SP-A1 and SP-A2 variants differentially enhance association of Pseudomonas aeruginosa with rat alveolar macrophages

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Mikerov, Anatoly N., Todd M. Umstead, Weixiong Huang, Wenlei Liu, David S. Phelps, and Joanna Floros. SP-A1 and SP-A2 variants differentially enhance association of Pseudomonas aeruginosa with rat alveolar macrophages. Am J Physiol Lung Cell Mol Physiol 288: L150–L158, 2005. First published September 17, 2004; doi:10.1152/ajplung.00135.2004.—Chronic airway inflammation caused by Pseudomonas aeruginosa is an important feature of cystic fibrosis (CF). Surfactant protein A (SP-A) enhances phagocytosis of P. aeruginosa. Two genes, SP-A1 and SP-A2, encode human SP-A. We hypothesized that genetically determined differences in the activity of SP-A1 and SP-A2 gene products exist. To test this, we studied association of a nonmucoid P. aeruginosa strain (ATCC 39018) with rat alveolar macrophages in the presence or absence of insect cell-expressed human SP-A variants. We used two trios, each consisting of SP-A1, SP-A2, and their coexpressed SP-A1/SP-A2 variants. We tested the 6A2 and 6A4 alleles (for SP-A1), the 1A0 and 1A alleles (for SP-A2), and their respective coexpressed SP-A1/SP-A2 gene products. After incubation of alveolar macrophages with P. aeruginosa in the presence of the SP-A variants at 37°C for 1 h, the cell association of bacteria was assessed by light microscopy analysis. We found 1) depending on SP-A concentration and variant, SP-A2 variants significantly increased the cell association more than the SP-A1 variants (the phagocytic index for SP-A1 was ~52–95% of the SP-A2 activity); 2) coexpressed variants at certain concentrations were more active than single gene products; and 3) the phagocytic index for SP-A variants was ~18–41% of the human SP-A from bronchoalveolar lavage. We conclude that human SP-A variants in vitro enhance association of P. aeruginosa with rat alveolar macrophages differentially and in a concentration-dependent manner, with SP-A2 variants having a higher activity compared with SP-A1 variants.

surfactant protein A variants; phagocytic index; cystic fibrosis

THE LUNG IS CONSTANTLY under assault from inhaled particles and microorganisms. Macrophages and alveolar lining fluid constitute the first line of defense against many inhaled pathogenic microorganisms. The lung has developed a complex defense system involving nonspecific innate immune mechanisms. These mechanisms may be of particular importance during the earlier stages of infection when the specific or adaptive immune response has not yet developed.

Some of the surfactant proteins are important components of the innate immune response and are involved in the regulation of inflammatory processes in the lung. Surfactant protein A (SP-A), a 34- to 36-kDa glycoprotein, plays a major role in the modulation of innate host defense in the lung (7, 16, 21, 41, 48, 52). SP-A has been shown to stimulate chemotaxis of macrophages (70), enhance phagocytosis (11, 34, 44, 46, 47, 50, 63), induce generation of reactive oxidants (61), regulate the nitric oxide production (1, 23), and influence the proliferation of immune cells (2, 38) and the production of proinflammatory cytokines (3, 27, 37, 39, 68, 69). SP-A can bind to receptors on the macrophage membrane (35). The interactions of SP-A with pathogens are complex. However, it has been shown that SP-A binds to the lipid A portion of rough LPS (62). It has also been reported that altered levels of SP-A are present during infection or when tissue damage occurs in the lung (7). Furthermore, genetically modified mice lacking SP-A are highly susceptible to pneumonia, probably because SP-A is not available to stimulate macrophage functions and aid in the clearance of microorganisms (8, 42–45).

Cystic fibrosis (CF) affects ~30,000 individuals in the US, and it is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. CF is associated with chronic infection and inflammation in the lung, along with the production of abnormally thick mucus and defects in mucociliary clearance. The microenvironment in the CF lung or other diseased lungs differs from that of normal lungs and in some cases may be more favorable for bacterial colonization. Pseudomonas aeruginosa, an opportunistic gram-negative bacilli form bacterium, often affects patients with compromised host defense. Interestingly, variability in pulmonary disease severity is observed among CF patients and even among CF patients homozygous for CFTR mutations (19, 33, 36, 51, 55). Thus it is probable that other modifier genes are responsible for the individual variability in the severity of pulmonary symptoms in patients with CF or with other lung disease.

