Acquired Immunity to Chlamydia pneumoniae Is Dependent on Gamma Interferon in Two Mouse Strains That Initially Differ in This Respect after Primary Challenge

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The role of gamma interferon (IFN-γ) in a Chlamydia pneumoniae mouse model was studied by in vivo neutralization in two inbred mouse strains. During primary C. pneumoniae infection, neutralization of IFN-γ increased both the numbers of bacteria and the pneumonia score in the lungs of C57BL/6 mice but not BALB/c mice. During reinfection, the bacterial counts in the lungs were increased by IFN-γ neutralization in both mouse strains. Thus, the effect of IFN-γ neutralization was dependent on the genetic background in primary infection. However, IFN-γ appeared to be equally important in both mouse strains during reinfection.

Chlamydia pneumoniae, the most common chlamydial pathogen affecting humans, causes a rather mild acute respiratory infection (14). It is probable that C. pneumoniae infection, like other chlamydial infections, can also result in chronic, persistent infection with several possible disease outcomes, such as coronary heart disease (5, 22). Chlamydia trachomatis is a prime example and a well-studied pathogen causing pelvic inflammatory disease and trachoma (2). In addition to animal models of these diseases, the C. trachomatis mouse pneumonitis (MoPn) variant has been used to study chlamydial pneumonia in mice. A mouse model has been developed also for C. pneumoniae (12, 27). For both C. pneumoniae and C. trachomatis, infection is common and no vaccines are available. In C. trachomatis MoPn and C. pneumoniae mouse models there is clear evidence of protective immunity seen as a more rapid clearance of bacteria in reinfection (4, 13), and the protective role of T cells seems to be different: the importance of CD8+ cells has been demonstrated both in the clearance of primary infection (20) and in protection against reinfection (18), while CD4+ cells, in the absence of CD8+ cells, may even enhance the bacterial growth in the lungs (20).

The mechanism underlying the protection mediated by the CD8+ cells in C. pneumoniae infection is still unclear. Cytotoxic T lymphocytes (CTL) specific for C. trachomatis have been demonstrated in C. trachomatis-infected mice (1, 21). However, there is no evidence of protective CTL activity against C. pneumoniae, and the course of primary infection is not altered in perforin-deficient mice (20). CD8+ cells may also function by secreting cytokines such as gamma interferon (IFN-γ) (7), and the protective role of C. trachomatis-specific CTLs has been reported to be dependent on IFN-γ production (15). The importance of IFN-γ in protection against C. trachomatis infection has been shown by several methods (in vivo depletion, administration of recombinant IFN-γ, and use of genetically modified mice) (11, 19, 24). C57BL/6 mice produce IFN-γ in response to C. pneumoniae primary infection (20) in contrast to BALB/c mice, which do not (17). Thus, there appears to be strain-specific differences in the importance of IFN-γ. Studies using genetically modified IFN-γ R−/− knock-out mice have also shown the importance of IFN-γ during C. pneumoniae infection in C57BL/6 mice (20). IFN-γ can be produced by several cell types, and it participates in many development and activation steps of different immune cells. Thus, the role of IFN-γ, particularly in acquired immunity, could not be studied by the knockout mouse approach, since the genetic modification would likely affect both the development of immunity in response to primary infection and its effect during reinfection. By contrast, in vivo neutralization of IFN-γ by specific antibodies, it is possible to target the deficiency on a specific phase, i.e., either on the primary infection or on reinfection.

In the present study, we therefore used the IFN-γ neutralization method to evaluate the role of IFN-γ in protection against either C. pneumoniae primary infection or reinfection in BALB/c and C57BL/6 mice. Monoclonal antibody to IFN-γ was administered intraperitoneally prior to and during infection, and the effect was evaluated using bacterial counts in the lungs and severity of pneumonia as parameters.

