Serum amyloid A (SAA) is the major acute phase protein in man and most mammals. We observed SAA binding to a surprisingly large number of Gram-negative bacteria, including Escherichia coli, Salmonella typhimurium, Shigella flexneri, Klebsiella pneumoniae, Vibrio cholerae, and Pseudomonas aeruginosa. The binding was found to be high affinity and rapid. Importantly, this binding was not inhibited by high density lipoprotein with which SAA is normally complexed in serum. Binding was also observed when bacteria were offered serum containing SAA. Ligand blots following SDS-PAGE or two-dimensional gels revealed two major ligands of 29 and 35 kDa that bound SAA when probing with radiolabeled SAA or SAA and monoclonal anti-SAA. Following fractionation the ligand was found in the outer membrane fraction of E. coli and was identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry to be outer membrane protein A (OmpA). OmpA-deficient E. coli did not bind SAA, and following purification of OmpA the protein retained binding activity. The ligands on other bacteria were likely to be homologues of OmpA because wild type, but not OprF-deficient, P. aeruginosa bound SAA.

During systemic inflammation the most notable change in protein synthesis involves hepatic synthesis of a set of proteins with roles in homeostasis or protection from the cause of the inflammation, most notably infection. Among these proteins the two that show the highest -fold increase are C-reactive protein and serum amyloid A (SAA).1 Human SAA is a family of proteins that includes two acute phase forms (SAA1 and SAA2) as well as a constitutive form (SAA4) (1). The acute phase forms have an approximate molecular mass of 12 kDa with no disulfide bonds and for which no structure has yet been elucidated and no clear function ascribed.

SAA is found in mammals and other vertebrates such as marsupials and fish (1) but more adventitiously in the sea cucumber, an echinoderm, where it is expressed in the coelomic epithelium and shows induction in response to lipopolysaccharide (2). In mammals during inflammation SAA1 and 2 are synthesized largely by hepatocytes and can comprise more than 2% of total protein synthesis (3), with the result that concentrations in plasma may increase from 1–5 μg/ml to approach 1 mg/ml (1). SAA of all isoforms associates with high density lipoprotein (HDL), in particular subfraction 3 (4). This interaction has lead to suggestions that the role of SAA may relate to one or more of the properties of HDL and lipid metabolism. It would in fact be surprising if responses were not seen when SAA becomes the major apolipoprotein, as is the case during severe inflammation. Indeed, a number of changes have been demonstrated (1), but studies have yet to demonstrate a convincing role. Alternative studies have examined direct effects of SAA on a number of immune cells. For instance, SAA has been reported to cause chemotaxis and activation of several cell types (5–7), but HDL can inhibit these responses (5, 6) leaving it uncertain whether this occurs in vivo. SAA has also been reported to induce a number of other cellular responses, including collagenase production (8) and induction of secretory phospholipase A2 (9). Previously described binding properties include interaction with a number of cell types such as platelets and T cells (10, 11) as well as binding to extracellular matrix glycoproteins such as laminin (11, 12) and the proteoglycans heparin and heparan sulfate (13).

In this report we have demonstrated that SAA binds to a range of Gram-negative bacteria and that this reactivity is not inhibited by HDL or serum. Ligand was bound when present in whole bacteria as well as in lysate preparations. Following fractionation of Escherichia coli the ligand was found in an outer membrane fraction and was shown by mass spectrometry to be outer membrane protein A. This is a protein found in almost all Gram-negative bacteria, which opens up the possibility that SAA may be a pattern recognition protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—SAA in this study was the isoform SAA1, prepared as described (14) with the modification that the first stage of purification on octyl-Sepharose 4B was performed in the presence of 10 mM EDTA. SAA was ~98% pure as determined by SDS-PAGE and comparable with recombinant human SAA (Peprotech). Recombinant human SAA is a chimeric protein comprising human SAA1 with 3 amino acid replacements/additions. SAA was dissolved in 4 M urea and dialyzed extensively against distilled water before addition to medium for experiments. SAA was radiolabeled with carrier-free 125I using the N-bromosuccinimide method (15) to specific activities between 70–120 kBq/μg. It was stored in PBS containing 1% bovine serum albumin and used within 48 h. SAA was freshly dialyzed from 4 M urea into distilled water o/n before incorporation into experimental media in all studies. HDL and SAA-depleted plasma were obtained by affinity chromatography from human plasma using columns generated from activated CH-Sepharose (Amersham Biosciences) coupled to affinity-purified goat polyclonal anti-SAA (provided by Prof. K. P. W. J. McAdam) or rabbit polyclonal anti-apoAI (Binding Site, Birmingham, UK) using protocols provided by the manufacturer.

