Evaluation of local and systemic immune responses induced by intramuscular injection of a *Mycoplasma hyopneumoniae* bacterin to pigs

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**Objective**—To evaluate immune responses induced by administration of *Mycoplasma hyopneumoniae* bacterin to pigs.

**Animals**—60 healthy 7- to 10-day-old cross-bred boars.

**Procedure**—Pigs were assigned to 1 of 4 pig groups (15 pigs/group): vaccinated, challenged; vaccinated, nonchallenged; nonvaccinated, challenged; nonvaccinated, nonchallenged. Vaccinated pigs received IM injections of a mycoplasma bacterin on days 0 and 14, whereas nonvaccinated pigs received saline (0.9% NaCl) solution. Pigs in the challenged groups were inoculated intratracheally with *M. hyopneumoniae* on day 42. Pigs were euthanized and necropsied 41, 44, 48, and 70 days after the first vaccination, and proportion of lung surface with pneumatic lesions was determined. Percentage of lymphocyte subpopulations and number of interferon-γ (IFN-γ) secreting lymphocytes in blood and tissues, cytokine and antibody concentrations in bronchoalveolar lavage (BAL) fluid, and serum antibody concentrations were determined.

**Results**—Vaccination against and infection with *M. hyopneumoniae* induced a local mucosal immune response in the respiratory tract of pigs. Proportion of lung surface with pneumatic lesions in vaccinated challenged pigs was reduced on day 70, compared with nonvaccinated challenged pigs. Vaccination stimulated the production of *M. hyopneumoniae*-specific IFN-γ secreting blood lymphocytes. Tumor necrosis factor-α concentration in BAL fluid on day 70 was increased in nonvaccinated challenged pigs, compared with vaccinated challenged pigs.

**Conclusions and Clinical Relevance**—Vaccination against *M. hyopneumoniae* induced local, mucosal, humoral, and cellular immune responses. Moreover, vaccination reduced the severity of lung lesions in challenged pigs, suggesting that mucosal antibodies, mediation of the inflammatory response, and cell-mediated immune responses are important for control of mycoplasmal pneumonia in pigs. (Am J Vet Res 2000;61:1384-1389)

Pneumonia caused by *Mycoplasma hyopneumoniae* an is an important economic problem affecting swine production worldwide. *Mycoplasma hyopneumoniae* is the etiologic agent of enzootic pneumonia and is 1 of the most commonly isolated agents from pigs with porcine respiratory disease complex. Results of recent controlled studies indicate that *M. hyopneumoniae* potentiates pneumonia induced by porcine reproductive and respiratory syndrome virus (PRRSV). Vaccination is an important intervention strategy used to control mycoplasmal pneumonia. Despite the induction of serum *M. hyopneumoniae*-specific antibodies by these vaccines, no direct correlation has been found between antibody concentration and protection against *M. hyopneumoniae* challenge. Several studies have evaluated cellular and humoral immune responses induced by vaccination or challenge. Cell-mediated immunity against *M. hyopneumoniae* in response to vaccination or challenge has been measured by determining the proliferative responses of lymphocytes following in vitro stimulation with *M. hyopneumoniae*-specific antigens. Results of these studies indicate that there was considerable variation among individual pigs, although proliferation of blood lymphocytes from vaccinated pigs was greater, compared with nonvaccinated pigs. However, to our knowledge, measurements of specific T-cell responses to mycoplasmal antigens have not been reported.

*Mycoplasma hyopneumoniae* infects the ciliated epithelial cells of the respiratory tract. Thus, a mucosal immune response may be important in the prevention and control of *M. hyopneumoniae*-induced pneumonia. A previous study found that antibody concentrations in respiratory tract washings did not correlate with protection against pneumonia. However, a study investigating the effect of vaccination on the potentiation of PRRSV by *M. hyopneumoniae* found that vaccination induced secretion of *M. hyopneumoniae*-specific antibodies into bronchoalveolar lavage (BAL) fluid, suggesting that these antibodies may be important in the prevention or resolution of mycoplasmal pneumonia. The purposes of the study reported here was to evaluate local mucosal and systemic immune responses against mycoplasmal membrane antigens induced by a commercially available *M. hyopneumoniae* bacterin and to determine the degree of protection against development of inflammation and pulmonary lesions following experimental challenge with *M. hyopneumoniae* in pigs.

