Immunoprophylaxis of Experimental *Mycoplasma pneumoniae* Disease: Effect of Route of Administration on the Immunogenicity and Protective Effect of Inactivated *M. pneumoniae* Vaccine

HARRY GREENBERG, CHARLES M. HELMS, MICHAEL B. GRIZZARD,* WALTER D. JAMES, ROBERT L. HORSWOOD, AND ROBERT M. CHANOCK

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014

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Formalin-inactivated *Mycoplasma pneumoniae* vaccine was administered subcutaneously or intranasally to hamsters to examine the effect of route of administration on immunogenicity and protective effect. Parenterally administered vaccine in the doses employed induced serum complement-fixing antibody formation, but did not significantly decrease the frequency of pneumonia following challenge with virulent *M. pneumoniae*. Intranasally instilled vaccine was ineffective in stimulating serum antibody, but did diminish the frequency of experimentally induced pneumonia due to *M. pneumoniae*. However, a greater degree of resistance was induced by intranasal infection with either wild-type organisms or the ts 640 attenuated mutant of *M. pneumoniae*.

*Mycoplasma pneumoniae* is a significant cause of lower respiratory tract disease in children and young adults (3, 4, 12). Indeed, among college students and military recruits *M. pneumoniae* accounts for approximately 50% of the cases of pneumonia observed. Efforts have therefore been directed toward the development of a vaccine to prevent *M. pneumoniae* disease.

The efficiency of inactivated *M. pneumoniae* vaccines studied in field trials has ranged from 45 to 66% for prevention of pneumonia due to *M. pneumoniae* and thus falls short of complete protection. In some cases, the failure of parenterally administered inactivated vaccines to provide complete protection against mycoplasmal disease may have been related to poor antigenicity (9). However, in two separate studies employing antigenically potent vaccines which stimulated levels of complement-fixing (CF) and metabolism-inhibiting antibodies comparable to those which develop after naturally occurring *M. pneumoniae* disease, a protective efficacy of only 66% was observed (15; R. P. Wenzel et al., Abstr. Intersci. Conf. Antimicrob. Ag. Chemother., 15th, Washington, D.C., Abstr. 374, 1975).

The failure of inactivated vaccines to provide complete protection of humans against pneumonia due to *M. pneumoniae* may be related in part to failure of antigen administered by the parenteral route to stimulate local, more superficial, protective mechanisms of the respiratory tract (2). Indeed, there is evidence suggesting that the route by which *M. pneumoniae* organisms are administered to experimental animals is a critical factor in determining whether resistance to subsequent virulent challenge is induced (10, 14, 15). Thus, whereas intranasal (i.n.) infection of hamsters with virulent *M. pneumoniae* organisms stimulated significant resistance to disease, previous parenteral immunization with the same organisms failed to provide a comparable degree of protection (10). Animals infected i.n. with attenuated strains of *M. pneumoniae* were also more resistant to challenge than were animals immunized by the parenteral route (10; C. Helms et al., unpublished data). In these studies the increased efficiency of the local route over the parenteral route of administration in stimulating immunity might also be related to the capacity of locally administered organisms to replicate and thus increase their antigenic mass within the respiratory tract. Parenterally administered organisms are presumably incapable of such replication. Local and parenteral administration of a nonreplicating *M. pneumoniae* antigen might permit a clearer definition of the role of route of administration of *M. pneumoniae* in the production of immunity. In this paper, the effect of route of administration of a nonreplicating antigen, i.e., inactivated *M. pneu-
niae vaccine, upon the immunological response of hamsters and upon the development of protective immunity is examined.

MATERIALS AND METHODS

Animals. Young adult (80-g) Syrian hamsters were supplied by Charles River-Lakeview, Newfield, N.J. Animals were observed for at least 1 week for morbidity and mortality prior to use.

