Immunological Reaction of Guinea-pigs Following Intranasal Mycoplasma pneumoniae Infection and Immunization with the 168 kDa Adherence Protein

By E. Jacobs,† M. Drews, A. Stuhlert, C. Büttner, P. J. Klein, M. Kist and W. Bredt

Institute for Medical Microbiology and Hygiene, and Institute for Pathology, University of Freiburg, D-7800 Freiburg, FRG

(Received 5 June 1987; revised 28 September 1987)

Humoral responses to Mycoplasma pneumoniae proteins, especially the 168 kDa protein, were demonstrated by Western blotting in sera and bronchial washings of all groups of infected or immunized guinea-pigs. However, infection was not prevented by these local and systemic antibodies. Hilar lymphocytes of infected and immunized guinea-pigs were stimulated in vitro by sonicated M. pneumoniae antigen and by the 168 kDa protein. Stimulation was significantly lower in animals which had been infected twice or had been preimmunized and challenged by infection. Histologically the most severe lesions were seen in the twice-infected group followed by the preimmunized group which was subsequently infected.

INTRODUCTION

The pathogenesis of Mycoplasma pneumoniae pneumonia is to a considerable extent determined by immunological reactions of the host (Fernald et al., 1979). In terms of the humoral response the 168 kDa adhesin protein (Feldner et al., 1982; Hu et al., 1982) appears to be the major immunogen (Jacobs et al., 1986), but this protein is possibly also involved in the pathogenesis of M. pneumoniae diseases. When monoclonal antibodies directed against this protein were given to hamsters together with an infective dose of M. pneumoniae, the lung lesion scores were reduced in the antibody-treated animals despite a normal colonization rate (Brunner et al., 1984). Apparently the antibodies interfered with the immune response against the homologous protein, thereby reducing the cellular infiltration of the lung. The isolation of the 168 kDa adherence protein in amounts sufficient for immunization (Jacobs & Clad, 1986; Jacobs et al., 1987) made it possible to study in more detail the effects of immunization with this protein antigen on the pathogenesis of M. pneumoniae infection.

METHODS

Mycoplasma strain, growth and preparation of suspensions. M. pneumoniae strain FH was grown in Roux bottles in Hayflick's medium (Hayflick, 1965). Glass-adherent cells were harvested after 48 h at 37 °C and stored at −70 °C for antigen preparation. For intranasal inoculation of guinea-pigs, freshly harvested mycoplasma cells were diluted with sterile phosphate-buffered saline (PBS) (0.14 M-NaCl, 0.01 M-sodium phosphate) pH 7.2 to an optical density of 0.15 at 660 nm (approx. 1 × 10⁸ c.f.u. ml⁻¹). For in vitro stimulation of lymphocyte cultures the standardized mycoplasma suspension was sonicated before use (Branson Sonifier Cell Disrupter B15, power 4 for 3 × 5 min, ice bath).

Isolation of the 168 kDa protein. M. pneumoniae cells were dissolved in electrophoresis sample buffer and heated for 3 min at 100 °C. Mycoplasmal proteins were separated by preparative SDS-PAGE as described previously (Jacobs & Clad, 1986). Gels were fixed and stained with Coomassie brilliant blue (Towbin et al., 1979). The

Abbreviation: PHA, phytohaemagglutinin.

0001-4217 © 1988 SGM
168 kDa band was cut out of the stained gel and was electroeluted (Elutrap, Schleicher and Schuell) for 8 h (Jacobs & Clad, 1986). The SDS was extracted according to Wessel & Flügge (1984) and the protein content of the extract was measured by the method of Peterson (1977). After evaporation of the solvent (CCl₄) the protein was stored at −70 °C. For in vitro stimulation the protein was dissolved in lymphocyte culture medium (RPMI, Gibco) to a protein concentration of 200 μg ml⁻¹. The purity and homogeneity of the isolated 168 kDa protein was checked by SDS-PAGE. The protein proved to be stable under these separation conditions. It possessed the expected 168 kDa band was cut out of the stained gel and was electroeluted (Elutrap, Schleicher and Schuell) for 8 h (Jacobs & Clad, 1986). The SDS was extracted according to Wessel & Flügge (1984) and the protein content of the extract was measured by the method of Peterson (1977). After evaporation of the solvent (CCl₄) the protein was stored at −70 °C. For in vitro stimulation the protein was dissolved in lymphocyte culture medium (RPMI, Gibco) to a protein concentration of 200 μg ml⁻¹. The purity and homogeneity of the isolated 168 kDa protein was checked by SDS-PAGE. The protein proved to be stable under these separation conditions. It possessed the expected mass of 168 kDa.