**Materials and Methods**

Pigs—Sixty cross-bred 7- to 10-day-old boars that were free of infection with swine influenza virus, PRRSV, and *M. hyopneumoniae* bacterin and to determine the production of *M. hyopneumoniae*-specific antibodies into bronchoalveolar lavage (BAL) fluid, and serum antibody concentrations were determined.
hyopneumoniae were obtained from a commercial herd and provided water and feed ad libitum throughout the study. Pigs were initially housed in 1 room, but after challenge with \( M \) \textit{hyopneumoniae}, pigs were housed in 4 rooms with equivalent lighting and ventilation systems. The study was conducted in accordance with the guidelines of the Iowa State University Institutional Committee on Animal Care and Use.

**Experimental design**—Pigs were randomly assigned to 1 of the 4 following treatment groups: nonvaccinated, nonchallenged \( (n = 15) \); nonvaccinated, challenged \( (15) \); vaccinated, nonchallenged \( (15) \); and vaccinated, challenged \( (15) \). Blood was collected into heparinized tubes from all pigs at 2-week intervals throughout the trial and prior to euthanasia. On day 0, when pigs were 13 to 16 days old, and on day 14, pigs in the vaccinated groups received IM injections of a commercially available \( M \) \textit{hyopneumoniae} bacterin, whereas those in the nonvaccinated groups received IM injections of saline (0.9% NaCl) solution. On day 42, pigs in the challenged groups were inoculated intratracheally with 10 ml of a 1:100 dilution of a tissue homogenate containing 10³ color changing units (CCU) of a derivative of \( M \) \textit{hyopneumoniae} strain 11 per ml (inoculating dose, 10⁴ CCU of \( M \) \textit{hyopneumoniae} strain 11/ml). Pigs in the 2 challenged groups (ie, nonvaccinated, challenged and vaccinated, challenged) were randomly assigned to be housed in 1 of 2 rooms after challenge, whereas pigs in the nonchallenged groups were assigned to 2 other rooms.

Two pigs from each treatment group were euthanatized and necropsied on day 41, the day before challenge. Data collected on day 41 were combined for the 2 nonvaccinated groups and the 2 vaccinated groups before analyses. On days 44 and 48, 4 pigs from each group were euthanatized and necropsied, and on day 70, 5 pigs from each group were euthanatized and necropsied.

**Necropsy**—Blood was collected into heparinized and serum tubes prior to euthanasia for isolation of lymphocytes and determination of serum \( M \) \textit{hyopneumoniae}-specific antibody concentrations, respectively. Necropsies were performed, as described. Briefly, the right rib cage was reflected, and a portion of lung was aseptically collected for isolation of \( M \) \textit{hyopneumoniae}. Swab specimens of the bronchi were submersed in RPMI medium with 0.1% fetal bovine serum (FBS) containing 100 \( \mu \)g of streptomycin/ml, 100 \( \mu \)g of penicillin G/ml, and 0.05 \( \mu \)g of gentamicin sulfate/ml. Number of interferon-\( \gamma \) (IFN-\( \gamma \)) secreting cells and percentage of \( B \) cells, CD4⁺, CD8⁺, and CD4⁺CD8⁻ T cells were determined by use of an enzyme-linked immunospot (ELISPOT) assay and flow cytometry, respectively.

