VACCINATION WITH THE MAJOR SECRETORY PROTEIN OF LEGIONELLA PNEUMOPHILA INDUCES CELL-MEDIATED AND PROTECTIVE IMMUNITY IN A GUINEA PIG MODEL OF LEGIONNAIRES' DISEASE

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Legionella pneumophila is a facultative intracellular bacterial pathogen that is the causative agent of Legionnaires' disease, a serious and often fatal form of pneumonia (1). L. pneumophila causes 80–85% of the cases of Legionnaires' disease (2). Patients with Legionnaires' disease develop both humoral and cell-mediated immune responses to L. pneumophila (3–5). Cell-mediated immunity appears to play a vital role in host defense. Activated human monocytes and alveolar macrophages inhibit the intracellular multiplication of L. pneumophila (5–9). Humoral immunity appears to play a lesser role in host defense (3–4).

The guinea pig is an excellent animal model for the study of Legionnaires' disease. When exposed to aerosols containing L. pneumophila, guinea pigs develop a pneumonic illness that is clinically and pathologically similar to Legionnaires' disease in humans (10–14).

Previous studies from this laboratory have demonstrated that guinea pigs immunized with a sublethal aerosol dose of wild-type L. pneumophila, or with an aerosolized dose of an avirulent mutant L. pneumophila, develop humoral and cell-mediated immune responses to wild-type L. pneumophila membrane antigens, and protective immunity against challenge with a lethal aerosol dose of L. pneumophila (15, 16). Moreover, previous work from this laboratory has demonstrated that guinea pigs immunized with an aerosolized sublethal dose of L. pneumophila develop a strong cell-mediated immune response to the major secretory protein (MSP)1 of L. pneumophila as demonstrated by in vitro lymphocyte proliferation (17). MSP is the most abundant protein released by L. pneumophila into growth medium (18). It is a protease with an apparent molecular weight of 39,000 whose enzymatic activity is inhibited by EDTA. An EDTA-inhibitable protease of comparable size has been isolated from another strain of L. pneumophila (19).

In the present study, we have examined the capacity of MSP to induce immune responses in the guinea pig. We shall demonstrate that vaccination with this protein induces humoral and cell-mediated immune responses. We shall also demonstrate

1 Abbreviations used in this paper: BEN, BisTris/EDTA/NaCl; CYEA, charcoal yeast extract agar; EYB, egg yolk buffer; LD, lethal aerosolized dose; MSP, major secretory protein; TNB, Tris/NaCl/BSA/sodium azide; TU, tryptic unit; YEB, yeast extract broth.
that, remarkably, vaccination with this single molecule of \textit{L. pneumophila} induces protective immunity against lethal aerosol challenge.

\textbf{Materials and Methods}

\textit{Media}. Each liter of yeast extract broth (YEB) was prepared with 10 g of ACES (Sigma Chemical Co., St. Louis, MO), 10 g yeast extract (Difco Laboratories, Detroit, MI), 0.4 g L-cysteine HCl (Fisher Scientific Co., Fairlawn, NJ), and 0.25 g ferric pyrophosphate (Sigma Chemical Co.) adjusted to a pH of 6.9 with 10 N KOH, and filter sterilized.

RPMI 1640 with l-glutamine (Gibco Laboratories, Grand Island, NY) was mixed with penicillin (100 U/ml) and streptomycin (100 \(\mu\)g/ml) (Gibco Laboratories). Egg yolk buffer (EYB) with or without 1% BSA (Miles Laboratories Inc., Naperville, IL) was prepared as previously described (1).

\textit{Agar}. Modified charcoal yeast extract agar (CYEA) was prepared as described (1). Modified CYEA without cysteine and tryptic soy agar with 5% sheep blood were used to assay for contamination of non-Legionella species.

\textit{Bacteria}. \textit{L. pneumophila}, Philadelphia 1 strain (serogroup 1), was grown in embryonated hen's eggs; harvested; tested for viability; tested for the presence of contaminating bacteria; passed one time only on CYEA; washed with EYB; flash frozen in aliquots of 10\(^{11}\) CFU/ml and stored at \(-70^\circ\)C (1). Before use in the aerosol inoculation system, a stock preparation of bacteria was diluted in EYB to the desired concentration.

