Neutrophils exhibit a dramatic enhancement of integrin-mediated cell adhesion in response to lipopolysaccharide (LPS). This response requires CD14 on the neutrophil and plasma proteins in solution. We have purified the factor from plasma that facilitates the adhesive response of neutrophil to LPS by using a combination of affinity and ion-exchange chromatography. Previous work has shown that the activity is associated with apolipoprotein A-I (apoA-I), and here we show that this activity is associated with an apoA-I-bearing complex of protein and phospholipid. Native polyacrylamide gel electrophoresis (PAGE) analysis showed a ladder of bands in the M, 200,000 region, and electron microscopy revealed round, indented particles of 11.4 ± 0.12 nm in diameter. Characterization of these particles revealed a density of 1.219–1.264 g/ml and ~10 molecules of lipid phosphate per M, 200,000 complex. SDS-PAGE showed that each of the bands seen in native PAGE was composed of several polypeptides. These were identified as apoA-I, LPS binding protein (LBP), and factor H-related proteins (FHRPs). Physical association of apoA-I, LBP, and FHRP in these particles was further confirmed using double immunodiffusion, and association of LBP and FHRP in plasma was confirmed by coimmunoprecipitation. FHRPs are the numerically dominant protein components in these particles, and all plasma FHRP-1 appears to be associated with these particles. We suggest that FHRPs may be the defining constituent of this novel "lipoprotein" particle.

Bacterial lipopolysaccharide (LPS, endotoxin) initiates profound responses in leukocytes. For example, polymorphonuclear leukocytes (PMNs) exhibit strongly enhanced integrin-mediated adhesion within 10 min of exposure to nanogram/ml concentrations of LPS (1–4). This adhesive response is thought to underlie the dramatic movement of PMN from the blood into tissues that accompanies endotoxemia (5–7). CD14 is a glycosylphosphatidylinositol (GPI)-linked protein present on monocytes and PMN that binds LPS (1) and plays a crucial role in mediating cellular responses to LPS (8). Blockade of CD14 with monoclonal antibodies strongly reduces many cellular responses to LPS (8, 9) and completely eliminates the adhesive response of PMN to LPS (1–3).

Spontaneous binding of LPS to CD14 occurs only slowly but can be dramatically enhanced by LBP (10), a lipid transfer protein present in plasma (1). As a result, PMNs exhibit little if any response to LPS alone, but responses are strong and rapid in the presence of LBP (1, 3). LBP acts catalytically to transfer single LPS molecules from LPS micelles to a binding site on CD14 (1). The catalytic action of LBP is consonant with its sequence similarity to cholesterol ester transfer protein and phospholipid transfer protein (11), well characterized plasma lipid transfer proteins. Cholesterol ester transfer protein and phospholipid transfer protein are both found on the surface of high density lipoprotein (HDL) particles (12, 13), and recent observations from our laboratory indicate that LBP is associated with apoA-I, the principal protein of HDL (14).

There are many minor subclasses of HDL with distinct physical characteristics and specialized functions. Examples include apolipoprotein J-containing lipoproteins (15), trypanosome lytic factor (16–18), and very high density lipoprotein (19–21). Here we have purified the proteins from plasma that facilitate CD14-dependent responses of PMN to LPS. We found that this activity is borne on a very high density particle composed of phospholipids, apoA-I, LBP, and FH-related proteins 1 and 2 (FHRP-1 and FHRP-2). Additional proteins yet to be characterized are also present on the particle. The physical properties of this particle are described herein.

**EXPERIMENTAL PROCEDURES**

Reagents—Fresh frozen normal human plasma (citrated) was obtained from the New York Blood Center (New York, NY). HiPAC-Aldehyde chromatographic resin was obtained from Chromatochem (Missoula, MT). Phosphatase (plasmaglobulin-free plasma phosphatase) was purchased from Calbiochem (San Diego, CA). Recombinant LBP and rabbit polyclonal anti-recombinant LBP were as described (1), monoclonal antibody against LBP (17G4) was a generous gift from Dr. David Emanuel (Indianapolis, IN). Polyclonal anti-FH antibody and anti-apoA-I antisera were obtained from Incstar (Stillwater, MN). 3D11, a mouse monoclonal antibody that reacts with FH and FHRP-1 (22), was a generous gift from Dr. Vesa Koistinen (Helsinki, Finland). RelPS was obtained from List Biological Laboratories (Campbell, CA). Goat anti-rabbit IgG and rabbit anti-mouse IgG conjugated with alkaline phosphatase were purchased from Bio-Rad, and rabbit anti-goat IgG was purchased from Incstar. Ouchterlony diffusion plates were purchased from The Binding Site (Birmingham, United Kingdom). Purified apoA-I and complement FH were purchased from Incstar and Calbiochem, respectively.