The human SP-A (hSP-A) locus consists of one pseudogene and two functional genes, SP-A1 and SP-A2, located on chromosome 10 (5) in opposite transcriptional orientation (25). More than 30 alleles for the SP-A genes have been characterized either partially or in their entirety (10). Four SP-A1 alleles (6A, 6A2, 6A3, 6A4) and six SP-A2 alleles (1A, 1A0, 1A1, 1A2, 1A3, 1A4) have been observed frequently (>1%) in the general human population and have been well characterized (10, 14, 15). The alleles for each SP-A gene are classified on the basis of nucleotide differences within the coding sequences (10, 14, 32). SP-A variants have been identified with both quantitative (regulatory) and qualitative (functional, biochemical, and/or structural) differences. Qualitative differences include differences in their ability to stimulate production of TNF-α by THP-1 cells, a macrophage-like cell line (28, 68, 69), ability to

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inhibit surfactant secretion (66), and differences in aggregation, oligomerization, or other characteristics (18, 57, 64, 66). Nucleotide or splice differences within regulatory [5'-untranslated region (UTR) and 3'-UTR] regions (13, 15, 32, 40, 54) have also been observed between the SP-A1 and SP-A2 genes and/or alleles. Quantitative differences include in vitro differences in basal mRNA levels and in response to dexamethasone (26, 67) and differences in the SP-A mRNA (17, 31) and protein (12, 24, 58) levels among individuals. The basis for the observed qualitative and quantitative differences may be genetic in nature (15, 26, 31, 40, 67).

In CF, the levels of SP-A and other surfactant components have been shown to be altered (20, 29, 53). Because SP-A enhances phagocytosis and SP-A variants have been identified with quantitative and qualitative differences, it is possible that the SP-A variant differences result in differences in their effectiveness to enhance association of P. aeruginosa with alveolar macrophages and thus may contribute to varied disease severity.

In this paper, we studied for the first time the ability of in vitro expressed SP-A1 and SP-A2 allelic variants to enhance association of P. aeruginosa with rat alveolar macrophages.

**MATERIALS AND METHODS**

**Reagents and Media**

Tryptic soy agar was obtained from Sigma (St. Louis, MO); RPMI 1640 (HEPES modification) and Dulbecco’s phosphate-buffered saline (PBS) was from Invitrogen (Life Technologies, Grand Island, NY). Sterile saline solution (0.9% NaCl) was purchased from Baxter Healthcare (Deerfield, IL). Bronchoalveolar lavage (BAL) fluid was obtained from patients with alveolar proteinosis (AP) who were undergoing routine therapeutic lavage.

**Growing and Preparation of Bacteria**

A nonmucoid P. aeruginosa strain (ATCC 39018) was obtained from American Type Culture Collection (ATCC, Rockville, MD). Bacteria were grown on tryptic soy agar plates overnight (20–24 h) at an optimal temperature of 30°C for this strain according to ATCC recommendations. Bacteria from plates were suspended in RPMI medium (RPMI 1640 HEPES modification, with L-glutamine) using a sterile bacteriological loop and spun at 250 g for 1 min to remove agar debris and large bacterial aggregates. The supernatant was then diluted with RPMI medium to a bacterial concentration of 1 \times 10^8 colony-forming units (CFU)/ml. The CFU value was determined with a calibration curve based on the optical density of a bacterial suspension at 660 nm. Spreading of the prepared bacterial suspension on agar plates was also performed to control for the quantification of bacteria. The bacterial suspension was used immediately for cell-association assays.

**Purification of SP-A from human BAL**

SP-A was purified (22) from the BAL fluid obtained from a patient with AP (AP BAL hSP-A) as described previously (27). The concentration of SP-A was determined with the Micro BCA Protein Assay Kit (Pierce, Rockford, IL) using RNase A as a standard, and the purity of the SP-A preparation was verified by SDS-PAGE followed by silver staining and by Western blotting.

**Preparation and Purification of hSP-A Genetic Variants Expressed in the Baculovirus-Mediated Insect Cell System**

hSP-A genetic variants (for SP-A1: 6A2, 6A4; for SP-A2: 1A0, 1A; and their coexpressed variants: 6A2/1A0, 6A4/1A) were produced using the baculovirus-mediated insect expression system as described previously (68). Briefly, SP-A variant expression was achieved with inoculation of a virus containing the SP-A variant gene into insect cells, growing in SF-900 II SFM medium (Invitrogen, Life Technologies, Grand Island, NY). The inoculated cells were cultured at 28°C with shaking at a starting density of 2 \times 10^6 cells/ml. Supernatants were harvested 72 h after inoculation, and proteins were purified by mannose-affinity chromatography. The purified SP-A variants were examined by silver staining by SDS-PAGE gels and by Western blotting. The protein concentration was determined using the Micro BCA Protein Assay and RNase A as a standard. We tested three independent protein preparations of SP-A variants 1A0, 6A2, and 1A0/6A2 and one preparation of 1A, 6A4, and 1A0/6A4.