\textit{Purification of MSP}. \textit{L. pneumophila} from a stock preparation was grown on CYEA plates, suspended in YEB, and inoculated into 2 L Erlenmeyer flasks containing 500 ml of prewarmed YEB. The optical density (OD) of the inoculated media at 540 nm on a spectrophotometer (Junior model 35; Perkin-Elmer Corp., Oak Brook, IL) was approximately 0.05. The bacteria were grown for \(\sim\)20 h at \(37^\circ\)C on a rotating platform at 120 rpm to an OD of \(\sim\)1.0. The culture was checked for the presence of contaminants by light microscopy, and demonstrated free of contaminants by inoculating CYEA, CYEA without L-cysteine, and sheep blood agar. The bacteria were then pelleted by centrifugation at 16,000 \(\times\) g for 10 min at 4\(^\circ\)C. The supernatant fluid was decanted and filtered sequentially through 0.45 \(\mu\)m and 0.2 \(\mu\)m Tuffryn low protein binding filters (Gelman Sciences Inc., Ann Arbor, MI) to remove residual bacteria. The filtrate was saturated with ammonium sulfate grade 1 to a level of 45\%, stirred 1 h at 4\(^\circ\)C, and the precipitate that formed was pelleted by centrifugation at 16,000 \(\times\) g for 35 min at 4\(^\circ\)C and discarded. The supernate was brought to 95\% saturation with ammonium sulfate, stirred gently overnight at 4\(^\circ\)C, and the precipitate that formed was pelleted by centrifugation as above. The supernatant was discarded and the MSP-rich pellet was resuspended in 0.025 M Bis Tris, 0.01 M EDTA, 0.15 M NaCl, pH 5.9 (BEN), and dialyzed at 4\(^\circ\)C overnight in a Spectrapor dialysis membrane (Spectrum Medical Industries, Los Angeles, CA) with a 6–8,000 mol wt cut-off against 1 L of BEN. The dialyzed MSP-rich fluid was then applied to a 50 \(\times\) 2.5 cm Sephacryl S-200 column (Pharmacia Fine Chemicals, Piscataway, NJ), eluted in BEN at a flow rate of 8 ml/h, and fractions containing MSP were identified by SDS-PAGE (20). These fractions were pooled and filter concentrated on ice with an Amicon Corp. (Danvers, MA) filter unit using a filter with a nominal mol wt limit of 30,000 (Millipore, Bedford, MA). The concentrated semipurified protein was applied to a 2.5 \(\times\) 13 cm Sepharose CL-6B column (Pharmacia Fine Chemicals). A gradient of 0.15–0.65 M NaCl in 0.025 M Bis Tris, 0.01 M EDTA, pH 5.9, was applied using a gradient maker (Pharmacia Fine Chemicals) with 2.5 bed volumes in each chamber, at a flow rate of 8 ml/h. Fractions containing only MSP were identified by SDS-PAGE, pooled, and filter concentrated on ice with an Amicon Corp. filter unit using a filter with a nominal mol wt limit of 30,000.

\textit{Mass of MSP}. A BCA protein assay (Pierce Chemical Co., Rockford, IL) was used to determine MSP mass, using BSA in BEN as a standard. Before analysis of mass, MSP was dialyzed at 4\(^\circ\)C overnight in a Spectrapor dialysis membrane with a 5–6,000 mol wt cut-off against 1 L of BEN.

\textit{Purity of MSP}. The purity of MSP was assessed by SDS-PAGE by applying 40 \(\mu\)g of purified MSP to a 12.5\% gel and staining with Coomassie Brilliant Blue R, and by applying 10 \(\mu\)g of purified MSP to a 12.5\% gel and silver staining (21).
Proteolytic Activity of MSP. A colorimetric assay was used to determine the proteolytic activity of MSP, with hide powder azure as the substrate and trypsin as the standard. Hide powder azure (50 mg) was dissolved in 2 ml diluting buffer consisting of 0.1 M sodium phosphate, 0.001 M zinc sulfate, 0.02% sodium azide, pH 6.0. Dilutions of MSP and trypsin dialyzed to diluting buffer, or diluting buffer alone as a blank, were added in 200-µl aliquots to the hide powder azure mix, and the mixture was rotated at 140 rpm at 37°C for 30 min. The mixture was then chilled to 4°C to stop the reaction and subjected to centrifugation at 1,400 g for 10 min at 4°C. The colored supernatant solution was aspirated, and the OD read at 595 nm on an ultrospec 4050 spectrophotometer (LKB Instruments, Inc., Gaithersburg, MD), setting the OD of the blank to zero. A standard curve of proteolytic activity (OD) versus mass for trypsin was constructed. One trypsinic unit (TU) was defined as the proteolytic activity of 1 µg of trypsin. The proteolytic activity of MSP was derived from the standard curve. The experiment was performed in duplicate.

Immunization of Guinea Pigs with MSP. MSP was dialyzed at 4°C overnight in a dialysis membrane with a 6-8,000 mol wt cut-off against 1 L normal saline and mixed with CFA adjuvant (Difco Laboratories) for the first injection, or IFA adjuvant (Sigma Chemical Co.) for the second injection 3 wk later. The injection sites of the guinea pigs were washed with 70% ethanol and the animals were then injected subcutaneously with 40, 10, 2.5, or 0.6 µg of MSP in a total volume of 200 µl. Control animals were sham immunized at the same time with normal saline only in CFA (first injection) or in IFA (second injection). One group of animals immunized with 40 µg of MSP in CFA was monitored daily for 5 d for fever, weight loss, or signs of illness.

Immunization of Guinea Pigs with a Sublethal Dose of L. pneumophila. Guinea pigs were immunized by exposure to aerosols of bacteria in the same lucite aerosol chamber described in detail in a previous study (15). Guinea pigs were immunized with an aerosol generated from a 10-ml suspension of L. pneumophila containing 2.5 × 10⁷ CFU/ml.

