Human Pulmonary Surfactant Protein (SP-A), a Protein Structurally Homologous to C1q, Can Enhance FcR- and CR1-mediated Phagocytosis*

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C1q, a subunit of the first component (C1) of the classical complement pathway, and the pulmonary surfactant protein SP-A are structurally homologous molecules, each having an extended collagen-like domain contiguous with a non-collagenous domain. It is the collagen-like region of C1q that binds to mononuclear phagocytes and mediates the enhancement of phagocytosis of opsonized particles by these cells. Because SP-A enhances the endocytosis of phospholipids by alveolar type II cells and alveolar macrophages, we examined whether these two molecules were functionally interchangeable. The phagocytosis of sheep erythrocytes opsonized with IgG or with IgM and complement was enhanced by the adherence of monocytes or macrophages, respectively, to SP-A. The enhanced response was dependent on the concentration of SP-A used for coating the surfaces, similar to that seen when monocytes were adhered to C1q-coated surfaces. Both the percentage of cells ingesting the opsonized targets and the number of targets ingested per cell increased with increasing concentrations of SP-A. No such enhancement was seen with cells adhered to albumin, iron-saturated transferrin, or uncoated surfaces. However, SP-A did not substitute for C1q in the formation of hemolytically active C1. C1q did not stimulate lipid uptake by alveolar type II cells or alveolar macrophages and had only a slight inhibitory effect on the binding of SP-A to alveolar type II cells. Thus, these results suggested that a function which requires interactions of both the collagenous and the non-collagenous regions (i.e., initiation of the classic complement cascade) could not be mimicked by a protein sharing structural macromolecular similarity but lacking sequence homology in the non-collagen-like region. However, SP-A could substitute for C1q in stimulating a function previously shown to be mediated by the collagen-like domains of the C1q molecule.

C1q is one of a small group of proteins that contain a region of collagen-like sequence contiguous with regions of non-collagenous amino acid sequences. SP-A, a major pulmonary surfactant protein (1), mannose-binding protein (2), core-specific lectin (3), acetylcholinesterase (4), and conglutinin (5) are the other proteins outside the collagens to have such a structure. C1q is a 460,000-dalton glycoprotein comprised of six subunits, each of which contains three distinct polypeptide chains (6). The NH2-terminal third of all three chains of C1q has an amino acid sequence and structure very similar to collagen (7), with repeating Gly-X-Y triplets and a high amount of proline and hydroxyproline. While C1q in serum is normally in association with C1r2s2 as the C1 complex, upon dissociation of C1r2s2 by C1 inhibitor after C1 activation by antibody-antigen complexes or other C1 activators (reviewed in Ref. 8), the collagen-like "tail" region of C1q becomes more exposed. We have shown that the region of C1q which is exposed upon dissociation of C1r2s2 from C1q can interact with specific cells of immunological importance: specifically B lymphocytes, monocytes, macrophages, polymorphonuclear leukocytes, and a small population of cells lacking B and T cell markers (9, 10). Other laboratories have demonstrated that lymphoblastoid cells, fibroblasts, and a variety of other cell types bind C1q (11–15). The physiologic significance of these receptors for C1q is currently unclear. However, surface-bound C1q stimulates oxidative metabolism in neutrophils (16), modulates FcR-mediated (17) and CR1-mediated (18, 19) phagocytosis in monocytes and macrophages, stimulates immunoglobulin production by B cells (20), and enhances fibroblast adherence (21).

