Secretory Immune Responses to *Mycoplasma pulmonis*

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Formalized *Mycoplasma pulmonis*, along with aluminum hydroxide as an adjuvant, was used to subcutaneously immunize rats in the vicinity of the salivary gland to examine the characteristics of the secretory immune response to this pathogen. The induction of specific antibody to this microorganism was detected in serum and the exocrine fluids, namely, saliva and lung lavage fluid. Both immunoglobulin G (IgG) and IgA isotype antibodies were detected in each of these fluids after primary and secondary local immunizations. Serum responses from immunized animals were significantly greater than in the control group, but a dose response was not observed in either IgG or IgA antibody at the dosages selected for immunization. Salivary IgG antibody responses peaked early after both the primary and secondary immunizations, exhibiting a clear dose response. Salivary IgA in immunized groups was significantly greater than that in the control group but displayed little dose-dependent kinetics, and, at the termination of the experiment, this response had not yet peaked. Lung lavage IgG and IgA were minimal after the primary immunization when the antibody was normalized to total protein but displayed dose-dependent kinetics after a secondary challenge. IgG peaked immediately after a secondary challenge, while IgA peak responses were observed only after 20 days. A positive correlation was noted between the serum, saliva, and lung lavage fluid IgGs after both primary and secondary immunizations and only after a secondary challenge for IgA. In this study we were able to elicit a secretory immune response, consisting of both IgG and IgA, which exhibited a dose-dependent characteristic in lung lavage fluid to this immunogen. Additionally, a positive correlation of antibody levels between saliva and lung lavage fluid suggests that saliva could be used as an indicator for monitoring specific antibody to *M. pulmonis* in lung lavage secretions without requiring invasive, deletcherious procedures.

Mucosal surfaces are continually challenged by a wide variety of pathogenic bacteria and viruses. These surfaces are bathed with products of the secretory immune responses, in particular secretory immunoglobulin A (IgA), which contribute largely to the defense of these surfaces (27). Secretory IgA and in some secretions IgG are the most frequently isolated immunoglobulins found in salivary, nasal, and respiratory secretions (15). Therefore, it is not surprising that a predominance of these isotopes of plasma cells are found in mucosal tissues (3). These antibodies are synthesized locally in response to antigenic stimulation at the mucosal surfaces and are secreted into the exocrine fluids (28).

It has been demonstrated that functional secretory immunoglobulins can be elicited against a broad array of antigenic substances through inhalation and ingestion. These include food and bacterial products, toxins, viruses, fungi, and protozoa (28). These findings have proven useful in the conceptual development of vaccines against many pathogenic microorganisms that utilize mucosal surfaces as the portal of entry for the establishment of disease. Specifically, mucosal vaccines have been or continue to be developed against poliovirus (29), influenza virus (1), *Vibrio cholerae* (37), *Haemophilus influenzae* (7), *Salmonella* sp. (31), *Streptococcus mutans* (22), and *Chlamydia trachomatis* (10). Some of these vaccines also use strategies for immunization which have shown that local stimulation of one mucosal surface can initiate the migration of locally sensitized B cells to distant mucosal surfaces and thus provide additional immunity to distant tissues of the common mucosal immune system (28).

*Mycoplasma pneumoniae* is an important pathogen of human beings that initiates lesions of both the upper and lower respiratory tracts (23). Clinical studies have identified acquired systemic (23) and secretory (4) immune responses to this pathogen. However, many of the characteristics of regulation of systemic and secretory immune responses to this microorganism have not yet been determined. To accomplish these studies, an animal model of this disease, murine mycoplasmosis, which is a respiratory disease of rodents caused by *Mycoplasma pulmonis*, has been utilized. This infection appears to spread readily via aerosols among susceptible murine colonies (14). Currently, animal colonies are monitored by the detection of specific antibody in serum that is elicited during infection with *M. pulmonis*, but it has been shown that animals can be infected and yet display seronegativity up to 4 weeks postinfection (5). In spite of these diagnostic antibody responses, data from Lai et al. (25) have indicated that the principal protective feature of immunity to respiratory lesions caused by *M. pulmonis* may be cell-mediated immune responses. The same report also noted that while different strains of rodents have different susceptibilities to *M. pulmonis*, other studies suggest that preexisting antibody can be protective in mice but not in rats (25). Recognizing that the mucosal surfaces are the site of initial colonization and infection, it could be hypothesized that secretory immune responses are capable of interfering with the pathogenesis of this microorganism.