ELISA for Antibody to MSP. The humoral immune response of guinea pigs to MSP was assessed by an ELISA assay. MSP in BEN was dialyzed at 4°C in a dialysis membrane with a 6-8,000 mol wt cut-off against 1 L of 0.01 M Tris, 0.1 M NaCl, 0.02% sodium azide, pH 7.2, diluted to a concentration of 5 µg/ml in this buffer, and applied (50 µl/well) to Nunc-Immuno Plates (Nunc Products, Thousand Oaks, CA). The plates were covered with an acetate plate sealer (Flow Laboratories Inc., McLean, VA), incubated overnight at room temperature, and stored at 4°C. The plates were washed five times with 0.01 M Tris, 0.1 M NaCl, 3% BSA, 0.02% sodium azide, pH 7.5 (TNB), blocked with this buffer for 1 h, and tapped dry. Guinea pig sera were diluted from 1/8 to 12048 in TNB, 50 µl of each dilution was added per well, and the plates were incubated for 1 h at room temperature with gentle shaking. The plates were washed five times with TNB, tapped dry, and 50 µl of a 1:10,000 dilution of alkaline phosphatase-conjugated goat anti-guinea pig IgG (whole molecule) (Sigma Chemical Co.) was added per well. The plates were incubated for 1 h at room temperature with gentle shaking, washed five times with TNB, and tapped dry. A reaction mix containing one p-nitrophenyl phosphate tablet per 5 ml of 20% diethanolamine was prepared with an alkaline phosphatase substrate kit (Bio-Rad Laboratories, Richmond, CA), and 125 µl added per well for 15 min. Blank wells were treated with reaction mix only. The reaction was halted with 50 µl per well of 0.6 M NaOH. The OD of wells was read at 405 nm on a Titertek Multiskan (Flow Laboratories) and a corrected OD was calculated by subtracting the mean of the OD of the blank wells from the OD of each experimental well. The threshold above which titers were considered positive was established in three independent experiments by measuring the OD of three different categories of control wells in each experiment. The control wells contained (a) MSP; no primary antibody; and conjugated antibody; (b) no MSP; primary antibody (six different sera tested) diluted from 1:32 to 1:1,024; and conjugated antibody; or (c) no MSP; no primary antibody; and conjugated antibody. For each of the three categories of control wells, a value equal to the mean of the corrected OD readings plus two times the standard deviation was calculated in each of the three experiments. The mean of these nine values was designated as the threshold, and experimental measurements above this threshold were considered positive. The titer of each animal's serum was expressed as the reciprocal of the highest dilution yielding a positive measurement. Negative controls...
were included on each experimental plate to monitor nonspecific reactions and positive controls were included to allow comparisons among plates. All sera were tested in duplicate, and the geometric mean of the reciprocal titers was calculated.

**Cutaneous Delayed-type Hypersensitivity Assay.** Guinea pigs were shaved over the back and flank, and administered 100 μl of various concentrations of MSP diluted in EYB, and 100 μl of control buffer (EYB) intradermally. The diameters of erythema and induration were measured at 24 h and 48 h after injection.

**Lymphocyte Proliferation Assay.** Splenic lymphocytes from guinea pigs immunized by injection with MSP or by sublethal infection, or from control guinea pigs, were obtained and purified, incubated with MSP in triplicate microtest wells for 2 d, and then tested for their capacity to incorporate [3H]thymidine as described (15).

**Studies of Protective Immunity.** Guinea pigs immunized with MSP and sham-immunized control guinea pigs were challenged 3 wk after the second immunization with a lethal aerosolized dose (LD) of *L. pneumophila*. This dose, which was generated from a 10-ml suspension of *L. pneumophila* containing 2.5 × 10⁶ CFU/ml, was the lowest dose that consistently resulted in the death of nonimmunized guinea pigs, and it was determined as previously described (15). In the first three experiments, the animals were challenged with 1 LD, and in the last two experiments, the animals were challenged with 2 LD. After a 7-d observation period, the survivors were quantitated, and differences in survival between immunized and control animals were evaluated by the Fisher's exact test, two-tailed.

**Quantitation of *L. pneumophila* in Pulmonary Tissue.** In two independent experiments, guinea pigs were immunized with MSP at 3-wk intervals or sham immunized (controls), as described above. 3 wk after the second immunization, the guinea pigs were challenged with 1 LD. At various time points 1–72 h after challenge, one immunized and one control animal were killed by hypercarbia. The animals were soaked first in 7x cleaning solution (Linbro Chemical Co., New Haven, CT) and second in 70% ethanol to decontaminate their skin, and then the right lung of each animal was removed by sterile technique. Each lung was placed in 10 ml of sterile EYB and ground thoroughly with a mortar and pestle as described (13). CFU in each lung was determined by plating dilutions of this lung suspension in triplicate on CYE A. The lung suspension was also cultured on sheep blood agar and CYE A without cysteine to check for the presence of contaminating bacteria; none of the lungs were contaminated with other bacteria.

**Results**

**Purification of MSP.** We purified MSP by a three-step process involving ammonium sulfate precipitation, molecular sieve chromatography, and ion-exchange chromatography. To assess the purity of MSP, we examined it by SDS-PAGE, loading 40 μg on a gel and staining with Coomassie Blue (Fig. 1), and loading 10 μg on a gel and silver staining (19). By both techniques, we obtained a single band of 39,000 apparent mol wt, suggesting a high degree of purity.

We assayed proteolytic activity at each step of the purification process (Table 1). The proteolytic activity per mass increased from 0.021 TU/μg in the unconcentrated supernatant to 1.6 TU/μg after ion-exchange chromatography, the final step in purification. The amount of proteolytic activity after ion-exchange chromatography indicated a 20% yield from the initial amount of proteolytic activity in the unconcentrated supernatant.

**Subcutaneously Administered MSP Is Nonlethal and Nontoxic to Guinea Pigs.** In five independent experiments, no animals died from subcutaneous immunization with MSP in CFA or IFA. Moreover, none of six other animals immunized subcutaneously with 40 μg of MSP in CFA and then monitored closely for 5 d exhibited fever, weight loss, or observable signs of illness.