Human (22, 23) and canine (24) SP-A have been cloned and the amino acid sequence derived from the sequenced cDNA. While slightly different sequences have been published for human SP-A (22, 23), it is yet unknown if these represent more than one gene for SP-A or genetic polymorphism. Like C1q, SP-A also has collagen-like sequence in the amino-terminal half of the protein, short NH2-terminal domains containing interchain disulfide bonds, and a break in the Gly-X-Y repeat pattern near the middle of the collagen-like sequence. At the amino acid sequence level these proteins are homologous in the collagen-like regions primarily due to the repeating Gly-X-Y triplet and a high percentage of proline/hydroxyproline. Voss et al. (25) recently showed that the macromolecular structure of SP-A is remarkably similar to the hexameric structure of C1q as visualized in the electron microscope. SP-A is suspected of having a role in the regulation of the level of lung surfactant, as it has been shown to enhance the uptake of lipids by isolated alveolar type II cells (26). Given the ability of C1q to modulate cellular phagocytic...
function and SP-A to enhance lipid uptake, we investigated the possibility that the common structural features of the collagen-like domains may provide a basis for common biological functions.

MATERIALS AND METHODS

Media, Reagents, and Antibodies—RPMI 1640 medium was purchased from Gibco and HL1 medium from Ventrex Laboratories (Portland, ME). Fetal bovine serum was purchased from Hyclone (Logan, Utah). L-Glutamine and gentamicin were obtained from M. A. Bioproducts (Walkersville, MD). The human serum albumin used in the phagocytosis assay and phorbol dibutyrate were purchased from Sigma. The human serum albumin used for the elutriation buffer was obtained from Travenol Laboratories, Inc., Glendale, CA. Medium and fetal bovine serum for the alveolar type I1 cell macrophage studies were obtained from the University of California Cell Culture Facility.

F(ab)\(^2\) fragments of a polyclonal goat anti-human C1q were prepared as described previously (10) except that octanoic acid was used to obtain the serum IgG fraction (27). The IgG fraction was affinity-purified by passage over Sepharose-C1q and elution with isothiocyanate. Anti-C1q antibodies were also obtained commercially from Atlantic Antibodies, Scarborough, ME (IgG fraction) and Genzyme (Boston, MA) (antiserum). Monoclonal antibodies to C1q were the generous gift of Drs. Verne Schumaker (University of California, Los Angeles) and Linda Curtis (Scrpps Clinic and Research Foundation, La Jolla, CA). Rabbit anti-SP-A antiserum was passed over Sepharose 4B-human serum and the IgG fraction isolated by DEAE-cellulose chromatography. Monoclonal antibodies to recombinant human SP-A were provided by Dr. Jan Marian and Jeanine Roderms (California Biotechnology, Incorporated, Mountain View, CA).

Protein Isolation—C1q was isolated from plasma-derived human serum by the method of Tenner et al. (28) modified as described.\(^2\) The preparations used were fully active as determined by hemolytic titration and homogeneous as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). SP-A was isolated from the lavage fluid of patients with alveolar proteinosis as described previously (26). Briefly, the surfactant was purified by density gradient centrifugation. The lipids were extracted with butanol. The butanol-insoluble proteins were extracted with 20 mM octylglycosylranoside to remove contaminating serum proteins. SP-A was solubilized in 5 mM Tris and dialyzed against the same solution. SP-A preparations were greater than 99% pure as assessed by densitometry.

The monomeric (34 kDa) and dimeric (68 kDa) forms of SP-A (43) from a representative preparation of SP-A are seen in Fig. 1. It has been previously determined that the NH\(_2\)-terminal (residues 1-22) amino acid sequence of SP-A from alveolar proteinosis patients is identical to that of normal human SP-A and that it has the ability to enhance lipid uptake by type II cells (22). For the binding assays, rat SP-A was isolated in an identical manner and iodinated with Enzyme (Bio-Rad).

Cells—Human peripheral blood monocytes were isolated by counterflow elutriation using a modification of the technique of Lionetti et al. (29) as described (30). Macrophages as defined here were elutriated monocytes that had been cultured in Teflon jars (Savillex Corporation, Minnetonka, MN) at 1 × 10\(^5\) cells/ml in HL-1 culture medium (a serum-free, defined medium) containing 2 mM L-glutamine and 10 \(\mu\)g/ml gentamicin. On days 2-6 of culture, macrophages were collected and washed three times in phosphate-buffered saline before use. Macrophages were assessed for the presence of myeloperoxidase by the use of a diaminobenzidine-based reaction (leukocyte peroxidase kit no. 391, Sigma). Freshly isolated alveolar type II cells were prepared by elastase digestion of rat lung. Cells were purified by differential adherence according to the methods of Dobbs et al. (31).