In this study, serum, saliva, and lung lavage secretions were examined for the presence of antibody to *M. pulmonis* after local immunization with formalized organisms. We also examined the correlation of antibody levels among the fluids to provide evidence for the autonomy of the secretory IgA responses as well as to determine whether salivary IgA

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could be used as an indicator of the antibody response to *M. pulmonis* in respiratory secretions.

**MATERIALS AND METHODS**

**Animals.** Two hundred male Fischer 344 rats, barrier raised and specific pathogen free [CDF(F-344)Cr1BR], were obtained from Harlan Sprague Dawley Inc. (National Institute of Aging Colonies, Indianapolis, Ind.) at 120 days of age. All rats were fed pelleted rat chow (Teklad LM 485) and water ad libitum and maintained in an Accredited Biohazard Facility at the University of Texas Health Science Center.

Upon arrival, each animal was weighed and baseline serum and saliva samples were obtained. These samples were screened for the presence of specific antibodies to *M. pulmonis* by both our methodology and that of Harlan Sprague Dawley Inc. and shown to be negative. The animals were then separated into five groups of 40 rats, with the lower-weight animals distributed equally among the five groups. Eight of these animals were bled and salivated, and five were sacrificed for lung lavage for each time point.

**Sample collection.** Animals were anesthetized by ether inhalation for saliva and serum collection. Saliva was obtained after a subcutaneous administration of pilocarpine nitrate (1 mg per 100 g of body weight; Sigma) and collected over a 15-min period. The volume of saliva was recorded, and the samples were stored on ice until processed. Saliva samples were processed the same day by centrifugation (16,000 × g for 20 min) to remove debris and stored at −80°C until tested. Blood was obtained through the retro-orbital plexus by using heparinized capillary tubes (Chase Instruments, Glens Falls, N.Y.), allowed to clot, and centrifuged at 2,500 × g to separate the serum. Serum samples were stored at −80°C until tested.

Lung lavage samples were obtained after euthanasia of the animals by using T-61 Euthanasia Solution (Hoechst-Roussel; 1 ml per 100 g of body weight). After the trachea was exposed, a small nick was made and a cannula was inserted into the lungs. The cannula was tied tightly onto the trachea with 3-0 silk sutures, and 3 ml of cold 0.85% NaCl containing 0.05% Tween 20 was lavaged through the lungs three times. After the third wash, the lavage fluid was collected (blood free) and stored on ice until processed. Lung lavage fluids were processed by centrifuging at 2,500 × g to remove debris and stored at −80°C until tested.

**Growth and antigen preparation of *M. pulmonis*.** *M. pulmonis* SA-F344 was generously supplied by Jerry Simecka of the University of Alabama at Birmingham. *M. pulmonis* was grown as previously described (11) and harvested when the medium turned orange-red. Initially, an aliquot of the medium from each culture bottle was Gram stained for assessment of bacterial or fungal contamination. The culture was then harvested by centrifugation at 16,000 × g to collect the *M. pulmonis* and washed three times with phosphate-buffered saline (PBS). The pellet was dislodged and broken into smaller clumps by expelling the slurry through gradually smaller-gauge sterile needles. Buffered formal saline was added to the slurry to a final concentration of 0.5% and incubated on a rotator overnight at 25°C. *M. pulmonis* was washed twice with PBS, the pellet was resuspended as described above, and a bicinchoninic acid protein determination (Pierce) was performed. These cells were then stored at 4°C until used.

**Immunoglobulin and antibody analysis.** Total levels of IgA and IgG were determined in the serum, saliva, and lung lavage samples by using a capture enzyme-linked immunosorbent assay (ELISA). Briefly, goat anti-rat antisera mono-specific for IgG (Jackson Laboratory) or IgA (Miles) were attached to microtiter wells as previously described (18). The addition of fluid samples was followed by the addition of biotinylated anti-rat IgG (Biodesign International) or IgA (2) and streptavidin-alkaline phosphatase (Zymed). Reactivity in the saliva and lung lavage samples was determined by using the Immunoselect ELISA amplification system (Bethesda Research Laboratories). Serum samples were developed by using *p*-nitrophenolphosphate (Sigma) as the substrate. The samples were compared with a previously calibrated rat reference serum (Bethyl Laboratories, Inc., Montgomery, Tex.).