Chromatography—Four units of fresh frozen plasma were thawed at room temperature, pooled, aliquoted, and frozen at −70 °C until analyzed further. To begin an experiment, aliquots were thawed at 37 °C and immediately placed on ice. Subsequent manipulation of the plasma...
Factor H-related protein-associated lipoprotein particle

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was done strictly either on ice or at low temperature (4 °C). Plasma was spun in a Sorvall SS-34 rotor at 17,000 rpm for 20 min and passed through a Sephadex G-25 (Pharmacia Biotech Inc.) column prior to chromatography. HiPAC-Aldehyde resin was packed into a HR 5/20 or 10/30 FPLC column (Pharmacia) and equilibrated with starting buffer (Dulbecco’s PBS without calcium and magnesium, 1 mM EDTA, and 0.05% sodium azide). Samples were loaded onto a Mono Q (Pharmacia) column at a rate of 2 ml/min. The column was washed with starting buffer until the absorbance (A280) came down to baseline line. Flow-through fractions were collected and stored for further analysis. Adsorbed material was eluted into 20 ml with 0.5 M ammonium acetate buffer, pH 3.0, immediately subjected to dialysis against 20 ml Tris, pH 8.0 or 8.5 overnight at 4 °C and loaded onto a Mono Q (Pharmacia) column at a rate of 2 ml/min. The column was washed with starting buffer until the absorbance (A280) came down to baseline line and eluted with a gradient of NaCl from 0 to 1 M at 4 ml/min with a fraction size of 5 ml. Each fraction was tested for activity by the PMN adhesion assay described below. In some experiments, the active fractions were chromatographed again on the Mono Q HR 5/5 column to obtain purer and/or a more concentrated sample. Ceramic hydroxyapatite (Bio-Rad) was packed in a FPLC HR5/5 column (Pharmacia), and chromatography was done using 10 mM potassium phosphate buffer, pH 6.8, as starting buffer. The sample was loaded, washed with starting buffer, and eluted with a gradient of potassium phosphate from 10–400 mM over 40 ml at a rate of 1 ml/min. One-ml fractions were collected.

All results shown used frozen plasma. Identical chromatographic elution patterns, SDS, and native PAGE profile and Western blotting images were obtained using plasma from freshly drawn blood (never frozen; data not shown).

Response of PMN to LPS—The enhancement of leukocyte integrin-mediated cell adhesion by LPS was measured using a two-step assay as described earlier (2). Briefly, freshly isolated PMN were labeled with 3–5 μM of carboxyfluorescein diacetate, succinimidyl ester (Molecular Probes, Eugene, OR); PMNs were then incubated with stimuli for 10 min at 37 °C. Stimuli usually consisted of a fixed dose of LPS (10 ng/ml) and varying doses of plasma or a fraction of plasma to be assayed. The PMNs were then washed and transferred to Terasaki plates coated with fibrinogen. Adhesion of the PMNs was determined by reading the fluorescence with a fluorescence plate reader (Cytofluor 2300; Millipore, Bedford, MA) before and after washing, and the percentage of PMNs adherent on the plate was calculated. All assays were done in triplicate.

Lipid Analysis—Lipid was extracted from the Mono Q-purified active sample with chloroform and methanol according to the method of Bligh and Dyer (23). Phospholipids were determined in the extracted sample according to the method of Ames and Dubois (24). Concentrations of triglycerides and total cholesterol (cholesterol plus cholesterol ester) were determined using an enzymatic method using a commercial kit (Sigma).