The type II cell preparations averaged 85-90% pure. Alveolar macrophages were isolated by lavaging rat lungs eight times with a buffered salt solution containing EGTA.

Phagocytosis Assay—Phagocytosis was assessed as described previously (18, 36). Sheep erythrocytes bearing IgG anti-sheep red blood cells (EA\(_{IgG}\)) or IgM anti-Forsmann antibody (EA\(_{IgM}\)) and C6b (EAC6b) were prepared as described previously (36). In experiments in which monocytes were cultured in HL-1, 10 ng/ml phorbol dibutyrate was added with the opsonized targets. This pretreatment of

\(^2\) K. R. Young, J. L. Ambrus, Jr., A. Malbran, A. S. Fauci, and A. J. Tenner, submitted for publication.

C1q

\[\text{SP-A}\]

\[\text{HL-1}\]

\[\text{EAlfi}\]

\[\text{EA,M}\]

\[\text{C4b}\]

\[\text{EAC4b}\]

\[\text{SP-A}\]

\[\text{E}\]

\[\text{E opsonized with IgM anti-Forssman}\]

\[\text{SP-A}

\[\text{Clq and 13 \(\mu\)g of SP-A were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions using a 5-15% gradient gel according to Laemmli (40). Molecular weights on right refer to standards (Bethesda Research Laboratories) run in the middle lane.}

HL-1-derived macrophages (which are less "activated" than serum-cultured macrophages) optimizes phagocytosis of complement-opsonized targets as elaborated previously (18). The number of E-targets ingested per 100 effector cells was defined as the phagocytic index, whereas the percentage of effector cells ingesting at least one E-target was defined as the percent phagocytosis. At least 200 effector cells were assessed per well, and each experiment used duplicate sample wells per condition. In some experiments, F(ab\(^\prime\))\(_2\) anti-C1q (in phosphate-buffered saline) was added to C1q- or SP-A-coated wells for 60 min at ambient temperature prior to effector cell addition. In each experiment, unopsonized E and E opsonized with IgM anti-Forsmann alone (E-IgM) were used as controls. These control E-targets were not ingested by macrophages or alveolar type II cells under any conditions.

Phospholipid Uptake Assay with Lung Cells in Suspension—Isolated alveolar type II cells or alveolar macrophages (2 × 10\(^4\)) were incubated in 1 ml of a minimal essential medium containing Krebs' improved salts. The cells were incubated at 37 °C for 60 min in room temperature air. Small unilamellar liposomes were prepared from surfactant-like lipids (Avanti Polar Lipids) consisting of dipalmitoylsphingomyelin, egg phosphatidylcholine, egg phosphatidylglycerol, and cholesterol in a molar ratio of 10:5:2:3. The liposomes were labeled with a trace amount of [\(^{14}\)C]dipalmitoylsphingomyelin. Proteins as designated were added to the cells after addition of liposomes. After an hour of incubation, the cells were separated from the media by centrifugation, washed twice by centrifugation, and analyzed for radioactivity and cell number. All of the methods are described in detail by Wright et al. (26).

Phospholipid Uptake with Adherent Lung Cells—Alveolar macrophages and isolated type II cells were reseeded in Dulbecco's modified Eagle's Medium (DME) containing 10% fetal bovine serum and 50 \(\mu\)g/ml gentamicin. Cells were plated in 24-well plates, at a density of 1 × 10\(^4\) cells/0.5 ml/well, that had been precoated with either SP-A or C1q or buffer (as in the phagocytosis experiments (18)). After 16 h, the cells were washed and 0.5 ml of DME containing 0.1% bovine serum albumin and liposomes with and without SP-A or C1q were added. After 60 min of incubation at 37 °C, cells were washed one time with DME and three times with Dulbecco's phosphate-buffered saline. Samples were solubilized in 1% sodium dodecyl sulfate and analyzed for radioactivity.