**Flow cytometry**—Blood and tissue lymphocytes were analyzed by use of flow cytometry according to a described protocol. Briefly, lymphocytes were diluted in PBS with 1% bovine serum albumin and 0.1% sodium azide (flow cytometry buffer) and placed in round-bottomed 96-well microtiter plates. Murine monoclonal antibodies (mAb) specific for porcine CD4 (mAb PT90A) and CD8 (mAb PT81B) molecules or matched isotype control antibodies were added to the appropriate wells, and plates were incubated for 30 minutes on ice. Cells were washed with flow cytometry buffer, followed by incubation for 30 minutes on ice with isotype-specific anti-mouse immunoglobulins conjugated to either fluorescein isothiocyanate (FITC) or R-phycocerythrin (PE) to allow for 2-color analysis of CD4⁺, CD8⁺, FITC-conjugated anti-swine IgG, which recognized heavy and light chains of immunoglobulin. Cells were again washed with flow cytometry buffer and fixed overnight in 2% paraformaldehyde. Samples were analyzed by use of flow cytometry at the Cell and Hybridoma Facility at Iowa State University.

**Enzyme-linked immunospot assay**—Numbers of IFN-\( \gamma \) secreting cells in blood and tissue lymphocytes were determined by use of an ELISPOT assay, as described. Briefly, 96-well microtiter plates were coated with 50 \( \mu \)l of mAb against porcine IFN-\( \gamma \) (mAb P2G10; 0.15 \( \mu \)g/ml) for 1 hour at 37°C, washed 3 times with sterile PBSS, and blocked with RPMI containing 10% FBS for 2 hours at 37°C. Cells were added to each well in complete medium alone (no stimulation) or with concanavalin A (ConA; 5 \( \mu \)g/ml) or \( M \) \textit{hyopneumoniae} antigen (10 \( \mu \)g/ml) in RPMI containing 10% FBS for 2 hours at 37°C. Cells were washed 3 times with PBSS containing 0.09% Tween 20 (PBST), incubated with 50 \( \mu \)l of polyclonal rabbit anti-porcine IFN-\( \gamma \) antibody for 1 hour at 37°C, washed 3 times with PBST, and incubated with 50 \( \mu \)l of peroxidase-conjugated donkey anti-rabbit IgG antibody for 1 hour at 37°C. Wells were washed 3 times with PBST, and 50 \( \mu \)l of 3,3',5,5'-tetramethylbenzidine membrane peroxidase added to each well for 10 minutes. The reaction was stopped with the addition of 100 \( \mu \)l of distilled water/well. Spots corresponding to IFN-\( \gamma \) secreting cells were counted, using a dissecting microscope, and data were reported as number of spot-forming cells per 2 \( \times \)10⁶ lymphocytes.
Analysis of bronchoalveolar lavage fluid—Porcine tumor necrosis factor-α (TNF-α) in BAL fluid was measured by use of a commercially available ELISA according to the manufacturer's directions. Concentration of M. hyopneumoniae-specific antibodies in BAL fluid was measured by use of a described ELISA. Briefly, microtiter plates were coated with a membrane preparation of M. hyopneumoniae clone 232-2A3 isolated from a pig inoculated with M. hyopneumoniae strain 11. Bronchoalveolar lavage fluid was tested undiluted. To determine the isotype of M. hyopneumoniae-specific antibodies, peroxidase-labeled goat anti-porcine IgA, IgG, and IgM antibodies (all heavy chain specific) were used. Optical density values determined for BAL fluid were analyzed by use of a general linear repeated measures mixed model ANOVA. The model included the fixed effects of vaccination group, challenge group by vaccination group by time interaction, and challenge group by vaccination group by time interaction and the random effects of room, vaccination group by room interaction, challenge group by vaccination group by time interaction, and residual. If the effects of vaccination group, vaccination group by time, or challenge group by vaccination group by time were significant ($P < 0.05$), then pairwise comparisons were made within the vaccination group by challenge group interaction among all possible combinations and between vaccinated and nonvaccinated treatment groups at each day. Significance was set at $P < 0.05$. Least-squares means were back-transformed to the original scale for presentation. Arithmetic means and SEM were calculated for variables determined for each vaccine-challenge group combination.