**Bacteria**—Streptococcus pneumoniae (serotype 3) and Pseudomonas aeruginosa strains (18S, a lab strain, and 7/1, a clinical strain) were kindly provided by Dr. S. H. Gillespie (Department of Microbiology, Royal Free Hospital, London) and Dr. T. Pitt (Central Public Health/
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Laboratory, Colindale, London), respectively. OmpA-deficient strain of E. coli was provided by Dr. R. Koebnik (Institute of Genetics, Martin-Luther-University, Halle, Germany) and OprF-deficient strain of P. aeruginosa was provided by Dr. H. E. Gilleland (Department of Microbiology and Immunology, Louisiana State University Health Sciences Center). Other organisms were obtained from P. Donachie (Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine). E. coli, P. aeruginosa, Salmonella typhimurium, Burkholderia cenocepacia, Vibrio cholerae, and Klebsiella pneumoniae were cultured in brain heart infusion broth at 37 °C for 18 h until stationary phase. Bacteria were harvested or washed with 2000 × g for 10 min. S. pneumoniae was cultured on blood agar plates in an atmosphere containing 10% v/v CO₂ at 37 °C.

SAA Binding Assays—Live bacteria (10⁶) were incubated with SAA at 0.01–5 μg/ml in PBS or plasma at various dilutions in PBS at 4 °C for 1 h followed by washing with PBS, followed by addition of 2 μg/ml monoclonal anti-human SAA (clone 513; Mississauga, Canada) or control for 1 h at 4 °C. Cells were washed three times, and F(ab')₂ goat anti-mouse IgG (Fluorescein isothiocyanate (Bio-Rad) diluted 1:50 was added for 1 h at 4 °C. Bacteria were washed three times and fixed with 4% (v/v) paraformaldehyde for 30 min at 4 °C and analyzed by FACs Calibur (BD Biociences). A minimum of 10,000 events were analyzed using Cell Quest software. In the time course experiment, bacteria were fixed with 4% (v/v) paraformaldehyde for 30 min and washed extensively before the binding assay.

Quantitation of radiolabeled SAA binding was performed by adding 170 μl of a 60:40 mixture of dibutyl phthalate/diethyl phthalate (Fischer Scientific) to 0.4 ml propylene tubes together with 100 μl of an upper aqueous layer of PBS containing 0.2% (v/v) bovine serum albumin. To this upper layer was added 2 × 10⁶ bacteria and 10⁻⁸-SAA at the concentration indicated. Following incubation at 4 °C for 1 h, or as stated, the bacteria were centrifuged through the oil layer (5 min, 12,000 × g) to a pellet for γ-counting. Aliquots of supernatant were counted to determine free SAA concentrations.

SAA Ligand Blotting—SDS-PAGE (10% acrylamide, 1:19 bisacrylamide) of bacterial lysates at 10⁵ bacteria/lane was ejected for 170 s in acrylamide:bisacrylamide) and 8 M dithiothreitol was performed. Gels were electroblotted onto polyvinylidene difluoride membrane and blocked with 1% (w/v) bovine serum albumin. Orange, tryptic digestion, and matrix-assisted laser desorption ionization time-of-flight analysis. Peptides were analyzed using Mascot (Dr. Lamont, University of Dundee).

OmpA Purification—OmpA was purified by sonication of E. coli BL21 in NaCl 0.15 M, 1 mg/ml E64, 2 mg phenylmethylsulfonyl fluoride, 1 mM pepstatin A, 1 mM EDTA and centrifugation for 15 min at 36,000 × g. This provided a pellet that was re-extracted in the same buffer and then extracted with 8 × urea, 1 mM EDTA, 25 μM phenylmethylsulfonyl fluoride, 1% Nonidet P-40 in 10 mM Heps, pH 7.4. Following centrifugation for 15 min at 30,000 × g the pellet was extracted with 8 × urea, 2 mM thiourea, 1 μg/ml E64, 2 mM phenylmethylsulfonyl fluoride, 0.1 μg/ml leupeptin in 10 mM Tris-HCl, pH 8.0, following by centrifugation at 36,000 × g for 15 min. The supernatant was further purified by anion exchange chromatography on a mono-Q HR5/5 column with loading buffer 8 × urea, 2 mM thiourea, and 15 mM Tris-HCl, pH 8.0. Elution buffer was the same with 0.3 M NaCl introduced and thiourea excluded.