\[\text{SP-A Binding Assays—Labeled SP-A (1 \(\mu\)g/ml) was incubated with rat alveolar type II cells in DME containing 0.1% bovine serum albumin (10\(^6\) cells adhered per well in 24-well plate) at 4 °C for 60 min. For some samples an excess of unlabeled SP-A (50 \(\mu\)g/ml) or collagen type IV (Sigma), fibronectin, or C1q (100 \(\mu\)g/ml) was included in the incubation media. Cells were washed one time with...}
DME and three times with Dulbecco's phosphate-buffered saline. Samples were solubilized in 0.1 M NaOH and analyzed for radioactivity.

Hemolytic Assays—C1q hemolytic activity was determined by the method of Kolb et al. (41). The assays of C1 activity reconstituted from purified C1q,Clr, and Cls were performed basically as described by Rapp and Borsos (37) with the modifications described by Tenner and Frank (42). These assays were performed with or without preincubation of SP-A with C1q, C1r, and Cls.

RESULTS

SP-A Enhances the Ingestion of IgG-opsonized Targets by Human Monocytes—Previous studies have demonstrated that extracellular matrix proteins (32), C1q (17), and collagen type IV (33), but not collagen type I (17), enhance the phagocytosis of opsonized particles by mononuclear phagocytes. Therefore, the ability of SP-A to mediate a similar effect on phagocytosis was assessed. Human peripheral blood monocytes purified by counterflow elutriation were allowed to adhere to Lab-Tek chamber wells that had been precoated with increasing concentrations of C1q, SP-A, or iron-saturated transferrin. Data from one of three similar experiments, presented in Fig. 2, demonstrate that surface-bound SP-A can enhance Fc receptor-mediated phagocytosis in a concentration-dependent manner. This enhancement was seen both as an increase in the percentage of cells ingesting the target particles and as an increase in the number of targets ingested per cell.

Enhancement of Complement (CR1) Receptor-mediated Phagocytosis by SP-A—Fragments of the complement protein C3 have long been known to be important opsonins (34). The C3b receptor, CR1, on monocytes and macrophages is also capable of interacting with the complement protein C4b (35). Monoclonal antibody to CR1 completely blocks the interaction of EAC4b with these cells under conditions identical to the phagocytosis assays used here (36). Recently it has been demonstrated that the interaction of C1q with mononuclear cells enhances CR1-mediated phagocytosis (18). Fig. 3 demonstrates that adherence of culture-derived macrophages to surfaces coated with different preparations of the SP-A protein also results in the enhancement of CR1-mediated phagocytosis. The enhancement of phagocytosis was dose-dependent and similar to that seen with C1q. No such enhancement was seen when cells were adhered to iron-saturated transferrin, bovine serum albumin, or human serum albumin (data not shown).

In an attempt to investigate the specificity of the cell interaction site on these structurally homologous proteins, we tested whether a polyclonal antibody to C1q, which had been previously shown to inhibit the C1q-induced enhancement of phagocytosis, could also inhibit the SP-A-induced enhancement of phagocytosis. Interestingly, in two separate experiments, while the F(ab')2 anti-C1q inhibited both the percentage of cells ingesting targets and the phagocytic index by 81.5 ± 3.5% and 88.5 ± 4.9%, respectively, no inhibition of SP-A-enhanced phagocytosis was detected in the presence of this F(ab')2 anti-C1q (Fig. 4). In addition, a monoclonal antibody which inhibits C1q-mediated enhanced phagocytosis did not affect the enhanced response generated by surface-bound SP-A.