Results

Lung lesions and bacteriologic evaluation—Necropsy evaluation on days 41, 44, and 48 revealed that a number of pigs in all groups had mild bacterial pneumonia; lesions detected at these times were not consistent with M. hyopneumoniae-induced pneumonia. Mycoplasma hyopneumoniae was not isolated from specimens collected from any pig during necropsies performed prior to challenge. However, H. parasuis was isolated from 9 pigs, Bordetella bronchiseptica from 10 pigs, and M. hyosynoviae from 4 pigs.

A significantly greater proportion of lung surface with pneumatic lesions was detected in the nonvaccinated pigs challenged with M. hyopneumoniae on day 70, compared with the other treatment groups (Table 1). Less than 1% of the lungs of vaccinated and challenged pigs had pneumatic lesions, indicating the efficacy of the vaccine. Although some evidence of bacterial pneumonia detected during the earlier necropsies was also apparent in pigs necropsied on day 70, lesions at this later date were less severe. The lung lesions observed in the nonvaccinated nonchallenged group on day 70 were consistent with lesions of bacterial pneumonia and not with lesions induced by M. hyopneumoniae.

Mycoplasma hyopneumoniae was cultured from lung specimens of 16 of 25 (64%) challenged pigs. The number of pigs with positive culture results was evenly divided between vaccinated and nonvaccinated groups. However, results of the direct fluorescent antibody assay were positive for only 6 of the 25 (24%) challenged pigs; 5 of these 6 pigs were in the vaccinat...

Table 1—Severity of lung lesions, determined by use of image analysis during necropsy, in pigs that received IM injections of saline (0.9% NaCl) solution (nonvaccinated) or a commercial Mycoplasma hyopneumoniae bacterin (vaccinated) on days 0 and 14 followed by challenge with M. hyopneumoniae on day 42.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 41* (a)</th>
<th>Day 44* (a)</th>
<th>Day 48* (a)</th>
<th>Day 70 (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonvaccinated, nonchallenged</td>
<td>0.71 ± 0.25 (4)</td>
<td>0.77 ± 0.69 (4)</td>
<td>0.13 ± 0.11 (4)</td>
<td>0.13 ± 0.13* (5)</td>
</tr>
<tr>
<td>Vaccinated, nonchallenged</td>
<td>0.36 ± 0.36 (4)</td>
<td>0.54 ± 0.43 (4)</td>
<td>2.07 ± 0.79 (4)</td>
<td>0.00 ± 0.00* (5)</td>
</tr>
<tr>
<td>Vaccinated, challenged</td>
<td>0.63 ± 0.45 (4)</td>
<td>3.40 ± 2.23 (3)</td>
<td>0.11 ± 0.08* (5)</td>
<td>0.00 ± 0.00* (5)</td>
</tr>
</tbody>
</table>

*Pneumonic lesions not consistent with M. hyopneumoniae-induced pneumonia; several pigs infected with Haemophilus suis, Bordetella bronchiseptica, or M. hyosynoviae. †Pneumonic lesions consistent with M. hyopneumoniae-induced pneumonia.

C = Data from 2 pigs in the vaccinated, challenged and vaccinated, nonchallenged groups combined with data from 2 pigs in the nonvaccinated, nonchallenged and vaccinated, nonchallenged groups, respectively.

Within a column, values with different superscript letters are significantly ($P < 0.05$) different.
ed challenged group. *Mycoplasma hyopneumoniae* was not detected in lung specimens from any nonchallenged pig by use of either bacteriologic culture or the direct fluorescent antibody assay.

**Lymphocyte numbers**—Flow cytometric analysis indicated that there were no differences in percentage of lymphocyte subsets in blood among treatment groups at any time during the trial. On day 70, we did detect an increase in the percentage of lymphocytes expressing surface immunoglobulins, suggestive of B cells, in the lungs of pigs in the nonvaccinated challenged group, compared with the other groups.