**Isolation of Rat Alveolar Macrophages**

Male pathogen-free Sprague-Dawley rats (250–300 g; Harlan, Indianapolis, IN) were used as a source for alveolar macrophages. All procedures involving animals were approved by Penn State University Institutional Animal Care and Use Committee. Animals were anesthetized with an intramuscular injection of a mixture of ketamine HCl (Ketaset; Fort Dodge Animal Health) and xylazine (XYLAJECT; Phoenix Pharmaceuticals, St. Joseph, MO). Alveolar macrophages were isolated from the BAL of the rats. Rats were killed and bled, the tracheas were cannulated, and the lungs were lavaged three times with 5 ml of sterile saline (0.9% NaCl) at room temperature. Lavages were collected in tubes on ice and centrifuged at 250 g for 3 min, and cell pellets were washed twice more with 10 ml of RPMI medium for 3 min at 250 g. Cells were counted with a hemacytometer and resuspended in RPMI medium at 1 \times 10^6 cells/ml. The viability of cells was determined by trypan blue dye exclusion, and only cell suspensions with >95% viable cells were used.

**Association of P. aeruginosa with Rat Alveolar Macrophages**

The cell-association assay was performed as described (6, 9, 50) with some modifications. Briefly, equal volumes (50 μl/50 μl) of suspensions of alveolar macrophages (10^6 cells per ml) and bacteria (10^5 CFU per ml) in RPMI medium were mixed at a proportion of alveolar macrophages:bacteria of 1:100. SP-A was then immediately added to the mixture. There was no preincubation of alveolar macrophages or bacteria with SP-A before the assay. The mixture was incubated for 1 h at 37°C for cell association. Throughout the 1-h incubation, the mixture was gently shaken end-over-end with the LABQUAKE shaker (Labindustries, Berkeley, CA) to provide mixing and to prevent adherence of macrophages to the surface of test tubes. One milliliter of ice-cold PBS was then added to stop phagocytosis. Alveolar macrophages were sedimented by centrifugation at 250 g for 8 min at 4°C. To remove unbound bacteria, alveolar macrophages were washed twice more with 1 ml of cold PBS followed by sedimentation at 250 g for 8 min at 4°C. Final suspensions of alveolar macrophages were resuspended in 200 μl of PBS and applied to slides with a cytocentrifuge. The slides were stained using the Hema-3 Stain Kit (Fisher Scientific, Pittsburgh, PA) for analysis by light microscopy.

Association of P. aeruginosa with Rat Alveolar Macrophages

The phagocytic index was calculated according to the following formula: phagocytic index = (average number of bacteria-positive cells + average number of bacteria-negative cells) / total number of bacteria. The phagocytic index for each experimental point was based on the assessment of 200 randomly selected macrophages (9) or 600–1,000 macrophages for the three to five experiments performed per study point or a total of 4,800 macrophages (2,600 for Fig. 2 and 2,200 for Fig. 3). The phagocytic index was calculated according to the following formula: the percentage of bacteria-positive macrophages (that associated with at least one bacterium) \times average number of bacteria per bacteria-positive macrophage (6). The values for the phagocytic index, for the number of bacteria-positive cells and for the average number of...
bacteria per bacteria-positive alveolar macrophages, was expressed as a percentage of the negative control (i.e., without SP-A), which was set to 100%.

Statistics

For comparison of the effect of SP-A variants at the same concentration, a paired t-test was performed, and for comparison of the effect of SP-A variants at different concentrations or for different variant trios, the two-sample t-test was used. Data were expressed as means ± SE for three or more independent experiments. Differences were considered significant when \( P < 0.05 \).

RESULTS

Detection of Live Bacteria, \( P. \) aeruginosa, Associated with Rat Alveolar Macrophages

To evaluate the effect of SP-A variants on the association of live \( P. \) aeruginosa with rat alveolar macrophages, we used a traditional light microscopy method. With this method, the attachment and internalization of bacteria (4) are assessed together. In this paper, we refer to these steps as cell association and used the phagocytic index, as described in MATERIALS AND METHODS and in Ref. 6, to determine relative levels of cell association among SP-A variants. Thus we assessed three measurements at the same time: phagocytic index, the number of bacteria-positive alveolar macrophages, and the average number of bacteria per bacteria-positive alveolar macrophages. We analyzed 200 randomly selected alveolar macrophages per each experimental point at \( \times 1,000 \) magnification under oil immersion (9). Figure 1 depicts bacteria associated with rat alveolar macrophages in the absence (Fig. 1A) or presence (Fig. 1B) of human SP-A from AP BAL. With the microscopic analysis, we were able to determine not only the number (percentage) of the bacteria-positive cells, but also how phagocytic they were (i.e., how many bacteria are associated with each cell). The negative control was set to 100%, and in each experiment we used relative data, expressed as a percentage of the negative control. Using this method, we tested (shown below) the level of association of \( P. \) aeruginosa with rat alveolar macrophages in the presence of in vitro expressed SP-A1, SP-A2, and their respective coexpressed SP-A1/SP-A2 variants.