C1q Does Not Enhance the Uptake of Phospholipids by Isolated Alveolar Type II Cells or Macrophages—Given the above effect of SP-A on mononuclear cell phagocytic activity and the previously established ability of SP-A to enhance uptake of phospholipids by alveolar type II cells and macrophages (26), we investigated the possibility that a common element in the structure of these two proteins may be suffi-

2 D. A. Bobak and A. J. Tenner, unpublished observations.
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Table I

Effect of SP-A and Clq on uptake of phospholipids by isolated alveolar type II cells and macrophages

| Protein | Type II cells | Macrophages |
|---------|---------------|-------------|
| No added protein | 100 | 100 |
| SP-A | | |
| 10 µg/ml | 261 (±55) | 364 (±73) |
| 30 µg/ml | 418 (±76) | 662 (±90) |
| Clq | | |
| 10 µg/ml | 94 (±7) | 129 (±18) |
| 30 µg/ml | 85 (±6) | 86 (±10) |
| 50 µg/ml | 97 (±5) | 90 (±5) |
| 100 µg/ml | 75 (±5) | 86 (±5) |

Table II

Inhibition of binding of SP-A to type II cells

| Protein | Binding (% inhibition) |
|---------|------------------------|
| SP-A | 74 (±5) |
| Clq | 15 (±5) |
| Collagen | 40 (±4) |
| Fibrinogen | 19 (±8) |

C1q resulted in maximal lysis (data not shown). In an alternative Cl assay (37) of the inhibition of C1 formation in which C1rSs was equimolar with C1q (rather than in excess as in the previous assay), SP-A consistently inhibited C1 hemolytic activity, suggesting competition of SP-A and C1q for C1rSs. However, this inhibition of hemolytic C1 formation was relatively weak in that a 20-fold excess of SP-A over C1q produced only 26–56% inhibition of the control hemolytic activity (i.e. in the absence of SP-A).

Analysis of Reactivity of Anti-C1q and Anti-SP-A with C1q and SP-A—Given the ability of SP-A to enhance the FcR- and CR1-mediated phagocytosis, we screened available polyclonal and monoclonal antibodies reactive with C1q or SP-A for their reactivity with SP-A and C1q, respectively. By dot blot analysis no antibody tested was found to cross-react with both proteins (Fig. 5). It is known that the affinity-purified anti-C1q is largely reactive with the collagen-like region of C1q, and that two of the monoclonals reactive with intact SP-A are not reactive with the collagenase-resistant fragment.

Discussion

C1q and pulmonary surfactant protein, SP-A, are structurally homologous proteins. The data presented in this paper indicate that in vitro SP-A can enhance FcR-mediated phagocytosis by monocytes and macrophages and CR1-mediated phagocytosis by macrophages, activities previously shown to be modulated by C1q as well as some components of extracellular matrix (32). However, SP-A can not substitute for C1q in the formation of hemolytically active C1, the recognition component of the classical complement pathway. C1q does not mimic the ability of SP-A to enhance the uptake of phospholipids by isolated alveolar type II cells or macrophages and has only a slight inhibitory effect on the binding of SP-A to alveolar type II cells. These observations are consistent with what is known about the homology between C1q and SP-A and the specific domains involved in the mediation of function by these proteins.

The homology between C1q and SP-A exists at the macromolecular level in that electron microscopy (25) demonstrated a nearly identical image of the two proteins as six globular domains connected by short arms to a common stem, and at the amino acid sequence level by repeating Gly-X-Y triplets and a high percentage of proline in the NH2-terminal region of the polypeptide chains. Excluding the common glycine residues in the collagen-like region of the molecules, 15, 21, and 20% of the amino acid residues of the C1q A, B, and C chains, respectively, are identical to the SP-A sequence. In contrast, the non-collagen-like region of C1q is not homologous to the non-collagenous region of SP-A. The isoelectric points of the intact proteins are also quite different, C1q being highly basic (pl > 10), whereas SP-A is acidic (pl = 4.6–5.0) (39). Thus, it is not surprising that SP-A does not cause complement-mediated hemolysis, as the globular (non-collagenous) region of C1q contains the interaction site for the immunoglobulin bound to the red cells. However, SP-A does interfere, albeit weakly, with the efficient assembly of C1, which involves the interaction of C1rSs with the collagen-like domain of C1q. The observation that C1q was unable to mediate phospholipid uptake by alveolar type II cells or alveolar macrophages is consistent with the recent evidence of Wright and colleagues (44) demonstrating that the collagenase-resistant fragment of SP-A (i.e. the portion lacking homology with C1q) binds to alveolar type II cells.