Density Determination—The density of the particle was determined by ultracentrifugation (25). Mono-Q-purified active samples were mixed with a series of various densities to yield final densities of 1.006, 1.019, 1.065, 1.119, 1.219, and 1.264 g/ml. The samples were centrifuged in a Beckman Ti 42 rotor for 5 h at 4 °C at 40,000 rpm, conditions calculated to bring lipoproteins to an equilibrium position (26). At the end of the spin, the top and bottom 30 μl of the 230-μl sample were analyzed for the presence of protein by BCA protein assay. As a control, purified human plasma low density lipoprotein (density < 1.060 g/ml) was analyzed in parallel.

Enzyme-linked Immunosorbent Assay—A sandwich assay for LBP was done as described previously (14). Briefly, plates were coated with anti-LBP monoclonal antibody 17G4 at 5 μg/ml of the 230-μl sample were analyzed for the presence of protein by BCA protein assay (Pierce). As a control, purified human plasma low density lipoprotein (density < 1.060 g/ml) was analyzed in parallel.

RESULTS

Incubation of PMN with 10 ng/ml LPS alone results in no enhancement of integrin-mediated adhesion. However, the addition of normal human plasma (NHP) enables a strong LPS- and CD14-dependent adhesion response (1–4), and the dose dependence of this effect of plasma is shown in Fig. 1B. We sought the plasma factors that mediate this response to LPS and have found a chromatographic resin (HiPAC-Aldehyde) that can purify the activity from NHP. NHP (5 ml) was loaded onto the HiPAC-Aldehyde column (HR 5/20), and the flow-through fraction was assayed for the ability to promote the adhesion of PMN to fibrinogen by LPS. Although it contained 99% of plasma proteins, it was completely devoid of activity. The column was eluted with low pH buffer, and more than 90% of the original activity was recovered in the eluate (Fig. 1 and Table I). When this process was scaled up to utilize 50 ml of NHP on a HR 10/30 column somewhat less activity (65%) was recovered. The eluate of the HiPAC-Aldehyde column was further fractionated by ion-exchange chromatography on Mono Q. Activity was recovered as a single peak, at about 150 mN NaCl (Fig. 2). Active fractions were pooled and analyzed. We confirmed that this purified fraction stimulated cells in an LPS-dependent fashion and that the activity was completely blocked.
by monoclonal antibody against CD14 (Fig. 2C). Table I shows that about 700-fold enrichment was achieved using two steps of chromatography.

Further chromatography on ceramic hydroxyapatite yielded a column profile with a single broad peak of protein and biological activity, and these peaks coincided closely (Fig. 3). SDS-PAGE gel profiles of the material in this peak appeared identical to those of the starting material (data not shown). This observation and additional analytical data described below suggest that the preparation obtained from the Mono Q column is relatively homogeneous.

On native PAGE gradient gels, the active purified fraction from plasma resolved into four or more discrete, evenly spaced bands in the interval from Mr 150,000–250,000 (Fig. 4B). A small and variable amount of protein was also observed in the Mr; 60,000 region. To verify that the species represented in the Mr 150,000–250,000 region of native gels are active in enabling responses of PMN to LPS, a native gel was run and cut into eight pieces; then electroelution of each gel piece was done as described under "Experimental Procedures." Assay of each eluted sample showed that all of the activity fell in the Mr 200,000 region (Fig. 5), confirming that activity was associated with species in this region. Isoelectric focusing analysis of the purified fraction showed a tight ladder of six to seven bands with pI of 6.5–7.3 (Fig. 4B). The simplicity of its isoelectric focusing and native PAGE profiles suggests that our preparation is relatively homogeneous. Electron microscopy of negatively stained samples revealed particles of discoid shape with an indentation in the center, some forming rouleaux (Fig. 4A). Measurement of 100 particles revealed an average diameter of 11.4 ± 0.12 nm. This size is consistent with the molecular weight estimation from native gels.

Our previous studies showed that the factor in plasma necessary for enabling responses of PMN to LPS is associated with

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**Table I**

Purification of a plasma factor that mediates PMN adhesion in response to LPS

| Total | Yield | Specific activity | Purification |
|-------|-------|------------------|--------------|
| mg prot. | % | units/mg | fold |
| NHP | 8500 | 100 | 0.0058 | 1 |
| HiPAC-Aldehyde | 83.3 | 90 | 0.54 | 93 |
| Desalting | 75 | 57.6 | 0.38 | 65 |
| Mono Q | 5.13 | 30 | 3.87 | 667 |

*One unit is defined as the amount of sample required for half-maximal PMN adhesion under standard assay conditions (see "Experimental Procedures").