**Splenic Lymphocytes from Guinea Pigs Immunized with Either a Sublethal Aerosol Dose of**
**Figure 1.** Purification of MSP. MSP was isolated from broth culture as described in the text, and preparations were examined by SDS-PAGE after each sequential stage of the purification process: ammonium sulfate precipitation (lane B), molecular sieve chromatography (lane C), and ion-exchange chromatography (lane D). Lane A contains molecular weight standards (bovine albumin, 66,000; ovalbumin, 45,000; glyceraldehyde-3-phosphate dehydrogenase 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; trypsin inhibitor 20,100).

*L. pneumophila* or MSP Proliferate in Response to MSP. To determine if a population of splenic lymphocytes from immunized guinea pigs recognize MSP, we examined lymphocyte proliferative responses of immunized and control guinea pigs to this molecule. In three experiments (Table II, part I), we compared four animals immunized with a sublethal aerosol dose of *L. pneumophila* with three control animals, and in four experiments (Table II, part II), we compared four animals immunized with MSP with four control animals. We performed the lymphocyte proliferation assay 3–5 wk after sublethal dose immunization, and 4 wk after the second immunization with MSP.

Immunized animals exhibited greater lymphocyte proliferative responses to MSP than control animals (Table II). Three of four guinea pigs immunized with a sublethal aerosol dose of *L. pneumophila* (Table II, part I) and all four guinea pigs immunized with MSP (Table II, part II) exhibited a higher stimulation index than their con-

| Purification stage                     | Protein     | Specific proteolytic activity | Total proteolytic activity | Recovery |
|----------------------------------------|-------------|-------------------------------|---------------------------|----------|
| 1. Unconcentrated culture supernatant  | 840,000     | 0.021                         | 17,640                    | 100      |
| 2. Ammonium sulfate precipitation      | 7,300       | 1.02                          | 7,446                     | 42       |
| 3. Gel permeation chromatography       | 3,367       | 1.3                           | 4,377                     | 25       |
| 4. Ion-exchange chromatography         | 2,150       | 1.6                           | 3,440                     | 20       |

MSP of *L. pneumophila* was purified from unconcentrated culture supernatant as described in the text. At each stage in the purification process, the mass of the preparation was measured by the BCA protein assay and the proteolytic activity was determined by the hide powder azure assay.
### Table II

**Lymphocytes from Guinea Pigs Immunized with either a Sublethal Aerosol Dose of *L. pneumophila* or MSP Proliferate in Response to MSP**

| Exp. | Immunized guinea pigs | MSP (0.5 μg/ml) | MSP (0.05 μg/ml) |
|------|------------------------|-----------------|-----------------|
| I.   | Guinea pigs immunized with a sublethal aerosol dose of *L. pneumophila* | | |
| A    | A1                     | 9.2             | 2.0             |
| B    | B1                     | 2.5             | 1.6             |
| C    | C1                     | 1.0             | 1.1             |
|      | C2                     | 6.2             | 4.0             |
| Mean ± SEM | 4.7 ± 1.8              | 2.2 ± 0.6       |
| Control guinea pigs | | | |
| A    | A2                     | 2.0             | 1.4             |
| B    | B2                     | 1.3             | 1.2             |
| C    | C3                     | 1.7             | 1.5             |
| Mean ± SEM | 1.7 ± 0.2              | 1.4 ± 0.1       |
| p<0.2 | <0.2                   | <0.2            |
| II.  | Guinea pigs immunized with MSP | | |
| D    | D1                     | 17.2            | 15.8            |
| E    | E1                     | 13.8            | 11.1            |
| F    | F1                     | 15.2            | 4.2             |
| G    | G1                     | 22.1            | 18.0            |
| Mean ± SEM | 18.7 ± 3.0             | 13.3 ± 3.2      | 8.2 ± 3.2       |
| Control guinea pigs | | | |
| D    | D2                     | 1.0             | 1.0             |
| E    | E2                     | 1.2             | 1.1             |
| F    | F2                     | 5.5             | 1.6             |
| G    | G2                     | 1.5             | 3.0             |
| Mean ± SEM | 4.6 ± 3.1              | 2.7 ± 1.0       | 1.4 ± 0.2       |
| p<0.05 | <0.01                  | <0.05           |

In three independent experiments (A–C), guinea pigs were immunized with a sublethal aerosol dose of *L. pneumophila* or not immunized (controls). In four independent experiments (D–G), guinea pigs were immunized subcutaneously with MSP (40 μg in CFA followed 3 wk later by 40 μg in IFA) or sham-immunized (buffer [saline] in CFA followed 3 wk later by buffer in IFA). Splenic lymphocytes were obtained 3–5 wk later and incubated (10^7/ml) in microtest wells at 37°C for 2 d without antigen or with MSP at the concentrations indicated. The lymphocytes were assayed for their capacity to incorporate [3H]thymidine, and stimulation indices were calculated.

* Stimulation index = (mean [3H]thymidine incorporation [cpm] of lymphocytes incubated with MSP)/(mean [3H]thymidine incorporation [cpm] of lymphocytes incubated without MSP).

p<0.05 value for difference between SI of immunized and control guinea pigs by t-test, two-tailed.
controls. The mean stimulation indices of sublethal dose-immunized guinea pigs in response to 0.5 and 0.05 µg/ml MSP were 4.7 and 2.2, respectively, compared with 1.7 and 1.4 for control animals (Table II, part I). Although the mean stimulation indices for the sublethal dose-immunized animals in response to 0.5 and 0.05 µg/ml of MSP were 2.8 and 1.6 times those of controls, respectively, this difference did not achieve significance by the $t$-test. The mean stimulation indices of MSP-immunized animals ranged from 8.2 to 18.7, depending on the concentration of MSP (0.05–5 µg/ml). In contrast, the mean stimulation indices of control animals ranged from 1.4 to 4.6 (Table II, part II). These differences were highly significant by the $t$-test ($p$ values ranged from $p < 0.01$ to $p < 0.05$; Table II, part II).