Antibody to *M. pulmonis* was determined in serum, saliva, and lung lavage samples by using an indirect ELISA. Formalized *M. pulmonis* (10 μg of protein/ml) was attached to the plates as previously described (17). Diluted samples were incubated, and then either goat anti-rat IgG or IgA was added. Subsequently, rabbit anti-goat IgG conjugated with alkaline phosphatase (Sigma) was added and the mixture was incubated overnight. Again, saliva and lung lavage samples were developed by using the Immunoselect ELISA amplification system as the substrate, and the serum substrate was *p*-nitrophenolphosphate. Controls used in this series of experiments were hyperimmunized rat sera and saliva samples that had been previously tested, pooled, and calibrated.

**Experimental protocol.** Animals were immunized subcutaneously in the salivary gland region with four doses (100, 50, 10, and 5 μg) of formalized *M. pulmonis* protein. The total volume of the sample injected was 0.2 ml that was injected in four increments of 0.05 ml. Previous results by us demonstrated that the antigen administered in this manner is not deposited directly into the salivary glands (19). PBS was used to immunize the control group (PBS group), and aluminum hydroxide (Alhydrogel; Accurate Chemical & Scientific Corp.) was used as an adjuvant for all immunizations. Samples of serum, saliva, and lung lavage fluid were collected 10, 30, 45, and 60 days after primary immunization. The remaining animals were then challenged with a second immunization (identical to the primary dose) on day 70, and samples were collected on 80, 100, 115, and 130 days after the start of the experiment.

**Statistical analysis.** Statistical analysis was performed by using the Statgraphics program (STSC). The mean and the standard error of the mean were calculated for the immune response of each group for a particular day and were compared with the values of their respective sham-immunized groups. An analysis of the difference between the groups was performed by using a Kruskal-Wallis analysis of variance, and correlation data were assessed by using the Spearman rank correlation analysis. Statistical significance for both of these analyses was set at *P* < 0.05.

**RESULTS**

**Peak antibody response levels to *M. pulmonis*.** Preliminary experiments were performed which demonstrated that constant IgA responses were elicited only when the *M. pulmonis* antigen was administered with Al(OH₃)₃ as an adjuvant (Table 1). As described in Table 1, lower doses of antigen (50 μg of protein) provided significantly greater antibody responses (*P* = 0.0155) in the presence of the adjuvant than those without.
TABLE 1. Comparison of salivary IgA response to *M. pulmonis* with and without the use of Al(OH)₃ as an adjuvant.

<table>
<thead>
<tr>
<th>Antigen dose of <em>M. pulmonis</em> (µg) or control</th>
<th>Salivary IgA response*</th>
<th>Immunization without adjuvant</th>
<th>Immunization with adjuvant</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>41 ± 6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>100</td>
<td>30 ± 3</td>
<td>47 ± 22</td>
<td>47 ± 22</td>
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<tr>
<td>50</td>
<td>22 ± 1</td>
<td>39 ± 8</td>
<td>39 ± 8</td>
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<tr>
<td>10</td>
<td>20 ± 3</td>
<td>37 ± 6</td>
<td>37 ± 6</td>
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<tr>
<td>5</td>
<td>ND</td>
<td>39 ± 4</td>
<td>39 ± 4</td>
</tr>
<tr>
<td>Control (PBS)</td>
<td>19 ± 2</td>
<td>19 ± 1</td>
<td>19 ± 1</td>
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* Mean ± standard error of the mean of antibody in ELISA units.

Primary responses of serum IgA demonstrated that all four experimental groups responded significantly greater than the control group (*P* < 0.05) (Fig. 1). Likewise, after the secondary immunization, serum IgA responses to *M. pulmonis* were again significantly elevated in all four immunized groups compared with the control group (*P* < 0.005) (Fig. 1). Additionally, each of the doses of *M. pulmonis* that was administered elicited a significantly greater secondary serum IgA antibody response compared with the primary immunization, although 50 µg of antigen appeared to provide optimal sensitization.