**Fig. 1.** HI-PAC-Aldehyde purification of the plasma factor that mediates PMN adhesion in response to LPS. NHP (8 ml) was applied to a HI-PAC-Aldehyde HR 5/20 FPLC column and eluted as described under "Experimental Procedures." Fractions were tested for the ability to mediate PMN adhesion in response to LPS. In A, a fixed dose of 2% NHP (P), HI-PAC-Aldehyde column eluate (E), or flow-through (F) was assayed in the presence or absence of 10 ng/ml LPS. Background adhesion with buffer alone (B) is also shown. In B, the dose dependence of the response mediated by 10 ng/ml LPS and starting NHP (●), eluate (●), and flow-through (▲) was measured after equivalent dilutions. Each data point is the average of three samples; bars, S.D. This result was seen in more than five experiments.

**Fig. 2.** Mono Q purification of the plasma factor that mediates PMN adhesion in response to LPS. In A, the eluate from a HI-PAC-Aldehyde column was loaded onto a Mono Q column after buffer exchange (into 20 mM Tris, pH 8.0). Elution was done using a NaCl gradient up to 1 M. In B, the histogram shows the results of a PMN adhesion assay performed with pooled pairs of fractions. Very little protein and no activity appeared in flow-through fractions. More than 90% of the activity applied was recovered from fractions 17–20. In the assay shown, the maximum adhesion with 2% NHP was 23.9 ± 2.3% and with buffer alone was 2.0 ± 0.6%. In C, active fractions from Mono Q were pooled and analyzed on a PMN adhesion assay in the presence or absence of monoclonal anti-CD14 antibody, 3C10. Each column represents: 1, no antibody; 2, 3C10; 3, 3G8, monoclonal anti-FcγIII antibody; and 4, without active Mono Q fractions. Anti-CD14 antibody, 3C10, completely blocked the activity of active Mono Q fractions.
apoA-I (14), and we thus sought evidence that our preparation contains lipids. Lipids were extracted, and phosphate analysis of the extract showed 13.8 nmol of phosphate per 318 nmol of protein. Measurement by enzymatic assay revealed, to our surprise, no detectable cholesterol or cholesterol ester (n = 3). Our assays would have detected as little as 1.7% cholesterol of total protein weight. Similarly, triglycerides were either not detected (n = 2) or were at the limit of detection (n = 1), suggesting that the particles contain less than 2.7% triglycerides of total protein weight. The density of our particles was determined as between 1.219 and 1.264 g/ml. This high density and high protein:lipid ratio of our particle is observed in very high density lipoprotein particles.

SDS-PAGE showed that our preparation contained several protein bands (Fig. 4B). These include a triplet of bands near Mr 85,000 (p85), bands of Mr 60,000 and Mr 50,000 (p60, p50), a Mr 38,000/35,000 doublet (p38/35), a Mr 30,000/28,000 doublet (p30/28), and a Mr 27,000 band (p27). The Mr 30,000/28,000 bands were seen in variable amounts, depending on the source of NHP. All of the other bands were reproducibly obtained in more than 10 separate preparations, although some minor variation was noted in relative intensity of each band. Electroleuted samples from the Mr 200,000 area of a native gel gave rise to an identical SDS gel pattern, except for the absence of p50 (Fig. 5, inset), indicating that each of the bands seen in the SDS-PAGE derive from Mr 200,000 particles except p50. Additionally, two-dimensional gel analysis, with native PAGE in the first dimension and SDS-PAGE in the second, showed that each of the native gel species in the Mr 150,000–250,000 range gave rise to an almost identical pattern of polypeptides in the second dimension (Fig. 6).

Thus, we have isolated a complex of lipid and protein that mediates responses of PMN to LPS. The properties of the complex are most similar to very high density lipoprotein. We have identified most of the polypeptides by a combination of immunoochemistry and protein sequencing.