Association of \( P. \) aeruginosa with Rat Alveolar Macrophages in the Presence of Human SP-A Variants

To assess the effect of baculovirus-mediated insect cell-expressed SP-A variants on cell association of \( P. \) aeruginosa, we used two trios of SP-A variants: one consisted of 1A\(^{0} \) (for SP-A2), 6A\(^{2} \) (for SP-A1), and their coexpressed 1A\(^{0} /6A^{2} \) variant and the other of 1A (for SP-A2), 6A\(^{4} \) (for SP-A1), and their coexpressed 1A/6A\(^{4} \) variant. SP-A variants of the same trio were expressed, purified, and tested at the same time. We assessed the effect of SP-A-mediated cell association by testing three concentrations of each SP-A variant: 25, 50, and 100 \( \mu \)g/ml. The hSP-A from AP BAL was used as a positive control at a single concentration of 25 \( \mu \)g/ml. The effect of hSP-A on cell association was always significantly different from the negative control (without SP-A) at \( P < 0.05 \). Higher concentrations of hSP-A (50 and 100 \( \mu \)g/ml) were not used because the very high level of cell association observed at these concentrations made it difficult to accurately assess the effect of hSP-A in microscope examinations.

SP-A variants 1A\(^{0} \), 6A\(^{2} \), and 1A\(^{0}/6A^{2} \). At the 25 \( \mu \)g/ml concentration, the 1A\(^{0} \) allele product, compared with the negative control or the 6A\(^{2} \) or the 1A\(^{0}/6A^{2} \) allele products, significantly increased the number of bacteria-positive cells, the number of bacteria associated, and the phagocytic index, whereas the activity of the 6A\(^{2} \) and the 1A\(^{0}/6A^{2} \) variants did not differ from negative control (Fig. 2).

At the 50 \( \mu \)g/ml concentration, the 1A\(^{0} \) allele product significantly increased the number of bacteria-positive cells and the phagocytic index, and 1A\(^{0}/6A^{2} \) significantly enhanced the values in all three tests compared with the negative control. Both the 1A\(^{0} \) and the 1A\(^{0}/6A^{2} \) variants were significantly more active than the 6A\(^{2} \) allele product for all three tests, and 1A\(^{0}/6A^{2} \) increased the number of bacteria in bacteria-positive cells and the phagocytic index significantly more than the 1A\(^{0} \) allele variant.

At the 100 \( \mu \)g/ml concentration compared with the negative control, the 1A\(^{0} \) allele significantly enhanced the values for all tests, 6A\(^{2} \) significantly increased the number of bacteria-positive cells and the phagocytic index, and the coexpressed variant 1A\(^{0}/6A^{2} \) significantly increased the number of bacteria-positive cells. The activity of the 1A\(^{0} \) allele variant was significantly higher compared with that of the 6A\(^{2} \) allele variant for all tests.

The hSP-A, as noted above, was used as positive control at 25 \( \mu \)g/ml in all variant tests. The results of the positive control indicate that the hSP-A was significantly more active than each of the three SP-A variants studied for all the tests performed, except for the average number of bacteria per bacteria-positive cells by 1A\(^{0} \) at 100 \( \mu \)g/ml. The data of the hSP-A positive control (at 25 \( \mu \)g/ml) for each variant concentration (25, 50, 100 \( \mu \)g/ml).
and 100 μg/ml) experiment were, respectively, as follows: 188 ± 13, 216 ± 10, and 205 ± 11 for the bacteria-positive cells; 256 ± 19, 250 ± 22, and 241 ± 16 for the number of bacteria; and 471 ± 36, 536 ± 34, and 495 ± 45 for phagocytic index tests.