**Guinea Pigs Immunized with Either a Sublethal Aerosol Dose of *L. pneumophila* or MSP Develop Cutaneous Delayed-type Hypersensitivity to MSP.** To determine if sublethal dose-immunized or MSP-immunized guinea pigs develop a cutaneous delayed-type hypersensitivity response to MSP, we injected them intradermally with various concentrations of MSP 3 wk after immunization and measured the extent of erythema and induration at the skin test sites. Both sublethal dose-immunized and MSP-immunized animals consistently had greater mean areas of erythema and induration than control animals (Table III).

Sublethal dose-immunized animals exhibited mean diameters of erythema and induration of 15.2 and 7.8 mm, respectively, 24 h after intradermal injection of MSP, compared with 2.0 and 0.0 mm, respectively, in control animals (Table III, Exp. A). Mean diameters of erythema and induration after intradermal injection of buffer control were minimal in both immunized and control animals.

MSP-immunized animals were skin tested at 24 and 48 h after injection with 100 µl of 1 µg/ml of MSP (Table III, Exp. B). At 24 h, MSP-immunized animals exhibited mean diameters of erythema and induration of 17.2 and 6.0 mm, respectively, compared with 3.3 and 1.0 mm, respectively, in control animals. By 48 h, skin responses in both groups of animals had waned, but the stronger responses of animals in the immunized group were still evident.

MSP-immunized and control animals were also skin tested with 100-µl injections of MSP over a range of concentrations (0.5, 5, and 50 µg/ml) (Table III, Exp. C). Both groups of animals exhibited dose-dependent skin responses, but at all three concentrations of MSP, the MSP-immunized animals exhibited much greater areas of erythema and induration than control animals.

In both types of experiments with MSP-immunized animals, intradermal injection of diluting buffer did not produce either erythema or induration in either immunized or control animals (Table III).

**MSP-immunized but not Sublethal Dose-immunized Guinea Pigs Develop a Humoral Immune Response to MSP.** To determine if guinea pigs immunized with either a sublethal aerosol dose of *L. pneumophila* or MSP develop a humoral immune response to MSP, we assayed anti-MSP antibody by an ELISA assay, using purified MSP as the capture antigen, guinea pig sera as the source of primary antibody, and alkaline phosphatase-conjugated goat anti-guinea pig IgG as the secondary antibody (Fig. 2). None of the sublethal dose-immunized guinea pigs developed a humoral immune response to MSP; all four such animals and three control animals had reciprocal antibody titers of <8 (Fig. 2 A). In contrast, all six MSP-immunized guinea pigs developed a strong humoral response to MSP; their titers ranged from 45 to >2,048.
MAJOR SECRETORY PROTEIN OF LEGIONELLA PNEUMOPHILA

Guinea Pigs Immunized with either a Sublethal Aerosol Dose of L. pneumophila or MSP

Develop Cutaneous Delayed-type Hypersensitivity to MSP

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**Table III**

**Guinea Pigs Immunized with either a Sublethal Aerosol Dose of L. pneumophila or MSP**

| Exp. | Status of guinea pigs | Number of guinea pigs | Skin reactions | Mean extent of erythema and induration in response to indicated concentration of MSP (µg/ml) |
|------|------------------------|------------------------|----------------|-----------------------------------------------------------------------------------|
|      |                        |                        |                | 1.0 (24 h) 1.0 (48 h) 0.0 (24 h) 0.0 (48 h)                                        |
| I.   | Guinea pigs immunized with a sublethal aerosol dose of L. pneumophila |                        |                |                                                                                 |
| A    | Immunized             | 3                      | Erythema       | 15.2 ± 2.3 1.0 ± 1.7                                                               |
|      |                        |                        | Induration     | 7.8 ± 1.0 0.0 ± 0.0                                                               |
|      | Control                | 3                      | Erythema       | 2.0 ± 2.0 1.7 ± 2.9                                                               |
|      |                        |                        | Induration     | 0.0 ± 0.0 0.0 ± 0.0                                                               |
|      |                        |                        | SI (Erythema)* | 7.6 0.6                                                                 |
|      |                        |                        | SI (Induration)* | ∞ -                                                                 |
| II.  | Guinea pigs immunized with MSP |                        |                |                                                                                 |
| B    | Immunized             | 3                      | Erythema       | 17.2 ± 4.5 11.8 ± 5.0 0.0 ± 0.0 0.0 ± 0.0                                         |
|      |                        |                        | Induration     | 6.0 ± 1.0 1.7 ± 2.9 0.0 ± 0.0 0.0 ± 0.0                                         |
|      | Control                | 3                      | Erythema       | 3.3 ± 2.9 0.0 ± 0.0 0.0 ± 0.0 0.0 ± 0.0                                         |
|      |                        |                        | Induration     | 1.0 ± 1.7 0.0 ± 0.0 0.0 ± 0.0 0.0 ± 0.0                                         |
|      |                        |                        | SI (Erythema)* | 5.2 ∞ -                                                                        |
|      |                        |                        | SI (Induration)* | ∞ -                                                                              |
| C    | Immunized             | 6                      | Erythema       | 23.3 ± 6.2 14.2 ± 4.6 9.0 ± 2.0 0.0 ± 0.0                                       |
|      |                        |                        | Induration     | 13.0 ± 8.4 6.8 ± 4.7 3.5 ± 3.7 0.0 ± 0.0                                       |
|      | Control                | 3                      | Erythema       | 8.3 ± 3.1 4.3 ± 0.5 0.0 ± 0.0 0.0 ± 0.0                                         |
|      |                        |                        | Induration     | 0.0 ± 0.0 0.0 ± 0.0 0.0 ± 0.0 0.0 ± 0.0                                         |
|      |                        |                        | SI (Erythema)* | 2.8 3.5 ∞ -                                                                    |
|      |                        |                        | SI (Induration)* | ∞ ∞ ∞ -                                                                         |