![FIG. 1. Peak serum antibody responses after immunization with formalized *M. pulmonis* (in micrograms of total protein). Peak primary responses are denoted by hatched bars, and peak secondary responses are denoted by solid bars. * indicates values are significantly different from those of the control (PBS) at *P* < 0.05.](http://iai.asm.org/journals/iai/v060/p329/fig1.png)

Similar findings were noted with serum IgG responses to *M. pulmonis*. All groups demonstrated significantly higher responses than the control group in both the primary and secondary responses. The secondary response was up to 10-fold greater than the peak primary response. A dose response of serum IgG production was noted only during the primary response (*P* < 0.05) (Fig. 1).

Salivary IgA to *M. pulmonis* was detected only in the 100-, 50-, and 10-µg dose groups during the primary response. A clear-cut anamnestic secondary response in salivary IgA production could not be demonstrated (Fig. 2). Moreover, all groups demonstrated a detectable IgG response to *M. pulmonis* in both the primary and secondary immune responses that was significantly higher than that of the control group (*P* < 0.05) (Fig. 2). Contrary to the case with salivary IgA and serum responses, there did appear to be a dose response in salivary IgG in both the primary and secondary responses. Additionally, the secondary response elicited levels of antibody at least twofold higher than the primary response.

![FIG. 2. Peak salivary responses after immunization with formalized *M. pulmonis* (in micrograms of total protein). Peak primary responses are denoted by hatched bars, and peak secondary responses are denoted by solid bars. * indicates values are significantly different from those of the control (PBS) at *P* < 0.05.](http://iai.asm.org/journals/iai/v060/p329/fig2.png)

Lung lavage antibody was normalized by comparing the antibody to *M. pulmonis* in ELISA units to the total immunoglobulin levels in the samples (either IgG or IgA), so the information is given as number of ELISA units per microgram of immunoglobulin. The only group that showed a significant elevation in peak primary IgA response was the 100-µg dose group (*P* < 0.05). However, significant dose-dependent elevations in peak IgA responses could be detected in the secondary responses in all groups, with increases ranging from 5 to 10 times that of the primary response (100- and 50-µg dose groups, *P* < 0.005; 10- and 5-µg dose groups, *P* < 0.05) (Fig. 3).
IgG antibody to *M. pulmonis* in the lung lavage fluid could not be detected in the primary response after any of the immunization doses. However, a secondary response was found in all groups that ranged in magnitude from 10 to 1,000 times higher than the primary response (100-, 50-, and 10-µg dose groups, *P* < 0.005; 5-µg dose group, *P* < 0.05) (Fig. 3).

**Antibody response kinetics.** Serum IgA to *M. pulmonis* peaked at various intervals during the primary response. However, in the secondary response, all groups responded within 10 days (day 80 of experiment) of the secondary challenge and generally increased with time through the end of the experimental period. As such, by day 130, all of the serum IgA responses of the groups remained at least 20-fold greater than baseline levels (Fig. 4).

Serum IgG antibody levels peaked late during the primary response period but reached maximum levels immediately after the secondary challenge (Fig. 4). The values started to decrease by day 100 after the booster immunization and fell to approximately 50% of the peak response by the completion of the experiment.

At higher doses of *M. pulmonis* antigen, the salivary IgA antibody response was uniformly higher than that of the control group (Fig. 5). However, the lowest dose of antigen (5-µg dose group) exhibited no response changes different from those of the control (PBS group). When saliva antibody data were normalized to the salivary protein data, there were few noticeable changes in the kinetics of the response; the graphs comparing the two are virtually identical (Fig. 5).

Salivary IgG responses were noted early in the primary response in the 100- and 50-µg dose groups and quickly dropped to background levels by day 30 (Fig. 6). After a secondary challenge with *M. pulmonis*, the IgG levels peaked early when higher doses were administered (100- and 50-µg dose groups), but responses to lower doses of antigen generally were not detected until 45 days postimmunization. As noted with salivary IgA, when the antibody data are normalized to total salivary protein data, the two graphs are virtually superimposable.