### Table 1. Lung lesion scores of different groups of guinea-pigs following intranasal infection with live M. pneumoniae (MP) or intranasal immunization with the 168 kDa antigen (168 Ag) or both treatments

The time interval between inoculations was 4 weeks if not noted otherwise. Each group consisted of three animals.

<table>
<thead>
<tr>
<th>Guinea-pig group</th>
<th>Inoculation schedule</th>
<th>Time of examination*</th>
<th>Lung lobes examined†</th>
<th>Median of lesion scores per group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st</td>
<td>2nd</td>
<td>3rd</td>
<td>RLL</td>
</tr>
<tr>
<td>1</td>
<td>MP</td>
<td>–</td>
<td>–</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>MP</td>
<td>MP</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>168 Ag</td>
<td>168 Ag</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>168 Ag</td>
<td>168 Ag</td>
<td>MP†</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0</td>
</tr>
</tbody>
</table>

* Weeks after last inoculation.
† RLL, right lower lobe; LUL, left upper lobe; LLL, left lower lobe. For definition of lesion scores see Methods.
‖ 2 weeks after second inoculation.

The proliferation test was done as a micromethod in flat-bottomed microtitre plates (Nunc Multidisk) using 1 ml of the lymphocyte suspension per well. Phytohaemagglutinin (PHA, 50 μg per well in 50 μl) (Wellcome), 50 μg sonicated M. pneumoniae protein (in 50 μl) or 10 μg 168 kDa protein (50 μl), respectively, was added to triplicate cultures. The cultures were incubated for 76 h (37 °C, 5%, v/v, CO₂ atmosphere). [methyl-³H]Thymidine, 0·5 μCi (18·5 M bq) per well, was added to the cultures 18 h before the cells were harvested onto a glass microfibre filter. The radioactive uptake was measured by liquid scintillation counting (2 ml Rotiszint 11, Roth; Betazint BF 8000, Berthold, FRG).

The thymidine uptake values of the lymphocytes from the different groups of experimental animals were compared and statistically analysed by the Student's t-test. The values for uninfected guinea-pigs (group 5) were considered as background.

Western immunoblotting. Serum and bronchial lavages of all guinea-pig lungs were tested for the presence of IgA, IgG and IgM to M. pneumoniae proteins using the Western immunoblotting method (Towbin et al., 1979). For this purpose M. pneumoniae proteins (as used for isolation of the 168 kDa protein) were separated by SDS-PAGE (Jacobs & Clad, 1986; Jacobs et al., 1987), and transferred to nitrocellulose filters (Schleicher & Schuell). The filters were incubated with 200 μl guinea pig serum in 1 ml 10% (w/v) skim milk in PBS for 1 h. After extensive washing steps, heavy-chain-specific anti-guinea-pig immunoglobulins conjugated with peroxidase (Miles Scientific Immunochromicals) were used to detect antigen–antibody complexes. 3,3'-Diaminobenzidine (Sigma) was used as substrate.
Histopathology. Tissue samples from the right lower lobe, left upper lobe and left lower lobe of the lungs of each guinea-pig were fixed and stained with haematoxylin and eosin by standard techniques. The histopathological parameters of the lung lesion scores were evaluated in a modification (H. Brunner, personal communication) of a procedure described elsewhere (Dajani et al., 1965): 0 = no infiltrates; 1 = 25% of the perivascular and/or peribronchial tissues infiltrated by mononuclear cells (either infiltration completely surrounding 25% of all bronchi/vessels in the area, or each bronchus/vessel infiltrated at about 25% of its circumference); 2 = 50% infiltration; 3 = 75%; 4 = 100%. One additional point was given for distinct alveolar infiltrates = maximum score of 5. Statistical analysis of the lung lesion scores was done by the Kruskal–Wallis test.

RESULTS

Western immunoblotting for antibodies to M. pneumoniae proteins

Serum antibodies against M. pneumoniae proteins or against the 168 kDa protein were regularly found after either infection with living organisms or immunization with the 168 kDa protein. The serum IgA antibody responses representative for all animals of the different groups are shown in Fig. 1. A distinct serum IgA response, mainly against the 168 kDa protein, developed after a single infection (group 1, Fig. 1, B). The spectrum of IgA specificity was considerably increased by reinfection (group 2, Fig. 1, C), with numerous additional proteins being recognized. A serum IgA response to the 168 kDa protein alone was demonstrated after immunization with this antigen (group 3, Fig. 1, D). A subsequent challenge infection (group 4, Fig. 1, E) intensified the antibody response to the 168 kDa adhesin without the production of detectable amounts of IgA antibodies against other M. pneumoniae proteins. In the bronchial washings of the twice-infected animals (group 2) a strong IgA response to many mycoplasmal proteins was seen (Fig. 1, F), whereas bronchial washings of animals preimmunized and