Comparison of SP-A2 and SP-A1 variant activities across all concentrations and tests reveals that the 1A0 allele product was always significantly more active than the 6A2 allele product. For the 25, 50, and 100 μg/ml concentrations, the activity of 1A0 or 6A2 was, respectively, as follows: the number of bacteria: 1A0: 129 ± 5, 155 ± 10, 177 ± 9; 6A2: 106 ± 6, 107 ± 6, 131 ± 3, the number of bacteria: 1A0: 129 ± 7, 109 ± 8, 179 ± 14; 6A2: 95 ± 8, 90 ± 10, 123 ± 11, and the phagocytic index: 1A0: 158 ± 9, 153 ± 6, 315 ± 24; 6A2: 98 ± 5, 94 ± 6, 162 ± 18. Comparison of the effect of coexpressed SP-A1/SP-A2 variant with that of single gene products revealed a higher level of 1A0/6A2 activity compared with that observed by each 1A0 or 6A2 variant alone at 50 μg/ml with regard to the number of bacteria and the phagocytic index.

The activity of each individual variant (1A0, 6A2, 1A0/6A2) appeared to be concentration dependent. The 1A0 SP-A variant increased the number of bacteria-positive cells, the number of bacteria per cell, and the phagocytic index significantly more at 100 μg/ml compared with the findings at concentrations of 25 or 50 μg/ml. The 6A2 allele variant increased both the phagocytic index and the number of bacteria-positive cells significantly more at 100 μg/ml compared with that at 25 or 50 μg/ml. The emerging picture for the less active member (6A2) appeared to be concentration dependent. The 1A0 SP-A variant increased the number of bacteria-positive cells, the number of bacteria, and the phagocytic index significantly more at 50 or at 100 μg/ml compared with the results at 25 μg/ml.

Comparison of SP-A variants 1A, 6A4, and 1A/6A4. At the 25 μg/ml concentration, 1A significantly increased the number of bacteria and the phagocytic index compared with the negative control (Fig. 3). At the 50 μg/ml concentration compared with the negative control, both 1A and 1A/6A4 significantly increased the value in each of the three tests, and 6A4 significantly enhanced only the phagocytic index. The activity of 1A was significantly increased compared with that of 6A4 with regard to the phagocytic index. The activity of the 1A/6A4 was higher than that for the 6A4 or 1A variant, with regard to the number of bacteria per cell and the phagocytic index.

At the 100 μg/ml concentration compared with the negative control, the 1A and 1A/6A4 variants exhibited a significantly higher activity in all three tests, and the 6A4 variant significantly increased the number of bacteria-positive cells. Also, 1A/6A4 increased the number of bacteria and the phagocytic index significantly more than 6A4.

hSP-A (at 25 μg/ml) was found to be more active compared with each of the three SP-A variants for bacteria-positive cells and phagocytic index tests. For the number of bacteria, the activity of 1A/6A4 was not significantly different from the positive control at all concentrations. The values of hSP-A...
serving as a positive control for each variant concentration (25, 50, and 100 μg/ml) experiment were, respectively, as follows: 204 ± 19, 230 ± 12, and 205 ± 13 for the bacteria-positive cells; 256 ± 28, 245 ± 22, and 246 ± 22 for the number of bacteria; and 517 ± 57, 559 ± 31, and 501 ± 44 for phagocytic index tests.

The activity of SP-A variants 1A and 1A/6A4 appeared to be concentration dependent. The 1A variant increased the phagocytic index at 100 μg/ml compared with that observed at 25 or 50 μg/ml, and at 50 μg/ml compared with that at 25 μg/ml.

Also, the coexpressed variant 1A/6A4 was more active at 50 μg/ml than at 25 μg/ml for both the number of bacteria and the phagocytic index tests. Comparison of variant activities across different concentrations reveals that the 1A variant enhanced the phagocytic index significantly (P < 0.05) more than the 6A4 variant only at the 50 μg/ml concentration (169 ± 7 for 1A and 124 ± 5 for 6A4).

Overall comparison of activity between SP-A1 and SP-A2 variants. Comparison of the overall phagocytic activity between SP-A1 and SP-A2 variants expressed by the baculovirus-mediated insect cell system revealed: 1) the activity of the 6A2 variant compared with the 1A0 variant (depending on the concentration used) was 75–84% for the number of bacteria-positive cells, 69–82% for the number of bacteria, and 52–63% for the phagocytic index; and 2) the activity of the 6A4 variant compared with the 1A variant (depending on the concentration used) was 89–95% for the number of bacteria-positive cells, 83–100% for the number of bacteria, and 74–95% for the phagocytic index (Table 1). Thus a greater difference in activity is observed between the 6A2 and 1A0 than between the 6A4 and 1A variants.