Primary lung lavage responses to *M. pulmonis* in both IgG and IgA were negligible. In contrast, secondary responses to the antigen peaked by day 100 for IgA and by day 80 for IgG (Fig. 7 and 8). The IgA response remained detectable over an extended period of time, whereas by day 130 the IgG responses in the 100-, 50-, and 5-µg dose groups had declined by at least 1 log from the peak response and the IgG response in the 10-µg dose group had declined almost 500 ELISA units from the peak levels. When lung lavage antibody responses were normalized to total protein rather than respective immunoglobulin levels, both IgG and IgA baseline responses were negligible. However, a clear-cut dose response could be visualized in both the IgG and IgA responses, with the highest responses being induced by the highest doses. As noted above, IgG peaked at 80 days and returned to near baseline levels by day 130. IgA responses were first noted at 100 days, and responses for the highest dose continued to climb until day 115. By day 115, the levels dropped to less than one-third of the peak response.

**Correlation of secretory immune responses.** Correlation
analyses were also performed to examine the relationship between IgA and IgG antibody responses in exocrine secretions from the oral cavity and respiratory tract. Similarities in antibody response patterns and levels between saliva and lung lavage fluids suggest that these secretions were related. In fact, a strong positive correlation was noted between the IgA levels in saliva and lung lavage fluid in the secondary response ($P = 0.0055$) (data not shown). Likewise, the IgG antibody responses in these secretions were also positively correlated ($P < 0.0001$). In contrast, there was no discernible relationship between lung lavage IgA and salivary IgG or lung lavage IgG and salivary IgA isotypes. Moreover, only the secretory IgG immune responses in saliva and lung lavage fluid were generally correlated with those responses noted in serum. However, the serum IgG and IgA responses were clearly related on the basis of the apparent antibody elicitation in both isotypes after local immunization with *M. pulmonis*.

**DISCUSSION**

Secretory immunity serves to protect the mucosal surfaces from disease by inhibiting adherence of bacteria to epithelial surfaces and by neutralizing bacterial products such as toxins and enzymes. Since many diseases are contracted through colonization of the mucosal surfaces, inhibiting colonization by immunization could protect these surfaces from the effects of disease. Recent studies using a mouse model with *V. cholerae* showed that disease progression could be eliminated by the presence of specific secretory IgA antibodies to bacterial components of *V. cholerae* when the animal was challenged with a lethal dose of the virulent microorganism (40). Similarly, murine respiratory mycoplasmosis is a respiratory infection of rats and mice initiated by the colonization of both the upper and lower respiratory tracts (12, 26). However, individual strains of mice and rats resolve the disease process differently. In mice, a humoral response appears to be necessary for the alleviation of the disease process and the prevention of future infection (6). In rats, cellular immunity appears to play the major role in the prevention of disease and the resultant lesions, and, although a humoral response can be detected during infection, it appears to have no effect in the progression of the disease (13). Additionally, other murine strains exhibit almost an innate resistance to the disease process, regardless of the dosage or strain of *M. pulmonis* (11, 12).

Immunological interference with infectious diseases has been frequently studied in murine models (24). These studies have included experimental designs to examine both systemic (32) and secretory (8) immunities. From these studies, clear evidence is available concerning the autonomy of the secretory immune response (28). In this regard, many of the
immunological investigations of \( M. \) pulmonis infections have concentrated on systemically derived immune responses that are expressed in the respiratory tract (6, 38). In this study, we used an immunization regimen that would be expected to elicit an immune response in the serum, saliva, and lung lavage fluids. Investigations of the secretory immune system have clearly demonstrated the existence of a common communication process among mucosal sites. As such, locally induced immune responses at one site can initiate the migration of antigen-sensitive B cells that migrate to and differentiate at distant sites (28). Thus, an attempt was made to correlate the presence of a secretory IgA response in the two exocrine secretions tested.