**Number of IFN-γ secreting cells**—On day 41 (the day before challenge), number of IFN-γ secreting cells in blood or any tissue evaluated did not differ among groups. However, on day 44, significantly more IFN-γ secreting cells were detected in blood obtained from pigs in the vaccinated nonchallenged group, compared with the nonvaccinated nonchallenged group (Table 2). On day 48, there were significantly more IFN-γ secreting cells in the blood of pigs in the vaccinated, challenged group, compared with all other groups. Conversely, on day 70, pigs in the vaccinated, nonchallenged group had a significantly greater number of IFN-γ secreting cells in blood, compared with the other groups. No other differences were observed among any of the groups or tissues.

**Analysis of BAL fluid**—We measured concentrations of TNF-α and IL-1 in BAL fluid as an indication of inflammation. On days 44 and 48, concentrations of TNF-α in all groups were below the detection limits of the assay. On day 70, concentration of TNF-α was significantly greater in the non-vaccinated, challenged group (88.1 pg/ml) than in all other groups; concentrations at this time in the other groups were below the detection limit of the assay. No differences in IL-1 concentrations were observed among groups on any day evaluated (days 41, 44, 48, and 70).

Low concentrations (mean OD450 nm ≤ 0.4) of *M hyopneumoniae*-specific IgM were detected in all groups at all times; concentration did not differ over time or among groups. On days 41, 44, and 48, concentrations of *M hyopneumoniae*-specific IgG and IgA were below the detection limit of the assay. On day 70, concentrations of specific IgG and IgA were significantly greater in BAL fluid from the vaccinated and challenged group, compared with the other groups (Table 3).

**Serum antibody concentrations**—All pigs were seronegative for *M hyopneumoniae* 5 days prior to the first injection of *M hyopneumoniae* bacterin or saline solution. Vaccinated pigs seroconverted approximately 2 weeks after the second vaccination (Table 4). Antibody concentrations decreased thereafter in the nonchallenged vaccinated pigs. The nonvaccinated challenged pigs remained seronegative throughout the trial. On day 70, serum concentration of *M hyopneu-

### Table 2
Mean (± SEM) number of blood lymphocytes in pigs that secreted interferon-γ (IFN-γ) in response to in vitro stimulation with *Mycoplasma hyopneumoniae* antigen.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 41 (n)</th>
<th>Day 44 (n)</th>
<th>Day 48 (n)</th>
<th>Day 70 (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonvaccinated, nonchallenged</td>
<td>4.33 ± 2.89 (4)</td>
<td>0.45 ± 0.21 (4)</td>
<td>1.33 ± 0.69 (4)</td>
<td>0.92 ± 0.62 (5)</td>
</tr>
<tr>
<td>Nonvaccinated, challenged</td>
<td>C</td>
<td>1.65 ± 1.23 (4)</td>
<td>0.25 ± 0.25 (4)</td>
<td>1.54 ± 0.55 (5)</td>
</tr>
<tr>
<td>Vaccinated, nonchallenged</td>
<td>6.35 ± 2.37 (4)</td>
<td>11.60 ± 8.73 (4)</td>
<td>2.50 ± 2.37 (4)</td>
<td>20.42 ± 2.4 (5)</td>
</tr>
<tr>
<td>Vaccinated, challenged</td>
<td>C</td>
<td>2.95 ± 1.91 (4)</td>
<td>7.37 ± 1.02 (3)</td>
<td>2.14 ± 0.71 (5)</td>
</tr>
</tbody>
</table>

*Pigs received IM injections of saline solution (nonvaccinated) or a commercial *M hyopneumoniae* bacterin (vaccinated) on days 0 and 14 followed by challenge with *M hyopneumoniae* on day 42.

See Table 1 for key.

### Table 3
*Mycoplasma hyopneumoniae*-specific antibody response on day 70 in bronchoalveolar lavage fluid obtained from pigs that received IM injections of saline solution (nonvaccinated) or a commercial *M hyopneumoniae* bacterin (vaccinated) on days 0 and 14 followed by challenge with *M hyopneumoniae* on day 42.