LBP—LBP is known to enable PMN to respond to LPS (3), and the presence of LBP in purified fractions was explored by Western blot analysis with antibodies raised against recombiant LBP. A Mr 60,000 band was detected in a position identical with that of purified recombinant LBP (data not shown) and with p60 of our sample (Fig. 7). An enzyme-linked immunosorbent assay for LBP showed that there were 12 μg of LBP in a 150 μg/ml preparation of the particle. This finding indicates that 8% of the protein in our preparation is LBP and suggests that LBP comprises the Mr 60,000 band in our preparation. Enzyme-linked immunosorbent assay analysis further showed that LBP was only found in the active fractions from the HiPAC-Aldehyde and Mono Q columns.

apoA-I—Recent experiments from our laboratory have shown that LBP and its biological activity are quantitatively retained on columns of anti-apoA-I (14). We, therefore, used Western blot analysis to determine if apoA-I was present in our purified fraction. A Mr 27,000 band (p27), which corresponds to the size of apoA-I, was recognized by anti-apoA-I, both in the fractions purified from the Mono Q column (Fig. 7) and in the samples electroleuted from the HiPAC-Aldehyde column. A monoclonal antibody, 3D11, directed against the C-terminal region of FH, has been shown to cross-react with FHRP-1 and Mono Q columns.

Factor H-related Proteins—p38/35 and p30/28 were characterized by N-terminal amino acid sequencing (Table II). The N-terminal sequence of both bands at p38/35 showed identity to the N terminus of FHRP-1, and the N-terminal sequence of p30/28 showed identity to that of FHRP-2. FHRPs are a family of abundant plasma proteins with unknown function, purified and cloned based on homology to complement FH (28). They show gel behavior identical to that reported for FHRP-1 and FHRP-2 (29). (b) Polyclonal anti-FH, which is known to cross-react with FHRP-1 and FHRP-2, recognized p38/35 and p30/28 in Western blot (data not shown). (c) A monoclonal antibody, 3D11, directed against the C-terminal region of FH, has been shown to cross-react with FHRP-1 (22, 31). We have
In PAGE, isoelectric focusing, and SDS (nonreducing)-PAGE as described in Experimental Procedures. Uniform, round, indented particles were seen (white arrow). Bar, 75.7 nm. Inset, enlarged images of single particles. In B, the Mono Q-purified fraction was analyzed by native PAGE, isoelectric focusing, and SDS (nonreducing)-PAGE as described under "Experimental Procedures." Silver-stained gels are shown.

Additional observations support the conclusion that FHRP-1 and FHRP-2 are associated with the particles described here. Western blots of plasma before and after chromatography on HiPAC-Aldehyde column showed that all of the FHRP-1 in plasma was retained in the column and was eluted with ammonium acetate, pH 3.0 (Fig. 8A). Western blot analysis of the entire Mono Q profile probed with anti-FH antibody showed bands consistent with FHRP-1 only in the fractions with biological activity (Fig. 8B). This finding suggests that FHRP-1 is quantitatively associated with the active species described here and may be a marker for this particle. The published purification of FHRP from NHP (29) used sequential chromatography of FHRP from NHP (29) used sequential chromatography and the proteins associated with a complex of phospholipid and other proteins in plasma and that this complex mediates responses of cells to LPS. Our findings both indicate the form of circulating FHRPs and suggest a role for FHRP in carrying and/or regulating the function of LBP.

**DISCUSSION**

FHRPs were discovered as proteins with sequence homology and antigenic cross-reactivity with complement FH (28). At the RNA level, at least six distinct transcripts with homology to FH can be detected by Northern blot analysis of human liver RNA (32). Thus far, three (FHRP-1, FHRP-2, and FHRP-3) have been cloned (30, 32–34). At the protein level, anti-FH antibody detects at least 10 bands in addition to FH in Western blots of plasma (28). Thus far, message has been linked to protein bands only for FHRP-1 and FHRP-2. No function has been described for FHRPs. FHRP-1 consists of five tandem repeats of a 60-amino acid motif known as the short consensus repeat. This motif is also found in complement regulatory proteins such as FH, complement receptor 1, and C4b-binding protein, in the adhesion proteins known as selectins, in the LPS-binding protein of horseshoe crab known as factor C (35), as well as in several other proteins (36). Published data have not described an association of FHRPs with lipoprotein, but a related protein composed of six short consensus repeats known as β2-glycoprotein I (also called apolipoprotein H) is known to associate both with HDL particles and with phospholipids (37, 38). Here we show that FHRP-1 and FHRP-2 are associated with a complex of phospholipid and other proteins in plasma and that this complex mediates responses of cells to LPS. Our findings both indicate the form of circulating FHRPs and suggest a role for FHRP in carrying and/or regulating the function of LBP.