No significant differences were found in the ratios of 6A2:1A0 variants or 6A4:1A at the different concentrations used in each of the three tests. This indicates that the relative value of SP-A1:SP-A2 activity, expressed as a ratio, remains unchanged at different SP-A concentrations tested. However, significant differences (Table 1) between 6A2:1A0 and 6A4:1A proportions were observed at 50 μg/ml for the number of bacteria-positive cells (75 and 89%, respectively) and at 25 μg/ml for

Table 1. Comparison of activity between SP-A1 (6A2, 6A4) and SP-A2 (1A0, 1A) variants

<table>
<thead>
<tr>
<th>Type of Test</th>
<th>SP-A, μg/ml</th>
<th>6A2:1A0, %</th>
<th>6A4:1A, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria-positive cells</td>
<td>25</td>
<td>83.93 ± 2.87</td>
<td>94.93 ± 4.24</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>75.02 ± 2.54*</td>
<td>89.40 ± 4.77*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>74.81 ± 5.41</td>
<td>93.12 ± 8.12</td>
</tr>
<tr>
<td>Number of bacteria</td>
<td>25</td>
<td>72.78 ± 3.52*</td>
<td>100.00 ± 6.31*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>81.94 ± 4.31</td>
<td>83.57 ± 6.26</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>68.93 ± 4.21</td>
<td>82.71 ± 8.64</td>
</tr>
<tr>
<td>Phagocytic index</td>
<td>25</td>
<td>62.58 ± 3.25*</td>
<td>95.50 ± 10.17*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>61.80 ± 5.28</td>
<td>74.13 ± 4.45</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>52.03 ± 6.1</td>
<td>75.95 ± 8.17</td>
</tr>
</tbody>
</table>

Values are means ± SE. The bacteria-positive cells, number of bacteria, and phagocytic index values were compared between surfactant protein (SP)-A1 and SP-A2 variants according to the following formula: SP-A1:SP-A2 (ratio) × 100% ± SE. The number of experiments for each test and concentration is shown in Figs. 2 and 3. *P < 0.05: significant differences between ratios 6A2:1A0 and 6A4:1A at the same concentration for the same test.
the number of bacteria (73 and 100%, respectively) and for the phagocytic index (63 and 95%, respectively) tests. It was estimated that the overall effectiveness of different SP-A variants compared with hSP-A (25 μg/ml) activity was 18–41% for the phagocytic index test, 37–60% for the number of bacteria test, and 53–70% for the number of bacteria-positive cell test (Table 2).

The observations made following comparison of the activity between SP-A1 and SP-A2 variants of different trios, shown in Table 3, indicate that 1) the 1A0 variant is more active than either 1A or 6A4, 2) 1A is more active than 6A2, 3) 6A4 is more active than 6A2, and 4) the coexpressed variant 1A/6A2 exhibits higher activity compared with 1A0/6A2. On the basis of the overall data presented in Table 3, the following order of activity emerges among single gene SP-A variants: 1A0 > 1A > 6A4 > 6A2.

**DISCUSSION**

Presently, it is not clear why some CF patients are more susceptible to pneumonia than others. Because of the important role of SP-A in lung host defense, its ability to increase phagocytosis of *P. aeruginosa*, and its natural genetic variability, we investigated the hypothesis that SP-A1 and SP-A2 genetic variants differ in their ability to enhance association of *P. aeruginosa* with alveolar macrophages. When in vitro expressed SP-A1 and SP-A2 allelic variants were tested for their ability to enhance cell association of *P. aeruginosa*, we observed in general that the SP-A2 variants are more effective than SP-A1 variants and also observed differences among SP-A variants. We speculate that these differences are accentuated in the CF lung microenvironment and could contribute to differences in the lung disease severity.

A traditional light microscopy method was used to assess differences in the ability of SP-A1 and SP-A2 gene products to enhance cell association. We assessed at the same time the percentage of active macrophages (i.e., associated with bacteria), as well as the level of macrophage activity by determining the number of bacteria each cell associates with (6). This assay is very sensitive because it enables one to account for every bacterium associated with each single cell analyzed. The light microscopy analysis allows for assessment of cell association. Cell association was determined by the phagocytic index measurements according to a previously described method and formula (6). The events we refer to in this paper as cell association, elsewhere (4) are referred to as attachment and internalization of bacteria (two steps of the phagocytosis process). As we published before (11), SP-A exerts similar stimulatory effects on both attachment and internalization of fluorescent (FITC-labeled) microspheres, gram-positive and gram-negative bacteria.