<table>
<thead>
<tr>
<th>Group</th>
<th>IgG</th>
<th>IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonvaccinated, nonchallenged</td>
<td>0.00 ± 0.00*</td>
<td>0.00 ± 0.00*</td>
</tr>
<tr>
<td>Nonvaccinated, challenged</td>
<td>0.07 ± 0.05*</td>
<td>0.01 ± 0.01*</td>
</tr>
<tr>
<td>Vaccinated, nonchallenged</td>
<td>0.01 ± 0.01*</td>
<td>0.00 ± 0.00*</td>
</tr>
<tr>
<td>Vaccinated, challenged</td>
<td>0.40 ± 0.10*</td>
<td>0.17 ± 0.05*</td>
</tr>
</tbody>
</table>

*Reported as optical density at 450 nm. See Table 1 for key.

### Table 4
*Mycoplasma hyopneumoniae*-specific antibody response on day 70 in serum of pigs that received IM injections of saline solution (nonvaccinated) or a commercial *M hyopneumoniae* bacterin (vaccinated) on days 0 and 14 followed by challenge with *M hyopneumoniae* on day 42.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 28</th>
<th>Day 41</th>
<th>Day 42</th>
<th>Day 44</th>
<th>Day 48</th>
<th>Day 70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonvaccinated, nonchallenged</td>
<td>0.002 ± 0.007*</td>
<td>−0.007 ± 0.014*</td>
<td>−0.001 ± 0.006*</td>
<td>0.006 ± 0.012*</td>
<td>0.004 ± 0.010*</td>
<td>0.010 ± 0.018*</td>
</tr>
<tr>
<td>Nonvaccinated, challenged</td>
<td>0.008 ± 0.006*</td>
<td>0.016 ± 0.014*</td>
<td>0.013 ± 0.006*</td>
<td>0.014 ± 0.012*</td>
<td>0.005 ± 0.010*</td>
<td>0.007 ± 0.010*</td>
</tr>
<tr>
<td>Vaccinated, nonchallenged</td>
<td>0.061 ± 0.058*</td>
<td>0.270 ± 0.090*</td>
<td>0.449 ± 0.095*</td>
<td>0.482 ± 0.097*</td>
<td>0.559 ± 0.082*</td>
<td>0.213 ± 0.116*</td>
</tr>
<tr>
<td>Vaccinated, challenged</td>
<td>0.515 ± 0.060*</td>
<td>0.215 ± 0.099*</td>
<td>0.412 ± 0.053*</td>
<td>0.286 ± 0.097*</td>
<td>0.348 ± 0.095*</td>
<td>0.882 ± 0.116*</td>
</tr>
</tbody>
</table>

*Reported as optical density at 450 nm. See Table 1 for key.
hyo pneumoniaspecific antibodies in the vaccinated and challenged group was significantly greater than in all other groups. Also, all pigs in this treatment group necropsied on day 70 were seropositive for *M. hyopneumoniae*.

**Discussion**

This study elucidated several mechanisms by which a commercial *M. hyopneumoniae* bacterin induces protection against mycoplasma pneumonia in pigs. Because *M. hyopneumoniae* attaches only to the cilia on epithelial cells in the respiratory tract, the hypothesis of this study was that a local mucosal and a cellular immune response would be important for control of clinical disease and lesion severity. To evaluate the protective mechanisms induced by the vaccine, a number of immunologic assays designed to measure humoral and cellular aspects of the immune response were used. Necropsies were performed at various times after vaccination and challenge, enabling us to evaluate temporal changes in the immune system response.

The pigs were obtained from a herd that was free of PRRSV and *M. hyopneumoniae*. Unfortunately, the pigs did have other health problems, which made the interpretation of a few aspects of the study difficult. The pigs had clinical signs consistent with polyserositis including swollen scrotum. No coughing was observed prior to *M. hyopneumoniae* inoculation. One pig from the vaccinated and challenged group died on day 6; lesions consistent with polyserositis were detected during necropsy. In several other pigs, mild serositis lesions, primarily in the ventral aspect of the peritoneum and scrotum, were detected during necropsy.