In one experiment (A), guinea pigs were immunized with a sublethal aerosol dose of L. pneumophila or they were not immunized (controls). In two other independent experiments (B and C), guinea pigs were immunized subcutaneously with MSP or sham immunized as in Table II. 3 wk later, all animals were skin tested with an intradermal injection of 100 µl of the indicated concentration of MSP, and the extent of erythema and induration was measured 24 h later in Exps. A and C and both 24 and 48 h later in experiment B. Data are the mean ± SD of the extent of erythema and induration for each group of animals.

* SI = stimulation index = [mean skin reactivity (mm) of immunized guinea pigs]/[mean skin reactivity (mm) of control guinea pigs].

while five of six control guinea pigs had titers of <16 (Fig. 2 B). A single control guinea pig exhibited a high titer to MSP; this was possibly due to the presence of a crossreactive antibody in this animal.

Thus, while MSP-immunized animals developed both a cell-mediated and humoral immune response to MSP, the sublethal dose-immunized animals developed only a cell-mediated immune response.

**Guinea Pigs Immunized with MSP Are Protected against Lethal Aerosol Challenge with L. pneumophila.** To determine if guinea pigs immunized with MSP are protected...
against lethal aerosol challenge, we immunized guinea pigs with 40 μg of MSP twice, 3 wk apart, and challenged them in five independent experiments. In the first three experiments, we challenged 14 control and 15 MSP-immunized guinea pigs with 1 LD of \textit{L. pneumophila}, and in the last two experiments, we challenged 12 control and 11 MSP-immunized guinea pigs with 2 LD of \textit{L. pneumophila}.

MSP immunization produced strong protective immunity in each of the 5 experiments (Table IV). Whereas none (0%) of 26 control animals survived challenge in the five experiments, 21 (81%) of 26 MSP-immunized animals survived challenge.

**Table IV**

| Exp. | Status of guinea pigs | Number guinea pigs surviving per number challenged (percent survival) | \(p^*\) |
|------|-----------------------|-------------------------------------------------|------|
| A    | Control               | 0/5 (0)                                         | 0.05 |
|      | Immunized             | 4/5 (80)                                        |      |
| B    | Control               | 0/5 (0)                                         | 0.02 |
|      | Immunized             | 5/6 (83)                                        |      |
| C    | Control               | 0/4 (0)                                         | 0.03 |
|      | Immunized             | 4/4 (100)                                       |      |
| D    | Control               | 0/6 (0)                                         | 0.03 |
|      | Immunized             | 4/6 (67)                                        |      |
| E    | Control               | 0/6 (0)                                         | 0.02 |
|      | Immunized             | 4/5 (80)                                        |      |
| Total| Control               | 0/26 (0)                                        |      |
|      | Immunized             | 21/26 (81)                                      | \(7 \times 10^{-10}\) |

In five independent experiments, guinea pigs were immunized subcutaneously with MSP or sham immunized as described in Table II. 3 wk later, the animals were challenged with 1 LD of \textit{L. pneumophila} (Exps. A, B, and C) or 2 LD (Exps. D and E), and survival was quantitated.

* Fisher’s Exact Test, two-tailed.
The difference in survival between MSP-immunized and control animals was highly significant ($p = 7 \times 10^{-10}$, Fisher’s exact test, two-tailed).

Of the MSP-immunized animals challenged with 1 LD, 13 (87%) of 15 survived, whereas of the MSP-immunized animals challenged with 2 LD, 6 (73%) of 11 survived. This difference was not statistically significant by the Fisher’s exact test, two-tailed.

Guinea pigs immunized with MSP first exhibited signs of illness, including fever, decreased activity, decreased feeding, and respiratory distress, 1 d after challenge, but they then recovered. Nonimmunized animals first exhibited these signs of illness 2 d after challenge, but they did not recover. All animals surviving by 7 d after challenge recovered fully from earlier signs of disease.

**Protection against Lethal Aerosol Challenge with L. pneumophila by Immunization with MSP is Dose-dependent.** To determine if lower doses of MSP could induce protective immunity in guinea pigs, we immunized animals with 40, 10, 2.5, or 0.6 µg of MSP, and then challenged them with 2 LD of L. pneumophila. As in previous studies, we immunized all animals twice, 3 wk apart, and sham-immunized control animals. We performed two independent experiments (Table V).

