL-12, is composed of a 40 kDa (p40) subunit and a 35 kDa (p35) subunit. IL-12p70 was undetectable after stimulation with all stimuli in both patients and controls (data not shown). The IL-12p40 subunit has no IL-12 activity but the dimer p40 has been shown to bind to the IL-12 receptor. After stimulation with LPS and viable or dead *L. pneumophila*, IL-12p40 levels increased compared with RPMI incubation alone (fig 2B). However, the levels of IL-12p40 were similar in patients and control subjects (LPS: 615 (74) pg/ml and 595 (92) pg/ml, respectively (*p*=0.87); viable bacteria: 178 (27) pg/ml and 133 (35) pg/ml, respectively (*p*=0.07); dead bacteria: 192 (28) pg/ml and 273 (63) pg/ml, respectively (*p*=0.33)). IL-12p40 was undetectable after stimulation with IFN-γ.

IFN-γ release after stimulation with LPS and IP-10 release after stimulation with IFN-γ were weakly correlated with severity of pneumonia (*p*=0.3, *p*=0.011 and *p*=0.3, *p*=0.037, respectively). TNF-α release after stimulation with LPS was not significantly correlated with severity of pneumonia (*p*=0.17, *p*=0.14).

**Complement receptor 3 expression on monocytes**

Thirteen representative patients and nine controls were randomly selected from the study group. Absolute number of monocytes were similar in patients and controls (*p*=0.31). No differences could be detected in CD18 and CD11b expression between patients and controls (mean fluorescence CD18 538 (54) in patients v 665 (106) in controls, *p*=0.45; mean fluorescence CD11b, 712 (81) v 834 (117), *p*=0.44).

**DISCUSSION**

Cellular immunity is pivotal in host defence against *L. pneumophila*. In vitro and in murine models a Th1 response, characterised by IFN-γ production, was found to be protective against replicative infection with *L. pneumophila*. Although a relative predominance of Th1 type cytokines was seen in patients with active LD, interpretation of these data is hampered by the fact that, during acute infection, circulating levels of inflammatory mediators are usually high. To identify impairments in cytokine mediated immunity in patients with LD, we measured levels of IFN-γ, IP-10, and TNF-α after whole blood stimulation with LPS, IL-12, IFN-γ, and *L. pneumophila* in patients who had LD a year earlier and in control subjects. IFN-γ levels were lower in former LD patients after incubation with LPS and IL-12. The response to IFN-γ, as measured by IP-10, as well as TNF-α production and IL-12p40 production was similar in patients and controls. Our data suggest that a decreased IFN-γ production capacity may be a risk factor for developing LD.

The control population consisted of partners or companions of the former LD patients who, together with the patients, had visited the flower show and thus were likely to have been exposed to *L. pneumophila* to a similar extent. However, serological investigations within the control group were not performed. In a serological survey among more than 700 exhibitors who were present during every day of the flower show, no *L. pneumophila* specific antibody response was detected.
show, the prevalence of serologically positive subjects was only 12%. This suggest that a seronegative status does not exclude exposure.

The IFN-γ response to LPS is presumably indirect as a result of LPS induction of lymphocyte activating monokines such as IL-12 and TNF that cause lymphocytes and natural killer (NK) cells to release IFN-γ. The impaired IFN-γ response to exogenous IL-12 and the increase in TNF-α and IL-12p40 after stimulation with LPS argue against the possibility that impaired monokine release is the sole explanation for impaired IFN-γ release in former LD patients. The data are also consistent with a possible defect in lymphocyte IL-12 binding capacity (IL-12R). To confirm the finding that IFN-γ productivity in patients with LD is impaired in comparison with controls, it would be interesting to perform additional tests with different non-specific stimuli in a dose response experiment.

IP-10 is a CXCR3 chemokine which binds to the CXCR3 receptor and specifically targets T lymphocytes and NK cells. It is important in the innate immune response to bacterial infection, probably by attracting CXCR3 positive Th1 cells to the site of inflammation, and it is largely IFN-γ dependent.

We found that IP-10 release after whole blood stimulation with IFN-γ was similar in patients and controls, suggesting that IFN-γ responsiveness is normal in former LD patients.

Phagocytosis of L. pneumophila by mononuclear phagocytic cells is mediated by CR1 and CR3 which selectively bind to the lower IFN-γ, have been described in immunocompetent individuals. It is not clear whether a circulating antibodies interfere with the outcome. It is not clear whether a

Supernatants from cultured monocytes of former patients after stimulation with non-specific stimuli such as LPS or phagocytosis is enhanced but, in the absence of complement or antibodies, L. pneumophila still binds to monocytes via CR1 and CR3. Expression of CR3 on mononuclear cells from patients and controls was not different, suggesting that the extent of CR3 expression is not involved in the susceptibility to L. pneumophila. However, other factors may be involved in phagocytosis of L. pneumophila, since bacterial growth after complement-independent attachment has been observed in guinea pig alveolar macrophages and cell lines.

Although former LD patients had less IFN-γ release after stimulation with LPS or IL-12 than controls, IFN-γ release was comparable after stimulation with live or heat killed L. pneumophila. In addition, patients had a higher TNF-α response after stimulation with live L. pneumophila than controls. This may be explained by a recall immune response. Mononuclear cells sensitised to L. pneumophila have been shown to persist for up to 20 months after an episode of LD. Supernatants from cultured monocytes of former patients stimulated with dead L. pneumophila contained more monocyte activating cytokines and inhibited multiplication of L. pneumophila significantly more than supernatants from monocytes from control subjects. A memory response involving IFN-γ has also been described after mycobacterial infection or vaccination. CD8+ lymphocytes from BCG vaccinated cattle produced more IFN-γ upon stimulation with BCG or PPD than lymphocytes from non-vaccinated cattle. Thus, because of a recall response in former LD patients, higher levels of IFN-γ may be produced after stimulation with L. pneumophila than after stimulation with non-specific stimuli such as LPS or IL-12 in comparison with controls. However, in this study we used whole blood for stimulation assays and we did not know about the presence of circulating antibodies to L. pneumophila in patients and controls 1 year after exposure. This means that we cannot exclude the possibility that antibodies to Legionella antigens interfere with the outcome. It is not clear whether a memory response protects against recurrent disease with L. pneumophila but, to our knowledge, no recurrence of LD has been described in immunocompetent individuals.

The inverse weak correlation between release of IFN-γ or IP-10 and the severity of LD is consistent with the idea that lower IFN-γ responses may be associated with worse LD. It has been shown that IFN-γ mediated inhibition of in vitro intracellular multiplication of L. pneumophila is dose dependent. Impaired IFN-γ production capacity after a non-specific stimulus such as LPS now and L. pneumophila at the time of infection may therefore not only increase the susceptibility to development of LD, but may also influence the outcome.

IFN-γ plays a pivotal role in the host defence against LD. We have shown that former LD patients have a reduced capacity to produce IFN-γ on stimulation with LPS or IL-12 compared with control subjects who were also exposed to L. pneumophila but did not develop the disease. These data suggest that an impaired capacity to produce IFN-γ may contribute to susceptibility to infection with L. pneumophila.

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