Inactivated M. pneumoniae vaccine. The M. pneumoniae vaccine employed (lot OSU-1A) was a protective immunity pneumonia glycolipid in 100% of hamsters and of hamsters and activated organisms which had been prepared and characterized as previously described (18). The vaccine was stored at −70°C. Intramuscular administration of 0.1 ml of OSU-1A vaccine was shown earlier to stimulate a significant rise in CF antibody to M. pneumoniae glycolipid in 100% of hamsters and guinea pigs (18).

In the present study the inoculum given to hamsters consisted of 0.2 ml of freshly thawed, undiluted OSU-1A vaccine (52.8 μg of protein).

M. pneumoniae organisms. A suspension of virulent M. pneumoniae (P1 1428 strain) at the second passage level in artificial medium (7 parts brain-heart infusion broth, 1 part yeast extract, and 2 parts horse serum) was used in all experiments. In previous studies 85% of hamsters developed pneumonia after i.n. administration of 10⁶ colony-forming units (CFU) of these organisms. A well-characterized temperature-sensitive mutant of M. pneumoniae derived from the virulent strain by chemical mutagenesis and designated mutant ts 640 was employed as a live attenuated vaccine (1, 19).

Intranasal inoculation procedure. After induction of anesthesia with sodium pentobarbital, hamsters were allowed to aspirate a 0.2-ml inoculum delivered to the external nares by use of a tuberculin syringe with a no. 25 needle. Animals were kept in supine position until recovery from anesthesia.

Measurement of growth of M. pneumoniae in vivo, histopathological techniques, and scoring of lesions. Methods used for quantitating M. pneumoniae organisms in hamster lungs and for preparing lung sections for histological examination have been described (1). Pulmonary lesions were graded on a scale of 0 to 8 by two independent observers. Scores of 1 through 5 reflected increasing degrees of bronchial disease. Up to three additional points were added for increasing degrees of interstitial disease. To both observers a score of 3 represented unequivocal histological evidence of pneumonia.

Measurement of CF Antibody. CF antibody was measured in serum by use of a purified glycolipid antigen, as described by Razin et al. (16).

Experimental protocol. A total of three experiments were performed in which groups of 20 to 30 hamsters were inoculated with inactivated M. pneumoniae vaccine or with living M. pneumoniae organisms. One group received a single dose of inactivated vaccine subcutaneously. A second group received three separate doses of inactivated vaccine i.n. at weekly intervals. A third group received a single i.n. inoculation of 10⁶ CFU of live, virulent M. pneumoniae organisms in a volume of 0.2 ml. In two experiments a fourth group was included which received a single i.n. dose of 10⁶ CFU of live ts 640 M. pneumoniae in 0.2 ml of mycoplasma medium. In each experiment control animals were included which had been inoculated with broth in an identical fashion to the experimental groups.

From 35 to 50 days after the last inoculation, at least 16 hamsters in each group were sacrificed and blood was obtained for serological study. The remaining animals were challenged i.n. with 10⁶ CFU of live, virulent M. pneumoniae. Ten days after challenge, animals were sacrificed, the lungs were cultured for M. pneumoniae, and lung tissue was fixed for histological examination.

RESULTS

Because of close comparability in experimental design, the results of three hamster experiments have been combined for presentation in Table 1.

Effect of route of administration on the immunogenicity of inactivated vaccine and comparison with infection with live organisms. Sera obtained from hamsters 35 to 50 days after subcutaneous immunization with the OSU-1A vaccine contained significantly elevated levels of serum CF antibody relative to the broth control group. The mean titer of CF antibody among hamsters parenterally inoculated with inactivated vaccine (1:8.6) was comparable to that of the group administered live ts 640 M. pneumoniae (1:14.9), but significantly less than that of the group infected (i.n.) with virulent M. pneumoniae (1:55.7). In contrast, animals which received inactivated vaccine by the i.n. route did not develop serum CF antibody although vaccine was given three times at weekly intervals. The mean titer of CF antibody in this locally vaccinated group (1:2.6) was comparable to that of the broth control group (1:2.5).