MSP induced protective immunity in dose-dependent fashion in both experiments. Combining the results of the two experiments, 73% of 11 animals immunized with 40 µg MSP survived challenge, 58% of 12 animals immunized with 10 µg MSP, 50% of 12 animals immunized with 2.5 µg MSP, 25% of 12 animals immunized with 0.6 µg MSP, and 0% of 12 sham-immunized animals (0 µg MSP). The 40-µg dose induced significant protection in both experiments, the 10- and 2.5-µg doses each induced significant protection in one of the two experiments, and the 0.6-µg dose did not induce significant protection in either experiment. When the results of both experiments were combined, the 40-, 10-, and 2.5-µg doses all produced significant levels of protection with $p = 0.0003$, $p = 0.005$, and $p = 0.01$, respectively, by the Fisher’s exact test, two-tailed. Even the 0.6-µg dose, the lowest dose

| Guinea pig status | MSP dose (µg) | Exp. A | Exp. B | Total |
|-------------------|--------------|--------|--------|-------|
|                   |              | Per number challenged | Percent | $p^*$  | Per number challenged | Percent | $p^*$  | Per number challenged | Percent | $p^*$  |
| Immunized         | 40           | 4/6    | 67     | 0.03  | 4/5    | 80  | 0.02  | 8/11    | 73     | 0.0003 |
| Immunized         | 10           | 2/6    | 33     | 0.5   | 5/6    | 83  | 0.02  | 7/12    | 58     | 0.005  |
| Immunized         | 2.5          | 1/6    | 17     | 1.0   | 5/6    | 83  | 0.02  | 6/12    | 50     | 0.01   |
| Immunized         | 0.6          | 0/6    | 0      | 1.0   | 3/6    | 50  | 0.09  | 3/12    | 25     | 0.1    |
| Control           | 0.0          | 0/6    | 0      |        | 0/6    | 0   |       | 0/12    | 0      |        |

In two independent experiments, guinea pigs were immunized subcutaneously with MSP or sham immunized as in Table II except that the dose of MSP ranged from 0.6 to 40 µg, as indicated. 3 wk later, the animals were challenged with 2 LD of L. pneumophila.

$p$ value for difference in survival between animals immunized with the indicated dose of MSP and control animals by the Fisher’s exact test, two-tailed.
tested, appeared to induce some protection, but the level of significance was not high ($p = 0.1$).

**Guinea Pigs Immunized with MSP Suppress Multiplication of L. pneumophila in the Lung.**

To determine the extent of *L. pneumophila* multiplication in the lung of MSP-immunized guinea pigs, we challenged five MSP-immunized and five control guinea pigs with 1 LD of *L. pneumophila*, killed them 1–72 h later, and determined CFU of *L. pneumophila* in the right lung. We performed two independent experiments (Fig. 3). At 1 h after aerosol exposure, immunized and control animals had similar numbers of *L. pneumophila* in their lungs in both experiments. By 24 h, CFU markedly increased (2 logs) in both immunized and control animals, but the rate of growth was the same so that both groups had comparable numbers of bacteria in the lung at this time point. Thereafter, CFU in immunized animals plateaued, whereas CFU in control animals continued to increase. By 48 h, CFU of *L. pneumophila* in controls was 0.6–0.7 logs greater than in immunized animals, and by 72 h, CFU of *L. pneumophila* in controls was 1.4 logs greater than in immunized animals.

**Discussion**

We have demonstrated that immunization of guinea pigs with the MSP of *L. pneumophila* induces a highly significant level ($p = 7 \times 10^{-10}$) of protective immunity against lethal challenge with an aerosolized dose of *L. pneumophila*. The lethal aerosol doses used in this study were much higher than those likely to be encountered by humans in nature. Remarkably, immunization with a single protein of *L. pneumophila* induces strong protective immunity against this complex intracellular parasite.

Previous studies from this laboratory have shown that immunization of guinea pigs...
pigs by the respiratory route with a sublethal dose of \textit{L. pneumophila} (15), or with an avirulent mutant strain of \textit{L. pneumophila} that survives but does not multiply in human monocytes (16), induces protective immunity. The present study demonstrates that immunization of guinea pigs by the subcutaneous route with MSP also can induce protective immunity.

MSP is a very potent inducer of protective immunity against lethal aerosol challenge. As little as 2.5 \(\mu\)g, injected twice over a 3 wk interval, was capable of inducing a significant level of protective immunity against 2 LD of \textit{L. pneumophila} (\(p = 0.01\), Fisher's exact test, two-tailed) and even two injections of 0.6 \(\mu\)g MSP appeared to induce some protection. In this series of experiments, over the range 0.6 to 40 \(\mu\)g of MSP, two injections of 40 \(\mu\)g MSP gave the highest level of protection.

Guinea pigs immunized with MSP and then challenged with a lethal dose of aerosolized \textit{L. pneumophila} limited \textit{L. pneumophila} multiplication in their lungs. In both immunized and control animals, \textit{L. pneumophila} multiplied in the lungs during the first 24 h after challenge. Thereafter, CFU in the lungs of control animals continued to increase, while CFU in the lungs of immunized animals plateaued. These results were similar to those obtained in studies of animals immunized with a sublethal dose of \textit{L. pneumophila} and control guinea pigs (15). These results suggest that protective immune defenses are marshalled during the first 24 h after challenge. This time period corresponds roughly to that required for lymphokines to activate human mononuclear phagocytes (6). Such activated monocytes and alveolar macrophages strongly inhibit the intracellular multiplication of \textit{L. pneumophila} (5–9).

Consistent with the idea that immunized guinea pigs marshall immune defenses during the first 24 h after challenge, immunized but not control guinea pigs developed signs of illness during this period. This may reflect an early immune response to \textit{L. pneumophila} in immunized animals, who generally recovered from the infection. In contrast, control animals developed signs of illness later after challenge and succumbed to the infection.