1. S. L. Robinson and A. J. Tenner, unpublished data.
2. J. R. Wright, unpublished data.
A third protein which is similarly homologous to C1q is the cation-dependent, mannose-binding protein/core-specific lectin (2, 3). Analysis by Drickamer et al. (2) has shown this protein to be homologous with SP-A in the non-collagenous as well as the collagen-like region. Mannose-binding protein binds to mannose-containing structures on pathogenic organisms and has already been shown to play a role in cellular mechanisms and has already been shown to play a role in cellular phagocytic functions. Mannose-binding protein, and, by analogy, SP-A, which also hosts. Similarly, Clq, in addition to its role in the initiation of immune cell function, can be speculated that the "recognition" of a specific target (Fc, mannose, phospholipid, etc.) is mediated by nonhomologous regions of the proteins, while the collagen-like regions mediate phagocytic functions.

Interestingly, in this study none of the antibodies to C1q or SP-A, including both polyclonal and monoclonal antibodies, cross-reacted with both of these proteins. Furthermore, neither the affinity-purified polyclonal anti-C1q nor a monoclonal anti-C1q known to interact with the collagen-like domain of C1q and to block the phagocytosis-enhancing effect of C1q inhibited the effect of SP-A on phagocytosis of either IgG or complement-opsonized targets. This would suggest that this monoclonal antibody and the polyclonal anti-C1q contain antibody that binds near, but not at, the cell binding residues of the C1q molecule, thereby sterically hindering ligand/receptor interaction. Alternatively, the cell interaction domains of C1q and SP-A may not be identical, or the proteins may act through different receptors. Nevertheless, while as yet there is no evidence that the cell interaction domains of any of these ligands are identical, the possibility exists that receptors for these molecules in the cell membrane may share common features and, in fact, may constitute a family of proteins.

It should be noted that, because neither C1q nor SP-A was required to be present on the particle being ingested in the phagocytic assays described above (Figs. 2 and 3), these molecules can function as activation ligands rather than merely as opsonic ligands which mediate binding of the targets to the cell, as discussed by Brown (32). Importantly, iron-saturated transferrin, which interacts with a cell membrane receptor, does not mediate this enhancement of function, indicating that this is not due to a non-specific ligand/receptor interaction (Fig. 2). Further definition of the specific receptor interaction domains on each of the proteins which share a macro-molecular structural homology and are able to trigger phagocytosis will ultimately determine the structural features required for a ligand to trigger this cellular function. Whether the involved structures are identical among the different proteins and whether the mechanism of signal transduction for each ligand/receptor system is similar should provide insight for future potential manipulations of these systems.

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Fig. 5. Dot blot analysis of reactivity of anti-C1q and anti-SP-A with C1q and SP-A. Purified human SP-A or purified human C1q was blotted onto nitrocellulose (amount applied indicated in the left margin of Blot A and on top of Blot B, except for columns 11 and 12). Antibodies indicated in the keys were added to the appropriate wells (in duplicate). Second antibodies were peroxidase-conjugated, and the blot was developed using horseradish peroxidase substrate 4-chloro-1-naphthol. (Black marks in bottom left-hand corners of A and B and top right-hand corner of A are ink position markers.) In Blot B, to control for C1q reactivity, no antigen (A), 500 ng of SP-A (B, C), 1000 mg of SP-A (D, E), 500 ng of C1q (F, G), or 1000 ng of C1q (H) was blotted to nitrocellulose and subsequently probed with the anti-C1q antibodies (column 11, Atlantic Antibodies anti-C1q; column 12, affinity purified anti-C1q) as described above.
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