To neutralize IFN-γ, 6- to 8-week-old female BALB/cHsd (Laboratory Animal Centre, University of Helsinki, Finland) and C57BL/6J (Bomholtgård Breeding and Research Centre Ltd., Ry, Denmark) mice were injected intraperitoneally with 1-mg doses of anti-IFN-γ monoclonal antibody (clone XMG 1.2; a kind gift from R. L. Coffman, DNAX Research Institute, Palo Alto, Calif.) that blocks biological activity of IFN-γ (3) 1 day before primary challenge or rechallenge, depending on which one was targeted, and every third or fourth day thereafter. IFN-γ-neutralized and untreated control mice were inoculated intranasally with 106 inclusion-forming units (IFU) of C. pneumoniae Kajaani 6 isolate in 40 μl of sucrose-phosphate-glutamate solution under light carbon dioxide (BALB/c) or Metofane (Pitman-Moore, Inc., Mundelein, Ill.) (C57BL/6) anesthesia. Rechallenge was given similarly, when the mice had cleared the primary infection (33 and 63 days after primary infection to BALB/c and C57BL/6 mice, respec-
dilution factors were taken into account, one inclusion seen by expressed as logarithmic values of IFU per lung. After the specific antibodies (Kallestad, Chaska, Minn.). The results are inclusions were counted under a UV microscope from cells to 10 IFN-\(\gamma\)-neutralized and untreated mice were sacrificed. The supernatants of mechanically homogenized right lungs 12 days after primary infection and 6 days after reinfection) 6 to 10 IFN-\(\gamma\)-neutralized and untreated mice were sacrificed. The supernatants of mechanically homogenized right lungs were cultured on Vero cell monolayers using centrifugation and cycloheximide, as described previously (17). Intracellular inclusions were counted under a UV microscope from cells stained with fluorescein isothiocyanate-conjugated Chlamydia-specific antibodies (Kallestad, Chaska, Minn.). The results are expressed as logarithmic values of IFU per lung. After the dilution factors were taken into account, one inclusion seen by microscopy corresponded to a \(\log_{10}\) value of 1 IFU/lung (the detection limit). If no inclusions were detected, a \(\log_{10}\) value of 0.5 was used for calculating means and statistics (using Mann-Whitney U test). In addition to the controls done in parallel with the IFN-\(\gamma\)-neutralized mice, accumulated culture data from several different primary infection and reinfection experiments are presented as a reference for general infection kinetics.

For histopathological scoring, the left lung that in mice consists of only one lobe was fixed in 10% buffered formalin. The whole left lung was cut transversely at an equidistance of 4 \(\mu\)m to three parts, representing the cranial, middle, and caudal left lung. The tissue was processed routinely, and the three parts were embedded in paraffin side by side into the same block in such a way that the three complete cross sections of the cranial, middle, and caudal lung could be evaluated on the same section. The sections were stained with hematoxylin and eosin and evaluated blindly under a light microscope by two pathologists.

The severity of pneumonia was described as minimal, mild, moderate, marked, and severe, for which arbitrary scores of 0, 1, 2, 3, and 4 were respectively assigned. The \textit{C. pneumoniae}-induced pneumonia is typically cranioventral in distribution, and in mild cases, pneumonic lesions are present only in the cranial part of the lung. As the severity increases, the pneumonic area extends to the middle lung, and in very severe cases, affects the whole lung. A locally extensive pneumonia with changes only in one of the three sections (the cranial part) affecting 10 to 50% of that section and about 5 to 25% of the whole cross-sectional area of the left lung was described as mild (1), and a nearly diffuse severe pneumonia affecting all three cross sections and 75 to 100% of the total cross-sectional area was described as severe (4). In moderate pneumonia (2) there were pneumonic changes in two cross sections affecting about 25 to 50% of the whole cross-sectional area of the lung. When there were changes in all three sections and about 50 to 75% of the whole cross-sectional area showed lesions, the pneumonia was described as marked (3). The differences between groups were tested with a nonparametric Mann-Whitney U test.

Mononuclear cells were isolated from mechanically homogenized lungs in the primary infection experiments, as described previously (17). For the flow cytometric analysis, 0.4 \(\times\) 10\(^8\) mononuclear cells were stained with 5 \(\mu\)l of phycoerythrin-conjugated anti-mouse mac-1/CD11b (M1/70.15) (Caltag, South San Francisco, Calif.). Rat IgG2b was used as a control for unspecific binding, and unstained cells were used for adjustment of FACScan (Becton Dickinson, San Jose, Calif.). Secretion of tumor necrosis factor alpha (TNF-\(\alpha\)), interleukin 10 (IL-10), and IL-5 was assessed with enzyme-linked immunosorbent assay from culture supernatants of the mononuclear cells alone (background) or stimulated with formalin-inactivated \textit{C. pneumoniae} (1 \(\mu\)g/ml) collected after 72 h incubation at 37°C in 5% CO\(_2\) atmosphere as described previously for IL-10 and TNF-\(\alpha\) in references 17 and 18, respectively. In an IL-5 enzyme-linked immunosorbent assay, 2 \(\mu\)g of anti-mouse and -human IL-5 (TRFK5, rat IgG1; Pharmingen, San Diego, Calif.) per ml was used as first antibody, 1 \(\mu\)g of biotinylated anti-mouse IL-5 (TRFK4, rat IgG2a; Pharmingen) per ml was used as second antibody, and recombinant mouse IL-5 (Pharmingen) was used as a standard. Results of the background control were subtracted from the final results.