Antibody to \( M. \) pulmonis was detected in all of the different secretions when the animals were immunized with formalinized mycoplasmas by using \( \text{Al(OH)}_3 \) as an adjuvant. Preliminary studies showed that immunization of animals with \( M. \) pulmonis without an adjuvant elicited detectable serum and lung lavage fluid responses, but the salivary response to this preparation was minimal (Table 1). Moreover, the response was brief and returned to near baseline levels by 45 days (data not shown). The use of formalin-killed microorganisms as immunogens in vaccines has a long history (38, 39). This type of preparation has been found to generally maintain protein and polysaccharide antigens in a form that induces antibodies that cross-react with the native molecule. The results of these studies indicate that formalinized \( M. \) pulmonis is an adequate immunogen that can elicit antibodies that recognize many soluble antigens derived from this organism (36a). Previous results from our laboratory have examined the activity of various adjuvants to enhance secretory immune responses. We have shown that \( \text{Al(OH)}_3 \) produced significant augmentation of salivary IgA responses when administered with antigen either orally or via parenteral injection (16 and unpublished observations). Additionally, studies in nonhuman primates demonstrate that this adjuvant enhanced salivary IgA responses to formalin-killed \( Porphryromonas gingivalis \) when the bacteria and adjuvant were provided orally in gelatin capsules (16). Similar results were observed in this system when \( M. \) pulmonis was used as the immunogen.

Dose-dependent responses to the formalinized \( M. \) pulmonis could be discerned in salivary IgG and lung lavage IgG and IgA when normalized to total fluid protein. In contrast, in serum responses, neither IgG nor IgA showed a clear dose response. We interpret these results as suggesting that the doses of formalinized \( M. \) pulmonis used were in excess of those necessary for deriving a dose response for serum antibody. However, since the saliva and lung lavage fluid secretory responses were of primary interest, it was necessary to increase the immunogen dose to produce a sufficient response in these secretions. Previous studies by us (20) and others (9, 36) have demonstrated the ability of both oral and parenteral injections of antigen to elicit substantial secretory IgA responses in rats. However, results from these studies indicated that higher doses of antigen were required for optimal oral stimulation and, in general, that local immuni-
zation tended to induce higher levels and more consistent response dynamics of salivary IgA. The principal difference in these routes of administration was that, most frequently, oral immunization resulted in minimal serum antibody responses in human beings, rats, mice, hamsters, and nonhuman primates (1, 7, 16, 19, 22, 34, 35). Consequently, M. pulmonis antigen appeared quite similar to other antigens utilized in these systems.

Peak IgG responses were detected by day 80 coincidently in the serum, saliva, and lung lavage fluids, suggesting that perhaps the majority of this response in the secretions is via serum-derived immunoglobulin. IgA could be detected as early as day 80 (10 days postimmunization) in the secondary response, continued to increase through day 115 in serum and lung lavage fluids, and was still rising in saliva at the termination of the experiment on day 130. Examination of the kinetics of the secondary responses to this antigen indicated an anamnestic IgA response in both secretions. This was most dramatic in the lung lavage fluid, which demonstrated an earlier onset of response, a significantly higher level, and a longer duration after the booster immunization than primary antibody responses in this secretion. The IgA response in saliva demonstrated both a higher level and a longer duration than primary responses in this secretion, but there was no noticeable evidence for an earlier onset of the response. These findings are similar to those previously published which describe anamnestic secretory IgA responses that are less clearly demarcated than classic serum immunoglobulin responses (19). Furthermore, the IgG responses in the secretions exhibited definitive evidence for immunologic memory contributing to the immune components at these mucosal surfaces.

As has been reported previously, active mucosal immune responses can often be detected in tissues that are not immediately adjacent to the site of vaccination. These findings resulted in the description of the common mucosal immune system and identified homing of IgA B cells within this system. Our data showed a positive correlation between serum, saliva, and lung lavage IgG responses (P < 0.001) and between saliva and lung lavage IgA responses (P < 0.05) in either the primary or secondary response. The relationship between respiratory, saliva, and serum IgG levels suggests that the majority of this isotype response locally is derived via serum immunoglobulin transudate into the secretions. Similar suggestions have been reported by Simecka et al. (33) with M. pulmonis infections in rats. Thus, it appears that a secretory immune response can be elicited to M. pulmonis in both salivary and respiratory secretions. Consequently, in these studies, active local immunization in the vicinity of the salivary gland also elicited secretory IgA responses in the bronchial lavage fluids. Similar findings have been reported by Cox and Taubman (9), who used a defined antigen to demonstrate distant mucosal priming for IgA responses. Thus, it appeared that saliva could be used to monitor the progression of the lung mucosal responses in lieu of invasive procedures to acquire respiratory secretions. Further studies will be required to examine the potential functional characteristics of this antibody.

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