Fig. 1. Anti-M. pneumoniae IgA antibodies in sera and bronchial washings of guinea-pigs using the Western immunoblot method against total M. pneumoniae protein. Load per track of gel, 80 µg protein. Sera from: A, control animals (group 5); B, once-infected animals (group 1); C, twice-infected animals (group 2); D, immunized animals (group 3); E, immunized and infected animals (group 4). Bronchial washing from: F, twice-infected animals (group 2); G, immunized and infected animals (group 4).
challenged by *M. pneumoniae* infection (group 4) again showed only a weak IgA response to the 168 kDa protein (Fig. 1, G). The IgA activity of the bronchial washings of animals in the other groups was below the detection limit.

The serum antibodies of the IgG and IgM classes reacted in Western immunoblotting in a comparable pattern (results not shown). Twice-infected animals (group 2) developed antibodies against many of the *M. pneumoniae* proteins, with the most intense reactions showing against the 168 kDa antigen. Immunization with the 168 kDa protein followed by *M. pneumoniae* infection (group 4) produced a lesser diversity of antibodies by a similarly prominent anti-168 kDa response.

**Proliferation of hilar lymphocytes**

Hilar lymphocytes were stimulated *in vitro* with PHA, sonicated *M. pneumoniae* cells and the purified 168 kDa protein, respectively. The [\(^3\)H]thymidine uptake of unstimulated lymphocytes of all groups was 800 ± 400 c.p.m. All lymphocytes from the different infection and immunization groups responded to PHA stimulation with a [\(^3\)H]thymidine uptake of about 1–2 × 10^5 c.p.m. The results of *in vitro* stimulation of lymphocytes by sonicated *M. pneumoniae* antigen and by isolated 168 kDa protein are shown in Fig. 2. Control lymphocytes (group 5) showed only minimal uptake of thymidine, not significantly different from that of the unstimulated lymphocytes, when they were stimulated with *M. pneumoniae* antigen or 168 kDa antigen.

The hilar lymphocytes of animals infected only once with *M. pneumoniae* (group 1) showed a 6.7-fold increase in thymidine uptake with respect to group 5 after stimulation with *M. pneumoniae* antigen and a 6.6-fold increase after stimulation with 168 kDa antigen. Lymphocytes of twice-infected animals (group 2) showed a smaller increase in thymidine uptake, of only 3.3-fold after *M. pneumoniae* antigen stimulation and of 3-fold after 168 kDa antigen stimulation as compared with group 5. The results with lymphocytes from the twice-infected group (group 2) were significantly different from the negative controls (*M. pneumoniae* antigen *P* < 0.001; 168 kDa antigen *P* < 0.01). The proliferation activity of lymphocytes from
168 kDa protein in *M. pneumoniae* infection

Fig. 3. Histopathological examination of guinea-pig lungs. (a) Lung section of a control animal (group 5), (b) scarce infiltrations around small vessels and of bronchus of an animal immunized with 168 kDa protein (group 3), (c) massive peribronchial and submucosal lymphocyte infiltrations of an animal immunized with 168 kDa protein and infected with *M. pneumoniae* (group 4). B, bronchus; V, venous vessel; A, arterial vessel; I, infiltration. Bars, 200 μm.
this group was significantly lower than that of lymphocytes of the once-infected animals ($P = 0.005$ for both antigens).

Lymphocytes of animals immunized with 168 kDa antigen (group 3) proliferated up to 30 times more after *M. pneumoniae* antigen stimulation and 13 times more after 168 kDa antigen stimulation than cells from the control animals (group 5) ($P < 0.001$). In contrast, uptake of thymidine by lymphocytes from the group of guinea-pigs immunized with 168 kDa antigen followed by *M. pneumoniae* infection (group 4) was in the range of the twice-infected animals (group 2), namely 3-7-fold after *M. pneumoniae* antigen and 2-fold after 168 kDa antigen stimulation. It was significantly lower than in group 1 (*M. pneumoniae* antigen $P = 0.02$; 168 kDa antigen $P = 0.001$) or in group 3 ($P < 0.001$ for both antigens).

The total number of isolated hilar lymphocytes harvested per animal was clearly different between the control group ($1.7 \times 10^7$) and *M. pneumoniae*-infected (group 1, $8.2 \times 10^7$) or reinfected (group 2, $11.2 \times 10^7$) animals. The highest number of lymphocytes ($23 \times 10^7$), however, was harvested in the group of animals immunized with the 168 kDa antigen (group 3). After infection of the similarly immunized group the number of lymphocytes was reduced to the level of the twice-infected animals (group 4, $11.3 \times 10^7$).