Intranasal inoculation of untreated mice with \textit{C. pneumoniae} resulted in a self-restricted infection without any clinical symptoms in either BALB/c or C57BL/6 mice, as shown previously (17, 20). Primary infection of BALB/c mice peaked during the first 2 weeks at 10\(^3\) IFU/lung and was cleared in a period of 3 to 4 weeks (Fig. 1A). The primary infection of C57BL/6 mice peaked somewhat later in the second week, at approximately 10\(^3\) IFU/lung, and was cleared in approximately 6 weeks (Fig. 1A). Neutralization of IFN-\(\gamma\) prior to and during the primary infection altered the bacterial counts in the lungs. In the BALB/c strain, less bacteria were isolated from the IFN-\(\gamma\)-neutralized mice during the first week (on day 6 the \(P\) value was 0.01), but during the second week of infection there was no difference between IFN-\(\gamma\)-neutralized and control mice (Fig. 1A).

In contrast, the numbers of bacteria were increased by a hundredfold in anti-IFN-\(\gamma\)-treated C57BL/6 mice at 12 days after primary \textit{C. pneumoniae} infection (\(P = 0.003\)) (Fig. 1A). The pneumonia score was significantly higher in IFN-\(\gamma\)-neutralized C57BL/6 mice on day 12 after primary infection than in untreated mice (Table 1). The pulmonary inflammatory infiltrate mainly consisted of macrophages and lesser numbers of neutrophils and lymphocytes. In addition, in the IFN-\(\gamma\)-neutralized mice there were moderate numbers of multinucleated giant cells and eosinophils, which were not present in the untreated mice. In BALB/c mice there were no differences between the IFN-\(\gamma\)-neutralized and untreated mice (Table 1). However, the lower pneumonia score in BALB/c mice than in C57BL/6 mice was partly due to the anesthesia method (our unpublished data).

The proportion of macrophages among the isolated pulmonary mononuclear cells, determined by flow cytometric analysis, was increased in IFN-\(\gamma\)-neutralized C57BL/6 mice, in comparison to the untreated mice, especially at 12 days after infection (33% versus 29% and 62% versus 21% at days 6 and 12, respectively). There were no differences in the proportions of macrophages in the IFN-\(\gamma\)-neutralized BALB/c mice versus controls (13% versus 16% and 10% versus 9%, at days 6 and 12, respectively). The pulmonary mononuclear cells isolated from IFN-\(\gamma\)-neutralized C57BL/6 mice at 12 days after infection expressed increased (by 330 pg/ml) \textit{C. pneumoniae}-induced TNF-\(\alpha\) production, compared to the untreated mice. In BALB/c mice, the TNF-\(\alpha\) production was reduced by 260 pg/ml in cells from IFN-\(\gamma\)-neutralized mice, compared to control. IFN-\(\gamma\) neutralization did not result in increased IL-10 or IL-5 production in response to \textit{C. pneumoniae} stimulation in either of the mouse strains (data not shown).

In both mouse strains, significant protection was acquired.
after primary infection, as shown previously (17, 20). During subsequent reinfection, less bacteria could be cultured and the infection was cleared in approximately 2 (BALB/c) and 3 (C57BL/6) weeks (Fig. 1B). The neutralization of IFN-γ resulted in a significant increase of the bacterial burden during reinfection \( (P \text{ values of 0.002 and 0.028 at day 6 in BALB/c and C57BL/6 mice, respectively, and a } P \text{ value of 0.04 at day 12 in BALB/c mice} \) (Fig. 1B). The pneumonia score was higher in the IFN-γ-neutralized C57BL/6 mice, but the difference was not statistically significant (Table 1). Again, no differences were detected in the pneumonia scores of BALB/c mice (Table 1).