The titer of CF antibody detected after parenteral administration of the inactivated vaccine was lower than that recorded by Somerson et al. (18). This difference may reflect a difference in sensitivity of the CF tests employed. However, such a variation should not vitiate the relative differences observed among the experimental groups in this study since all the sera were assayed in one test.

Effect of route of administration of inactivated vaccine on resistance to disease. Hamsters previously immunized parenterally with inactivated vaccine showed no evidence of protection when challenged intranasally with live, virulent organisms. Thus, despite moderate levels of serum antibody, these animals devel-
Table 1. Protective efficacy of live or inactivated *M. pneumoniae* organisms administered by intranasal (i.n.) or subcutaneous (s.c.) route

<table>
<thead>
<tr>
<th>Immunization of hamsters</th>
<th>Route of inoculation</th>
<th>Reciprocal of serum CF antibody titer (geometric mean)</th>
<th>No. of sera examined</th>
<th>Percent which developed pneumonia (lesion score ≥ 3)</th>
<th>No. of hamsters</th>
<th>Mean lung lesion score ± SE</th>
<th>Titer of organisms in lung (mean CFU per g, log_{10})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broth</td>
<td>i.n., s.c.</td>
<td>26 2.5 ± 1.1</td>
<td>30</td>
<td>90</td>
<td>4.9 ± 0.4</td>
<td>5.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Inactivated vaccine</td>
<td>s.c.</td>
<td>21 8.6 ± 1.3b,c</td>
<td>20</td>
<td>90</td>
<td>4.1 ± 0.4</td>
<td>5.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Inactivated vaccine</td>
<td>i.n.</td>
<td>25 2.6 ± 1.1</td>
<td>30</td>
<td>53d</td>
<td>3.3 ± 0.5c</td>
<td>6.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>ts 640, <em>M. pneumoniae</em> (10^8 CFU)</td>
<td>i.n.</td>
<td>16 14.9 ± 1.4b,c</td>
<td>10</td>
<td>10</td>
<td>1.1 ± 0.3c</td>
<td>4.0 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Wild-type <em>M. pneumoniae</em> (10^8 CFU)</td>
<td>i.n.</td>
<td>22 55.7 ± 1.3b</td>
<td>30</td>
<td>17</td>
<td>1.7 ± 0.2c</td>
<td>3.5 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

a Hamsters sacrificed 10 days postchallenge.
b Significantly elevated relative to broth control group (P < 0.01, t test).
c Significantly reduced relative to group receiving wild-type organisms i.n. (P < 0.01, t test).
d Significantly reduced relative to group receiving inactivated vaccine s.c. (P = 0.05, Fisher exact test).
e Significantly reduced relative to broth control group (P ≤ 0.01, t test).
f Significantly reduced relative to broth control group (P < 0.01, Fisher exact test).

opned pneumonia with a frequency comparable to that of the broth control group (90%). In contrast, i.n. administration of inactivated vaccine induced resistance which was evident from a reduction in frequency of pneumonia (53%) and severity of lung lesions (mean lung lesion score of 3.3). Resistance to pulmonary disease was observed although these hamsters failed to develop serum CF antibody after immunization. Furthermore, the hamsters vaccinated i.n. were protected although this type of immunization did not suppress growth of the challenge organism in the lungs.

Animals immunized i.n. with live virulent *M. pneumoniae* or the attenuated ts 640 mutant were significantly protected from development of disease after challenge with virulent organisms, as indicated by the low frequency of pneumonia (10 to 17%) and the low mean lung lesion score (1.1 to 1.7). Resistance to development of pulmonary pathology was associated with a significant suppression of growth of the challenge organism. Animals previously infected with wild-type or ts 640 organisms did not have pulmonary lesions at the time of challenge; however, *M. pneumoniae* was still present in the lungs (10^{8.3} to 10^{8.9} CFU/g) at that time. Thus, the reduction in titer of organisms in the lungs post-challenge represents an underestimation of the resistance of these hamsters.