**Histopathology**

The histological examination of the lungs of the control animals (group 5) showed no lymphocyte infiltration (Fig. 3a). In contrast, the lung tissues of twice-infected animals were densely infiltrated, with lymphocytes and histiocytes around the walls of small bronchi and around small vessels. Guinea-pigs immunized with 168 kDa protein showed rather scarce lymphocyte infiltrations around small vessels and focal peribronchiolar infiltrations with lymphocytes (Fig. 3b). Extensive lympho-histiocyte infiltrations in the submucosa and deeper parts of the wall of a greater bronchus were seen in animals after immunization with the 168 kDa protein followed by infection with *M. pneumoniae* (group 4, Fig. 3c). Similar infiltrations were also found around small vessels.

The lung lesion scores of each group (Table 1) were calculated and compared with the control group. The most severe lung lesions were found in animals which had been infected twice with *M. pneumoniae* (group 2), followed by the group with a single *M. pneumoniae* infection (group 1) and the group with combined 168 kDa protein immunization and *M. pneumoniae* infection (group 4). Animals immunized only with the 168 kDa protein (group 3) were least affected. Due to the small number of animals in each group, only the difference between controls (group 5) and the other groups was statistically significant in the Kruskal–Wallis test ($P = 0.024$).

**DISCUSSION**

In our experiments the humoral response after single or repeated infection was intense in all immunoglobulin classes and was directed against a variety of proteins of *M. pneumoniae*. However, in contrast to reinfection without preceding immunization, the challenged animals which were preimmunized with 168 kDa protein developed only a minor antibody response against the other proteins. Apparently the strongly immunogenic 168 kDa protein to some extent influenced the antibody response against other protein antigens of *M. pneumoniae*.

The strong local and systemic response against the 168 kDa protein caused by immunization did not, however, provide protection against reinfection. On the contrary, repeated infection as well as infection after intranasal immunization produced severe lesions, and the only successful reisolations of *M. pneumoniae* were obtained in these groups (one of three animals per group; data not shown). This finding is not unexpected, since inoculation of hamsters with mycoplasmas treated with adherence-inhibiting monoclonal anti-168 kDa antibodies did not affect colonization (Brunner *et al.*, 1984). These results suggest that local and systemic immunity against the adhesin may not be sufficient to inhibit infection with *M. pneumoniae*. The intended use of this protein for local immunoprophylaxis against *M. pneumoniae* infections (Fernald, 1979; Razin, 1985) may therefore not be as promising as previously expected.
Lung hilar lymphocytes of twice-infected or of preimmunized and subsequently infected guinea-pigs were significantly less stimulated by specific antigens than those of once-infected or immunized animals, respectively. This effect may be interpreted in two ways: (i) the second contact with the infecting agent or with one of its major antigens had immunosuppressive effects, or (ii) all lymphocytes capable of specific stimulation had migrated from the lymph nodes to peripheral lung tissues. The first explanation would be in agreement with observations of prolonged tuberculin anergy during M. pneumoniae infection (Biberfeld & Sterner, 1976). However, in our study the reactivity of the cells to a non-specific mitogen (PHA) was not affected. Moreover the intense humoral response did not indicate an impaired helper cell function of T-lymphocytes. The second possibility, namely the depletion of lymph nodes of M. pneumoniae-reactive lymphocytes, could not be proven or disproven, because sufficient amounts of purified lymphocytes from peripheral lung tissue could not be obtained. Preliminary immunohistochemical studies using anti-guinea-pig immunoglobulins to detect B cells showed only weak staining in lung infiltrates of infected animals, suggesting that the infiltrates comprised mostly T-cells (results not shown).

The similarity of the lymphocyte reaction patterns in twice-infected animals and in animals preimmunized and infected indicates that immunization with the purified 168 kDa protein can to some extent mimic primary infection with living organisms. The adherence protein of M. pneumoniae seems to be involved in pathogenesis in two different ways: (i) it mediates contact with the infecting agent or with one of its major antigens had immunosuppressive effects, or (ii) all lymphocytes capable of specific stimulation had migrated from the lymph nodes to peripheral lung tissues. The first explanation would be in agreement with observations of prolonged tuberculin anergy during M. pneumoniae infection (Biberfeld & Sterner, 1976).

The work was supported in part by grant 01 ZR 171/1 of the Bundesministerium für Forschung und Technologie. The secretarial help of Ruth Thomas is gratefully acknowledged.

REFERENCES