Moreover, for several reasons, conditions that more closely mimic in vivo conditions were used. When heat-killed *P. aeruginosa* bacteria were used, hSP-A from BAL did not enhance phagocytosis (60). This result could be associated with changes in the surface properties of the bacteria resulting from heat killing. When live FITC-labeled *P. aeruginosa* were used, SP-A had a stimulatory effect on phagocytosis (34, 47). Although after the FITC-labeling procedure the bacteria were still alive, this method involves a long labeling and washing time and then a prolonged period of storage of bacteria at −80°C in the presence of glycerin. The prolonged handling procedure and storage of labeled bacteria may lead to increases in the numbers of dead bacteria, depending on the length of storage. In our preliminary experiments (data not shown), we found that the viability of FITC-labeled bacteria was considerably reduced during their storage at −80°C in glycerin. Moreover, labeling procedures can cause possible modifications of the bacterial surface and surface receptors or cause a reduction in bacterial virulence. Labeled bacteria may be different from unlabeled with respect to their properties, including their binding to macrophages and their binding by different SP-A variants. Because SP-A has been shown to interact with both bacteria and macrophages, we opted in this study to mimic, to the greatest extent possible, the native conditions and avoid any potential modifications of both bacteria and macrophages. Therefore, the present findings may be more reflective of in vivo occurrences due to the use of live, nonlabeled, freshly isolated *P. aeruginosa* rather than use of heat-killed or FITC-labeled bacteria (34, 47, 60) as discussed above.

The continuous presence of SP-A during the assay may better reflect in vivo conditions during the contact of macrophages with pathogenic bacteria in the lungs and may also contribute to a maximal level of phagocytosis. Of relevance for the latter, a recent study indicated that hSP-A from AP BAL may enhance phagocytosis by THP-1 cells by two different mechanisms, by SP-A binding of the target to enhance its recognition and its subsequent phagocytosis and by a direct SP-A stimulatory effect on the phagocyte itself (11). Moreover, assessing three related measurements, the number of bacteria-positive alveolar macrophages, the number of bacteria per each positive alveolar macrophage, and the phagocytic index, is likely to provide some insight into phagocytosis events. For example, we observed that the concentration-dependent increased activity of the less active member (6A2) of the 1A0, 6A2, 1A0/6A2 trio appeared to be due to the increase of the bacteria-positive cells, suggesting a concentration-dependent binding of SP-A to the target cell (macrophage) or bacterium. Interestingly, previous studies (18) have shown that the thermal structural stability of the SP-A1, 6A2 allele product, is lower than that of the SP-A2. It is possible therefore that because of its lower structure stability, higher concentrations of 6A2 are required for adequate cell binding, and this may be expressed at higher protein concentrations as increased number of bacteria-positive cells.

### Table 2. Comparison of activity between each SP-A variant and hSP-A at a concentration of 25 μg/ml

<table>
<thead>
<tr>
<th>SP-A Variant</th>
<th>Compared with hSP-A</th>
<th>Bacteria-Positive Cells, %</th>
<th>Number of Bacteria, %</th>
<th>Phagocytic Index, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A0</td>
<td>70.44±6.73</td>
<td>60.25±5.53</td>
<td>40.78±4.04</td>
<td></td>
</tr>
<tr>
<td>6A2</td>
<td>58.46±4.26</td>
<td>43.53±4.30</td>
<td>25.17±2.08</td>
<td></td>
</tr>
<tr>
<td>1A0/6A2</td>
<td>52.77±3.65</td>
<td>37.02±4.74</td>
<td>18.50±1.65</td>
<td></td>
</tr>
<tr>
<td>1A</td>
<td>57.20±5.74</td>
<td>47.78±5.05</td>
<td>27.46±3.89</td>
<td></td>
</tr>
<tr>
<td>6A4</td>
<td>54.04±4.42</td>
<td>48.30±7.06</td>
<td>26.45±5.06</td>
<td></td>
</tr>
<tr>
<td>1A/6A4</td>
<td>56.18±1.85</td>
<td>60.22±10.57</td>
<td>33.80±5.89</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. The bacteria-positive cells, number of bacteria, and phagocytic index values were compared between each SP-A variant tested and the positive control human (h) SP-A at the same concentration, according to the following formula: SP-A variant:hSP-A (ratio) × 100% ± SE. The number of experiments for each test is shown in Fig. 2.
Interestingly, although the 1A0 variant exhibited higher activity compared with 1A, the coexpressed variant 1A0/6A2 was less active than the 1A/6A4. We speculate that the activity level for the coexpressed 1A0/6A2 and 1A/6A4 variants is modulated by the SP-A1 variant present in the SP-A1/SP-A2 oligomer. 6A4 exhibited higher activity than 6A2 when an overall comparison of the activity among the SP-A variants was carried out (Table 3). However, further experimentation is needed to better understand differences between SP-A1 or SP-A2 allele variants. The functional diversity among SP-A variants may have significance for host defense differences in the lung, whether the reference is made to the activity of the single gene products or the coexpressed products, and points to the importance of concentration and genotype. Of relevance, the differences in the regulation of SP-A1 and SP-A2 expression (30, 49, 56) have already been observed, and these regulatory differences may result in different proportions of SP-A1/SP-A2 among individuals. Moreover, quantitative or qualitative differences among SP-A variants may be further altered (e.g., magnified) in different lung microenvironments, as these may differ in various lung diseases. Thus, under some circumstances, the differences among SP-A variants may become significant factors in the success or failure of the first line of defense, which in turn may promote or attenuate unwanted complications (i.e., infection). A change in pH in lung microenvironment may, for example, alter the functional ability of SP-A. The airways in CF are acidified, and the pH decreases even more after an infective exacerbation (59). The phagocytic ability of SP-A, as assessed by its ability to promote uptake of microspheres by THP-1 cells, has been shown to be pH dependent (11). Therefore, the decrease in pH in CF or other diseased lungs may compromise the phagocytic activity of SP-A. However, it is currently unknown to what degree SP-A1 and SP-A2 activity is altered in response to changes in pH.