Both guinea pigs immunized with a sublethal dose of \textit{L. pneumophila} and guinea pigs immunized with MSP developed a cell-mediated immune response to MSP. However, only MSP-immunized animals developed a humoral immune response to MSP. Both groups of immunized animals exhibited strong protective immunity. This suggests that antibody against MSP is not important to host defense but that a cell-mediated immune response to MSP may be. Although previous studies indicate that cell-mediated immunity is of primary importance to host defense against \textit{L. pneumophila} after natural infection, it is possible that anti-MSP antibody is also playing a role in the protective immunity induced by MSP immunization.

That both sublethal dose-immunized and MSP-immunized guinea pigs develop a cell-mediated immune response to MSP, but only MSP-immunized animals develop a humoral immune response to this molecule, suggests the possibility that antigen processing of MSP differs according to how the molecule is presented to the immune system. In guinea pigs immunized by sublethal dose, MSP produced by \textit{L. pneumophila} within mononuclear phagocytes may be processed intracellularly such that only small linear determinants are presented to lymphocytes. Such determinants may induce cellular immune responses, which are frequently directed toward unfolded linear epitopes, but not humoral immune responses, which are frequently directed toward a tertiary structure of nondenatured antigen (22–24). In contrast,
in guinea pigs immunized subcutaneously with MSP, both primary and tertiary structures may be presented to lymphocytes, allowing both cell-mediated and humoral immune responses.

If, indeed, native or processed MSP is released by mononuclear phagocytes or presented on their surface, then the immune system would have a mechanism for detecting intracellular \textit{L. pneumophila}. Thus, immune lymphocytes that recognize MSP epitopes might proliferate and release lymphokines such as IFN-\(\gamma\) that are capable of activating the infected mononuclear phagocyte and endowing it with the capacity to inhibit the multiplication of intracellular \textit{L. pneumophila} (8, 9). By analogy, secretory molecules of intracellular pathogens may in general allow the immune system to detect parasites sequestered in an intracellular site and thereby to defend the host against them. Along these lines, the intracellular bacterial pathogens \textit{Listeria monocytogenes} and \textit{Mycobacterium tuberculosis} release molecules into broth culture that stimulate a cellular immune response (25, 26).

The finding that the MSP is capable of inducing protective immunity against \textit{L. pneumophila} infection was arrived at by (a) establishing an animal model suitable for studying cell-mediated and protective immunity (15); (b) systematically examining \textit{L. pneumophila} molecules for their capacity to induce cell-mediated immunity (17); and (c) testing such immunostimulatory molecules for their capacity to induce protective immunity. This approach might similarly reveal protective antigens of other intracellular pathogens for which cell-mediated immunity plays an important role in host defense.

A vaccine against an intracellular pathogen consisting of a secretory molecule potentially has two important advantages over a conventional vaccine, consisting of whole organisms or their surface components. First, it may more readily allow the immune system to detect pathogens sequestered in an intracellular site, as noted above. Second, it should be less likely to induce antibody to surface molecules of the pathogen. Such antibody might promote uptake of the pathogen into mononuclear phagocytes, where the organism multiplies, and thereby be counter-productive to host defense.

Summary

We have examined the capacity of the major secretory protein (MSP) of \textit{Legionella pneumophila} to induce humoral, cell-mediated, and protective immunity in a guinea pig model of Legionnaires' disease. MSP was purified to homogeneity by ammonium sulfate precipitation, molecular sieve chromatography, and ion-exchange chromatography. The purified MSP was nonlethal and nontoxic to guinea pigs upon subcutaneous administration.

Guinea pigs immunized with a sublethal dose of aerosolized \textit{L. pneumophila} or a subcutaneous dose of MSP developed a strong cell-mediated immune response to MSP. Such guinea pigs exhibited marked splenic lymphocyte proliferation and cutaneous delayed-type hypersensitivity to MSP in comparison with control animals.

Guinea pigs immunized with MSP also developed a strong humoral immune response to MSP, as assayed by an ELISA. The median reciprocal antibody titer was 362 (range 45 to >2,048) for immunized animals compared with <8 for controls. In contrast, guinea pigs immunized with a sublethal dose of \textit{L. pneumophila} failed to develop anti-MSP antibody.

Guinea pigs immunized with MSP and then challenged with a lethal aerosol dose
of *L. pneumophila* exhibited highly significant protective immunity in each of five consecutive experiments. MSP induced protective immunity in dose-dependent fashion (40 > 10 > 2.5 > 0.6 µg MSP); vaccination with two doses of as little as 2.5 µg MSP induced significant protective immunity (*p* = 0.01, Fisher's Exact Test, two-tailed). Altogether, 21 (81%) of 26 animals immunized with 40 µg MSP survived challenge compared with 0 (0%) of 26 sham-immunized control animals (*p* = 7 × 10⁻¹⁰, Fisher's Exact Test, two-tailed). MSP-immunized but not control guinea pigs were able to limit *L. pneumophila* multiplication in their lungs.

This study demonstrates that (a) guinea pigs sublethally infected with *L. pneumophila* develop a strong cell-mediated immune response to MSP; (b) guinea pigs immunized with MSP develop a strong humoral and cell-mediated immune response to MSP; (c) guinea pigs immunized with MSP develop a very high level of protective immunity to lethal aerosol challenge with *L. pneumophila*; and (d) MSP-immunized animals are able to limit *L. pneumophila* multiplication in their lungs.

MSP, an extracellular protein of an intracellular pathogen, has potential as a vaccine for the prevention of Legionnaires' disease. Secretory molecules of other intracellular pathogens may also have vaccine potential.

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