RESULTS

Using centrifugation of bacteria through a phthalate oil layer, we demonstrated that ¹²⁵I-labeled purified human SAA1 bound strongly to a variety of Gram-negative bacteria in suspension, including E. coli, S. typhimurium, Shigella flexneri, P. aeruginosa, and K. pneumoniae. In contrast, little binding was seen to the Gram-positive organisms S. pneumoniae (serotype 3) or Staphylococcus aureus or to certain Gram-negative organisms such as B. cepacia (Fig. 1a). Other Gram-negative bacteria also bound, such as Serratia marcescens and V. cholerae. The unencapsulated form of S. pneumoniae R36A also failed to bind. When radiolabeled SAA was added to the bacteria and bound radiolabel separated from free by washing the bacteria, the recovered radiolabel had a molecular mass of 12 kDa. A recombinant version of human SAA (similar to SAA1) containing 3 amino acid substitutions also bound to the same range of bacteria using the same methodology (data not shown). Binding was rapid and reached equilibrium within 15 min (Fig. 1b). When nonspecific binding (obtained by addition of 20-fold excess unlabeled SAA) was subtracted from the specific binding, saturation of binding was approached at the highest concentrations of SAA added (Fig. 1c). Scatchard analysis indicated a dissociation constant of ~1 × 10⁻⁷ M for P. aeruginosa. FACS analysis for SAA binding using monoclonal anti-human SAA (Fig. 2a) confirmed these results and showed unlabeled SAA also bound to bacteria. Using this approach and various concentrations of SAA to examine binding to P. aeruginosa, maximal binding was observed at ~1 μg/ml (Fig. 2b). Calculations of K₅ for these data using double reciprocal plots revealed a value of ~5 × 10⁻⁸ M, similar to that observed using radiolabeled SAA. Affinity of SAA binding varied according to the bacteria, with E. coli and V. cholerae having apparently higher affinity than some other bacteria such as P. aeruginosa. Thus the concentration of SAA required for half maximal binding was within physiological normal levels of SAA and did not require acute phase levels.

Because SAA circulates in HDL, HDL was purified by affinity chromatography using antibody to apolipoprotein-A1 and was introduced into radiolabeled SAA binding assays using the phthalate oil technique such that SAA would represent ~15% of total protein, typical of a strong acute phase response. The presence of HDL did not significantly decrease the binding of SAA to P. aeruginosa (Fig. 3). If HDL were purified by ultracentrifugation then this also failed to cause significant inhibition of the binding of SAA to the same organisms despite the presence of low levels of SAA in the normal plasma from which the HDL was generated (data not shown). Following this, we demonstrated that SAA in plasma binds to bacteria using FACS. The amount of SAA binding from acute phase sera, which originally contained 100 μg/ml SAA and was diluted in PBS 10-fold so that it contained 10 μg/ml SAA (Fig. 4b), was greater than seen with normal sera diluted in the same way so
that it contained 0.5 μg/ml SAA (Fig. 4a). This was consistent with dose responses obtained with purified SAA. When the SAA was depleted from these sera by passage through an anti-SAA column, the SAA-depleted plasma showed reduced binding to \textit{P. aeruginosa} compared with both normal and acute phase plasma from which it was derived (Fig. 4, a and b). Failure to completely remove SAA following passage through the affinity column (SAA was reduced to 65 or 15 ng/ml in depleted acute phase or normal serum, respectively, as measured by enzyme-linked immunosorbent assay) led to the residual SAA being sufficient to generate low levels of binding. Again this was consistent with the dose response for purified SAA (Fig. 2b). Similar results were obtained using another strain of the same organism.

**FIG. 1.** Radiolabeled SAA binds to various Gram-negative bacteria. \(a\), \({}^{125}\text{I}-\text{SAA} (1 \mu\text{g/ml})\) was incubated with \(2 \times 10^8\) live bacteria (\textit{S. pneumoniae}, \textit{S. aureus}, \textit{B. cepaciae}, \textit{E. coli}, \textit{K. pneumoniae}, \textit{P. aeruginosa 18S}, \textit{S. typhimurium}, and \textit{S. flexeri}), centrifuged through a phthalate oil layer, and the pellet counted. Mean ± S.E. of four determinations. \(b\), \(2 \times 10^8\) fixed \textit{P. aeruginosa 18S} were incubated with \({}^{125}\text{I}-\text{SAA} (1 \mu\text{g/ml})\) and binding analyzed by phthalate oil technique at various times up to 1 h at 4 °C. Mean ± S.E. four replicates. \(c\), dose response of specific binding of \({}^{125}\text{I}-\text{SAA}\) to \(2 \times 10^8\) \textit{P. aeruginosa 18S} (C) or 7/1 (C). Binding was examined by phthalate oil technique following incubation for 1 h at 4 °C. Each point represents the total counts minus counts in the presence of 20-fold excess unlabeled SAA and represents the mean of four replicates.