**DISCUSSION**

When living *M. pneumoniae* organisms are administered i.n. to golden Syrian hamsters, a disease is produced which closely resembles pneumonia seen in humans with regard to temporal, poral sequence, pathology, and serological events (8). From in vivo and in vitro studies, employing the hamster model of pneumonia due to *M. pneumoniae* and from clinical studies, much has been learned of the pathogenesis of this important disease (9).

A critical element in pathogenesis appears to be the attachment of *M. pneumoniae* by a specialized terminal process of ciliated epithelial cells of the lower respiratory tract (6, 7). This attachment, perhaps in concert with the elaboration of a toxin, results in disruption of ciliary function and cellular architecture (5–7, 17, 21). Subsequent inflammatory and immune events are confined to the immediate local area of infection and may be mediated indirectly by T cells (11). Indeed, it has been suggested that serum antibody and antigen-reactive cells in circulation merely reflect local immunopathological processes occurring in the respiratory tract (11). Thus, *M. pneumoniae* disease appears to begin as a superficial infection of the respiratory tract. Local immune processes should therefore be of paramount importance in preventing disease due to this organism.

Protection from experimentally induced *M. pneumoniae* disease in humans has been correlated with high levels of respiratory tract secretory antibody (2) and high levels of serum metabolism-inhibiting antibody (3). Studies to date suggest that secretory antibody correlates better with resistance to experimental challenge with *M. pneumoniae* than does serum antibody; however, the relative contribution of serum and secretory antibodies to protection requires further study (2). There is evidence
that optimal protection from experimental challenge is obtained when both local and systemic antibodies are present (2). Thus, it is significant that i.n. administered live virulent organisms or attenuated temperature-sensitive mutants of \textit{M. pneumoniae} stimulate both local and systemic antibodies (2, 13).

Stimulation of serum CF antibodies in hamsters by inactivated vaccine was dependent on the route of administration of antigen. Parenteral vaccine induced moderate levels of serum antibody, but locally administered vaccine was far less effective in this regard. Previously, serum antibody level was shown to correlate poorly with protection in experimental \textit{M. pneumoniae} disease (10). In keeping with these earlier findings, it was observed that parenterally administered inactivated vaccine, despite inducing moderate levels of serum antibody in hamsters, had no significant protective effect. On the other hand, the same vaccine given i.n., although it did not stimulate detectable serum antibody, produced a significant reduction in the occurrence of experimental disease.

Although i.n. administration of inactivated vaccine to hamsters prevented development of pneumonia after challenge, growth of the challenge organisms in the lungs was not suppressed. Similar paradoxical findings have previously been reported by others (10). Possibly, local immune processes may under certain circumstances inhibit the ability of \textit{M. pneumoniae} organisms to produce disease independently of the ability of the organism to replicate. Such a circumstance might arise if local antibodies were capable of binding to the specialized attachment process of \textit{M. pneumoniae}, preventing attachment of the organism to the ciliated epithelium and thereby preventing the initiation of pathogenic mechanisms leading to disease (5–7, 17).

It should be noted, however, that the protective effect induced by locally administered inactivated vaccine was not as marked as that observed after i.n. infection with either virulent organisms or a temperature-sensitive attenuated mutant of \textit{M. pneumoniae}. Presumably, the greater resistance induced by infection can be attributed to the greater antigenic mass provided by replicating organisms. Since immune-competent cells are present in both the upper respiratory tract and the lungs, the question remains as to which level or levels of the respiratory tract must be stimulated to afford the most effective protection against \textit{M. pneumoniae} disease. Additional studies will attempt to delineate the effects of separately stimulating the upper and lower segments of the respiratory tract with \textit{M. pneumoniae}.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


17. Sobelslavsky, O., B. Prescott, and R. M. Chanock. 1968. Adsorption of \textit{Mycoplasma pneumoniae} to neuraminic