The exact etiopathogenesis of the pneumonia observed in pigs euthanatized before challenge was not known. Necropsies were performed at various times after vaccination and challenge, enabling us to evaluate temporal changes in the immune system response.

The pigs had clinical signs consistent with polyserositis including swollen scrotum. No coughing was observed prior to *M. hyopneumoniae* inoculation. One pig from the vaccinated and challenged group died on day 6; lesions consistent with polyserositis were detected at necropsy. In several other pigs, mild serositis lesions, primarily in the ventral aspect of the peritoneum and scrotum, were detected during necropsy. The exact etiopathogenesis of the pneumonia observed in pigs euthanatized before challenge was not known, as several bacterial pathogens were isolated from these pigs. On the basis of bacteriologic culture results, *M. hyopneumoniae* and *M. hyosynoviae* were isolated from several pigs. The effect of pneumonia and serosal disease induced by these pathogens on the efficacy of, and the local immune response induced by, the *M. hyopneumoniae* vaccine appeared to be minimal, especially in pigs that were necropsied on day 70. The effect of exposure to *M. hyosynoviae* on the immune responses measured in this trial is unknown. However, cross-reactivity of serum antibodies has not been reported between *M. hyopneumoniae* and *M. hyosynoviae*.

Our results revealed several findings about local immune responses induced by an intramuscularly administered *M. hyopneumoniae* vaccine. The efficacy of the mycoplasma vaccine was indicated by the reduction of severity of pneumonia lesions. Mean proportion of lung surfaces with lesions consistent with *M. hyopneumoniae*-induced pneumonia was 7.58 in the nonvaccinated challenged group and 0.11 in the vaccinated challenged group on day 70 (28 days after challenge). Accordingly, the *M. hyopneumoniae*-specific immune responses described in the present study are thought to be representative of the immune response induced by this vaccine.

Flow cytometric analyses were performed on lymphocytes isolated from blood collected periodically throughout the trial. The purpose of these analyses was to evaluate the effect of vaccination and *M. hyopneumoniae* challenge on blood lymphocyte subpopulations. Flow cytometry does not measure a specific *M. hyopneumoniae* response. On the basis of results of these analyses, we could find no evidence of nonspecific suppression or expansion of any lymphocyte subpopulation as a result of the vaccination or challenge.

Flow cytometric analyses were also performed on lymphocytes isolated from the tissues obtained during necropsy. We found an increased number of lymphocytes expressing surface immunoglobulins, which is consistent with the characteristics of B cells, in the lungs of nonvaccinated challenged pigs on day 70. The presence of B cells is consistent with earlier data from our laboratory and appears to be typical of lymphocyte populations observed in the lungs of pigs with pneumonic lesions induced by *M. hyopneumoniae*.17 The lack of increased numbers of B cells in the vaccinated and challenged group indicates that the vaccine inhibits the proliferation of lymphocytes in response to *M. hyopneumoniae* infection. We did not determine whether the increase in B cells was a nonspecific inflammatory response to the mycoplasma organisms or a specific response by the immune system against the pathogen.

To evaluate the cell-mediated immune response induced by vaccination or challenge with *M. hyopneumoniae*, ELISPOT assays to identify IFN-γ-secreting cells were performed. The ability of a mycoplasma vaccine to induce IFN-γ secretion by lymphocytes is an important and interesting discovery. Pigs vaccinated against and challenged with *M. hyopneumoniae* had the greatest number of IFN-γ-secreting cells on day 48, whereas on day 70, the vaccinated nonchallenged pigs had the greatest number of these cells. These results suggest that IFN-γ-secreting cells may be important in mediating a protective immune response against mycoplasmal pneumonia. Interferon-γ secreting cells are important for the activation of macrophages as well as the promotion of a cell-mediated immune response.

Our results suggest that activated T-cells producing IFN-γ may be important in the resolution of *M. hyopneumoniae* infection.