The effect of anti-IFN-γ antibody treatment during primary infection differed between the two mouse strains studied; neutralization of IFN-γ exacerbated the infection only in C57BL/6 mice. The differences in the effect of IFN-γ neutralization indicate the effect of genetic background on the initial immunity, as seen during primary infection. Macrophages have been
shown to be able to produce IFN-γ in response to, for example, mycobacterial stimulation (8a). Thus, IFN-γ production by macrophages may be one factor affecting the type of initial immunity in the two mouse strains. Different inbred mouse strains have been shown to develop different types of immune responses during many intracellular infections, and this is often reflected in differences in their susceptibilities to infection (9, 26). It is interesting that although BALB/c mice appear not to be dependent on IFN-γ during primary C. pneumoniae infection, they do not develop a typical Th2 type response either, since no increment in IL-10 or IL-5 production was detected even after IFN-γ neutralization. Since the in vivo neutralization technique probably did not lead to a total deficiency of IFN-γ, it may be that clearance of primary C. pneumoniae infection in BALB/c mice is either IFN-γ independent or only very small amounts of IFN-γ are needed. In BALB/c mice, lesser numbers of bacteria were recovered from the lungs of IFN-γ-deficient mice than control mice at 6 days after infection. This effect could be a demonstration of the efficacy of IFN-γ-independent mechanisms in naive BALB/c mice. Neutralization of IFN-γ increased the number of macrophages and the production of TNF-α in the mononuclear cell fraction isolated from the lungs of C57BL/6 mice. IFN-γ neutralization increased the severity of pneumonia in C57BL/6 mice but not in BALB/c mice. In addition, multinucleated giant cells were present in the lungs of IFN-γ-neutralized C57BL/6 mice. These cells are usually formed by fusion of epithelioid cells or immature macrophages after, for example, long-lasting stimulation by T cells and/or ineffective processing of intracellular bacteria (8). In our infection model, it may be that neutralization of IFN-γ had a negative effect on macrophage function. The present findings during primary infection are in good accordance with previous data showing an IFN-γ-independent cellular response in BALB/c mice (17), while C57BL/6 mice have been shown to be dependent on IFN-γ-mediated protection mechanisms (20). During reinfection, a Th1 type immune response has been detected also in BALB/c mice. Neutralization of IFN-γ exacerbated the infection in both mouse strains. A similar exacerbation of C. pneumoniae reinfection by IFN-γ neutralization is also seen in outbred NIH/5 mice (our unpublished data). As we have shown earlier that the acquired immunity in BALB/c mice is dependent on CD8+ cells (18), the results of this study suggest that they may at least partially function through IFN-γ production. However, actively IFN-γ-producing cells have also been demonstrated in CD8-depleted mice, in which the acquired immunity seen during reinfection is abolished (18); thus, IFN-γ alone is probably not the only effector mechanism in acquired immunity. In conclusion, these results suggest that the importance of IFN-γ during primary C. pneumoniae infection depends on the genetic background of the mouse. However, in acquired immunity, detected during reinfection, the importance of IFN-γ overrides the initial differences.

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**REFERENCES**


**TABLE 1. Effect of IFN-γ neutralization on the pneumonia score in the two mouse strains after primary infection and reinfection with *C. pneumoniae***

<table>
<thead>
<tr>
<th>Mouse strain and treatment</th>
<th>Primary infection</th>
<th>Reinfction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 6</td>
<td>Day 12*</td>
</tr>
<tr>
<td>BALB/c</td>
<td></td>
<td></td>
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<tr>
<td>Untreated control</td>
<td>0.8 ± 0.5</td>
<td>1.0 ± 0</td>
</tr>
<tr>
<td>IFN-γ neutralization</td>
<td>0.2 ± 0.5</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td>C57BL/6</td>
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<td></td>
</tr>
<tr>
<td>Untreated control</td>
<td>2.3 ± 0.8</td>
<td>2.0 ± 0.8*</td>
</tr>
<tr>
<td>IFN-γ neutralization</td>
<td>2.3 ± 0.5</td>
<td>3.2 ± 0.8*</td>
</tr>
</tbody>
</table>

* Values are means ± standard deviations.
* Significant difference between these groups (*P* = 0.045) as calculated by two-tailed Mann-Whitney U test.
** ND, not determined.”

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