**FIG. 2.** SAA binds to Gram-negative bacteria. \(a\), binding of SAA to various bacteria was determined by FACS. Bacteria were incubated with (open histogram) or without 1 μg/ml SAA (filled histogram). Binding was demonstrated using anti-SAA and fluorescein isothiocyanate-labeled F(ab')₂ anti-mouse IgG. 20,000 events were captured by FACS. \(b\), binding of SAA at various concentrations to \textit{P. aeruginosa 18S} was determined as above and expressed as mean fluorescence intensity.

**FIG. 3.** HDL does not inhibit SAA binding to bacteria. Radiolabeled SAA (50 ng/ml) was incubated with \textit{S. typhimurium} or \textit{P. aeruginosa} or without (control) bacteria in the presence (open bars) or absence (filled bars) of HDL at 6-fold protein excess. Bound SAA was separated from free by centrifugation of bacteria through a phthalate oil layer, counted, and expressed as molecules/bacterium. Data show mean ± S.E. \((n = 8)\).
SAA Binds to OmpA

We then sought to identify the bacterial ligand responsible for SAA binding. Ligand blotting of whole bacterial lysates following SDS-PAGE with $^{125}$I-SAA revealed bands at $29$ and $35$ kDa for *P. aeruginosa* but not *S. pneumoniae* (Fig. 5a). Bands of similar molecular mass were observed for various Gram-negative bacteria, including *E. coli*. The same specificity was observed if unlabeled SAA and monoclonal anti-SAA were used to determine binding to ligands (data not shown). *E. coli* was fractionated by sonication, followed by freeze/thawing, centrifugation, and extraction with urea/thiourea buffer. The various fractions of bacteria were examined by ligand blotting, and ligand was identified mainly in the outer membrane fractions. The purified fraction of outer membrane revealed that ligands of 29 and 35 kDa had a pI of $4.5-5.5$ (Fig. 5b), whereas no spots were observed in the absence of SAA. Because this fraction contained major bands at 29 and 35 kDa by protein staining, matrix-assisted laser desorption ionization time-of-flight analysis of these ligands from one-dimensional gels revealed the ligand identity as the surface protein OmpA since this was indeed the major ligand on *E. coli* (Fig. 8). The failure of the OprF-deficient bacteria to bind suggested that this was indeed the major ligand on *P. aeruginosa*.

Fig. 4. SAA in plasma binds to bacteria. *a*, SAA in normal plasma binds to *P. aeruginosa*. Depletion of SAA from plasma reduces SAA binding to bacteria. *P. aeruginosa* 18S was incubated without plasma (open dotted histogram), normal plasma containing SAA at 0.5 $\mu$g/ml (open histogram), or SAA-depleted normal plasma (15 ng/ml SAA; shaded histogram). *b*, SAA in acute phase plasma binds to *P. aeruginosa*. Depletion of SAA from acute phase plasma reduces SAA binding to bacteria. *P. aeruginosa* 18S was incubated without plasma (open dotted histogram), with acute phase plasma containing SAA at 10 $\mu$g/ml (open histogram), or with SAA-depleted acute phase plasma (63 ng/ml SAA; shaded histogram).

Fig. 5. SAA binds to ligands at 29 and 35 kDa in lysates and outer membrane fractions. *a*, SAA binds to 29- and 35-kDa ligands in bacterial lysates separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Lysates of *S. pneumoniae* (lane 1) and *P. aeruginosa* (lane 2) were boiled for 2 min. Blots were probed with $^{125}$I-SAA (5 $\mu$g/ml). *b*, ligand was found in an outer membrane fraction of *E. coli*. The pellet obtained after extraction of the outer membrane fraction with 8M urea was re-extracted with 8M urea, 2M thiourea, and 4% CHAPS. Following centrifugation the supernatant was found to contain ligand as demonstrated at 29- and 35 kDa with pI 4.5-5.5 by two-dimensional electrophoresis and blotting with SAA (5 $\mu$g/ml) and immunodetection with monoclonal anti-SAA and horseradish peroxidase-conjugated goat Fab’2 anti-mouse IgG using chemiluminescence. Control blots without SAA revealed no comparable staining.

We were interested to know whether this protein ligand was also responsible for binding to other bacteria. OmpA is expressed in *S. typhimurium*, *V. cholerae*, and *K. pneumoniae*, which is likely to account for the binding of SAA seen with these bacteria. Many other Gram-negative bacteria have homologues of this protein (16–18). In *P. aeruginosa* the homologue is OprF, and we thus investigated the OprF-deficient mutant and wild type for SAA binding using FACS analysis (Fig. 8). The failure of the OprF-deficient bacteria to bind suggested that this was indeed the major ligand on *P. aeruginosa*.