The results of BAL fluid analyses were informative with respect to the local immune response induced by the vaccine and the inflammatory response induced by challenge with *M. hyopneumoniae*. Results of earlier studies indicated that BAL fluid IL-1 concentrations increase following challenge with *M. hyopneumoniae*.17 Interleukin-1 concentrations varied among pigs within each group in the present study, which made it difficult to detect significant differences among groups. However, TNF-α lesions, which agrees with results of a previous study.17 We did not detect an increase in TNF-α concentrations on day 41 in pigs with bacterial pneumonia caused by a pathogen other than *M. hyopneumoniae*. Nonvaccinated challenged pigs had significantly more TNF-α in BAL fluid on day 70 than the pigs in the other groups; TNF-α concentrations in the other 3 groups were less than the detection limit of the assay. We did not detect TNF-α in BAL fluid of vaccinated challenged pigs at any time, indicating that vaccination...
helped protect the lung from inflammation associated with M hyopneumoniae infection. Increase in concentrations of proinflammatory cytokines, including TNF-α, are thought to be one possible mechanism for the potentiation of PRRSV-induced pneumonia by M hyopneumoniae. Results of a previous study performed in our laboratory indicate that vaccination against M hyopneumoniae reduced the potentiation of PRRSV-induced pneumonia by M hyopneumoniae. This may be because the vaccine prevents an increase in lung TNF-α concentrations in response to challenge with M hyopneumoniae, thus impeding potentiation of PRRSV-induced pneumonia.

Previously we had shown that protection against clinical mycoplasmal pneumonia was not directly correlated with serum antibody concentrations induced by vaccination against M hyopneumoniae. However, the results of the study reported here suggest that an intramuscularly administered vaccine resulted in an enhanced local mucosal immune response following challenge, as demonstrated by the significantly increased concentrations of M hyopneumoniae-specific IgG and IgA in the BAL fluid of vaccinated challenged pigs. Because BAL fluid is in close contact with M hyopneumoniae organisms attached to the cilia of the respiratory tract, antibodies in this compartment may be an important mechanism of defense against clinical disease.

Serum concentrations of M hyopneumoniae-specific antibodies decreased significantly over time in the vaccinated nonchallenged group, such that 2 of the 3 pigs in this group necropsied on day 70 were seronegative. However, all 5 challenged pigs necropsied on day 70 continued to have detectable concentrations of specific serum antibodies. None of the nonvaccinated nonchallenged pigs seroconverted during the trial, confirming that biosecurity was maintained. Nonvaccinated challenged pigs also failed to develop M hyopneumoniae-specific antibodies by day 70.

Mycoplasma hyopneumoniae remains an important pathogen to the swine industry. Understanding the protective capabilities of mycoplasmal vaccines increases our understanding of the pathogenesis of M hyopneumoniae, which is still largely unknown. Results of this study suggest that IM injection of a commercial M hyopneumoniae bacterin induces secretion of M hyopneumoniae-specific antibodies and suppresses secretion of TNF-α into BAL fluid and induces the formation of IFN-γ secreting cells in blood. These findings suggest that a local mucosal humoral and a systemic cell mediated immune response are important in controlling mycoplasmal pneumonia in pigs.

\footnote{RespirSure Mycoplasma hyopneumoniae bacterin, Pfizer Animal Health, New York, NY.}
\footnote{SEM-IPS image analyzing system, Zeiss, Thornwood, NY.}
\footnote{Histopaque 1077, Sigma Chemical Co, St Louis, Mo.}
\footnote{VMRD, Pullman, Wash.}
\footnote{Immunonoll B, Dyantech, Chantilly, Va.}
\footnote{Gift of Dr. Federico Zuckermann, Department of Pathobiology, College of Veterinary Medicine, University of Illinois, Urbana, Ill, and the Jackson Immunoresearch Laboratory, West Grove, Pa.}
\footnote{Kirkgaard and Perry Laboratory, Gaithersburg, Md.}

**References**