It has been proposed that the trimeric form of hSP-A consists of two SP-A1 molecules and one SP-A2 molecule (65). However, the ratio of SP-A1:SP-A2 mRNA among individuals differs considerably from the anticipated 2:1 ratio (31). If the mRNA levels are assumed to reflect protein levels, the variable SP-A1:SP-A2 mRNA ratio indicates that single gene products exist, and these may form functional homotrimers and/or homooligomers. Recent data support the latter notion. Single gene products have been shown to be functional in several assays, namely, in their ability to modulate cytokine production (68, 69), inhibit surfactant secretion (66), and enhance cell association (present study). Invariably, in all the functions tested, the SP-A1 variants exhibited lower activity than the SP-A2 variants. Studies of circular dichroic spectroscopy revealed differences between SP-A1 and SP-A2 in the midpoint transition melting temperature, with the SP-A2 being structurally more stable (18). Amino acid differences among SP-A alleles are located within the signal peptide, the collagen-like, and the carbohydrate recognition domain (CRD) regions of SP-A, and thus for functional studies, the collagen-like and CRD differences are most relevant. Although the CRD region is very important for the biological properties of SP-A, the SP-A1 (6A2) and SP-A2 (1A0) variants do not differ in the CRD region. Therefore, differences in the activity of SP-A1 and SP-A2 could be associated with other factors. The major SP-A1 and SP-A2 amino acid differences are located in the collagen-like domain. At amino acid 85 in the collagen domain, SP-A1 has a cysteine, whereas SP-A2 has an arginine. The extra cysteine of SP-A1 at position 85 may be involved in the formation of an SP-A intertrimeric or intratrimeric disulfide bond and account, in part, for differences in the oligomerization pattern between SP-A1 and SP-A2 variants (66). This, in turn, may reflect and account for the observed functional differences. We found recently that the majority of SP-A2 oligomers exist in the dimer and trimer forms, whereas the majority of SP-A1 were found in larger molecular forms (i.e., trimers and hexamers) (18, 66). However, it is unknown whether the smaller size SP-A2 oligomers are responsible for the observed higher activity. It is also possible that the substitution of Arg85 to Cys85 in the collagen domain of SP-A1 can affect the local stability on the triple helix and render the SP-A1 molecule less stable than SP-A2 (18). Whether differences in thermal stability, oligomer size, or other play a role in the observed functional differences between SP-A1 and SP-A2 and whether SP-A1 and SP-A2 gene products utilize different mechanisms to stimulate phagocytosis remain to be determined. However, what appears to clearly emerge is that SP-A variants exhibit an extensive diversity in their functional capability and that this, either alone or in response to other microenvironmental factors, may play an important role in disease susceptibility and contribute to the health or disease status of the individual.

In summary, our results show for the first time that the SP-A2 gene products are more effective in stimulating associ-
ation of *P. aeruginosa* with alveolar macrophages than the SP-A1/SP-A2 gene products (or homooligomers) and the SP-A1/SP-A2 gene products (or heterooligomers). We speculate that individuals whose lungs contain a higher amount of SP-A1 than SP-A2 allele products will in general be more susceptible to bacterial pneumonia caused by *P. aeruginosa*. Therefore, it is possible that knowledge of the SP-A genotype and the overall relative amounts of SP-A1/SP-A2 in the lung may help to predict the disease severity potential among different individuals.

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**GRANTS**

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**REFERENCES**