DISCUSSION

The OmpA/OprF family are conserved proteins among the Enterobacteriaceae; related members are found in almost all Gram-negative bacteria (16–18). They are involved in the maintenance of structural membrane integrity and likely porin activity. The OmpA/OprF molecule consists of a periplasmic C-terminal region that is involved in peptidoglycan binding and anchoring of the outer membrane and an N-terminal domain arranged as a 170-amino acid S-transmembrane $\beta$-barrel (19). The C-terminal region is highly conserved as is the trans-
membrane region of the N-terminal domain (20). However, the external loops are proposed to be highly mobile and extend beyond the outer membrane (21) and are highly variable within the Enterobacteriaceae (19). However, several studies have indicated that an alternative minority conformer of the protein exists as a 16-stranded transmembrane form with a pore size of ~1 nm with regions of the conserved C-terminal domain presented externally (22–24). Because OmpA has been reported to exist at up to 100,000 copies/bacterium it may well be that the SAA binds to the C-terminal region, which is only surface expressed in ~2–3% of the total OmpA (22). The failure to bind to B. cepacia may be related to the lack of an OmpA/OprF member or to a divergent sequence because a close homologue was not observed by sequence analysis of the bacterial genome. Studies should now be directed to determining which part of the ligand is responsible for binding.

Although studies to examine SAA binding to bacteria have been restricted in the report to SAA1, the fact that recombinant SAA also bound suggests that it would be interesting to examine SAA2 for its ability to bind to bacteria. Human SAA2 only differs from SAA1 in a few amino acids (~7, depending on isoform). The constitutive form SAA4 could have similar properties, although these proteins are less easy to work with because of greater hydrophobicity and reduced solubility. OmpA is involved in a number of bacterial functions in addition to linking peptidoglycan to the outer membrane. These include having porin activity, albeit inefficiently. Properties vary between bacteria (16). It is not clear what role OmpA plays in invasion and survival within cells; it may depend on the context, because OmpA-deficient bacteria have been reported to survive better in macrophages (25) and it has been shown that neutrophil elastase can attack OmpA (26). However, OmpA has been shown to be required for interaction and for crossing the brain microvascular endothelial cell barrier (27, 28), and OmpA may reduce complement-mediated attack (29). Further studies will be needed to determine whether this interaction has a role in innate immunity. It will be informative to investigate whether the binding of SAA to various bacteria will lead to increased or decreased responses because OmpA has been reported to activate macrophages, dendritic cells (30, 31), and endothelial cells through a grp96-like receptor (27, 28). However, SAA could equally generate responses through receptors that have been reported for SAA. Currently four potential candidates for the SAA receptor have been suggested, including Tanis (32) (a membrane selenoprotein), RAGE (33) (the receptor for advanced glycation end product that binds to amyloidogenic forms of SAA), and FPRL1 (5) (a chemotactic 7-transmembrane receptor). Most recently SR-B1 was found to be capable of interacting with SAA both as a free molecule and as a component of HDL (34, 35). These do not represent the only potential ways in which SAA might link bacteria with host cells as binding is also reported to laminin and vitronectin (11) and interactions with the integrin receptor αIIbβ3 on platelets have
been reported (10). It is clear though that some of these interactions would be predicted to have innate immune consequences for innate immune cells such as macrophages and neutrophils.

Previous studies showed that SAA synthesis in the normal non-inflamed state was largely epithelial (36) and that SAA was induced in mouse intestinal epithelium in response to normal mucosal enteric bacteria (37). Thus SAA is produced in a site that needs protection against Gram-negative bacteria. The interaction of SAA with bacteria could have a direct effect on bacteria or bacterial adherence. But because SAA has been reported under some conditions to activate immune cells, the question of whether SAA opsonization has any ability to help in clearing bacteria or bacterial adherence. But because SAA has been reported under some conditions to activate immune cells, the question of whether SAA opsonization of bacteria changes the responses of host immune cells becomes an important one. In particular, an obvious line of investigation will be to examine whether SAA opsonization has any ability to help in clearing bacteria by phagocytes, which is primarily important for an efficient response to combat bacterial infection.

Thus we have shown that SAA binds strongly to a range of Gram-negative bacteria and that the ligand OmpA may well be a pathogen-associated molecular pattern because it is conserved across the Gram-negative bacteria. The important features of this reaction are that it occurs in the presence of HDL and has sufficiently high affinity to occur at normal concentrations of SAA.

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