FHRP-1 is the dominant protein component of the particle described here. FHRP-1 appears severalfold more abundant than either apoA-I or LBP, both in silver-stained (Fig. 4C) or Coomassie-stained (data not shown) gels. The relative abundance of FHRPs over LBP can also be inferred from the finding that essentially all plasma LBP (data not shown) and FHRPs (Fig. 8) are found in these particles, but the reported plasma level for FHRP-1 (40 μg/ml; Ref. 29) is 8-fold greater than that reported for LBP (5 μg/ml; Ref. 39). Additionally, Ouchterlony double diffusion analysis suggests that although all LBP and all apoA-I in the purified particles are associated with FHRPs, not all of the FHRPs are associated with either LBP or apoA-I (Fig. 9). This observation is most compatible with the hypothesis that LBP and apoA-I are present on a subpopulation of particles that share FHRPs as a common constituent. Since FHRPs are the dominant species in these particles and since FHRPs are found only in these particles, we will refer to them as "FHRP-associated lipoprotein particles" (FALPs). FALPs represent a very small proportion of plasma lipopro-
tein. By assuming a 30% yield in purification, we calculate that these particles contain no more than 0.9% of plasma phospholipid and 0.7% of plasma apoA-I. They are thus a minor subpopulation of lipoproteins and unlikely to play a large role in the transport of bulk nutrients. FALPs also exhibit considerable structural heterogeneity. This is best seen in native PAGE separations, which reveal several distinct species (Fig. 4B). The structural basis of this heterogeneity is not clear at this time. It is important to point out that we have not identified the triplet of bands at Mr ~ 85,000, and these relatively abundant proteins may play an important role in the structure and heterogeneity of FALPs.

The precise function of FALPs is not clear at this time, but the association with LBPs suggest that FALP proteins could affect LBP action. Interaction of LBP with plasma proteins and modulation of LBP action by plasma proteins are suggested by previous work. We have shown that plasma enables responses to LPS with characteristics different from those of purified LBP. Under defined conditions, an LPS-dependent response mediated by certain chromatographic fractions of plasma can be strongly enhanced by the addition of other chromatographic fractions, suggesting that multiple components may participate in presentation of LPS to cells in plasma (40). Moreover, addition of the protease inhibitor Pefabloc SC strongly blocks LPS activation of PMNs mediated by plasma, whereas activation mediated by purified LBP is not affected by this compound (40). These observations prompted us to suggest that in whole plasma, LPS is presented to cellsurface CD14 by the combined action of several proteins and proposed the name “septin” to describe this activity. The results described here support the close interaction of LBP with other proteins and suggest that components of FALPs are candidates that may serve to regulate its activity. This suggestion is strengthened by preliminary studies indicating that Pefabloc SC blocks the ability of FALPs to enable a response of PMNs to LPS (data not shown). On the other hand, it is now clear that the activity we termed septin may result from the combined activity of soluble CD14, lipoprotein particles, and FALPs. Since these individual contributors have pre-existing names and functions not uniquely related to

### Table II

| N-terminal amino acid sequence from p38/35 and p30/28 compared to FHRP-1 (32, 33) and FHRP-2 (30) |
|------------------------------------------------|
| FHRP-1 | EATFCDFPKINHGL |
| TP38   | EATFXDFPKINHGL |
| TP35   | EATFXDFPKINHGL |
| FHRP-2 | EAMCFDFPKINHGL |
| TP30   | EAMFXDFPKINHGL |
| TP28   | EAMFXDFPKINHGL |

* X represents an undetermined amino acid.
endotoxin and sepsis, we believe term "septin" is unnecessary and have discontinued its use.

In summary, we have purified a novel complex of protein and phospholipid that bears LBP and FHPRPs. The function of this particle is currently under study.

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