Human Alveolar Macrophage Fibronectin:

Synthesis, Secretion, and Ultrastructural Localization during

Gelatin-coated Latex Particle Binding

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ABSTRACT   Human pulmonary alveolar macrophages synthesized and secreted several characteristic high molecular weight proteins for at least 7 d in vitro. Immunoprecipitates of medium and cell lysates from metabolically labeled cultures with specific anti-human plasma fibronectin IgG contained one major labeled polypeptide of molecular weight 440,000 (unreduced) or 220,000 (reduced). An identical polypeptide in conditioned medium from radiolabeled macrophages bound specifically to gelatin-Sepharose, demonstrating that alveolar macrophages synthesized and secreted a molecule immunologically and functionally similar to fibronectin. Fibronectin was the major newly synthesized and secreted polypeptide of freshly harvested alveolar macrophages. Pulse-chase experiments revealed that newly synthesized fibronectin was rapidly secreted into medium, ~50% appearing by 1 h and 80% by 8 h. Immunoperoxidase staining using antifibronectin F(ab')$_2$-peroxidase conjugates revealed the majority of immunoreactive fibronectin to be intracellular, localized to endoplasmic reticulum and Golgi apparatus. No extracellular matrix fibronectin was visualized, and cell surface staining was rarely seen, usually appearing only at sites where cells were closely apposed and not at sites of macrophage-substrate attachment. Similar immunostaining of fibroblast cultures revealed cell surface-associated fibrillar fibronectin. Ultrastructural localization of fibronectin during binding and phagocytosis of gelatin-coated and plain latex particles revealed fibronectin only on gelatin-latex beads and at their cell binding sites. Neither plain latex beads nor their cell membrane binding sites stained for fibronectin. These results demonstrate that fibronectin is a major product of human alveolar macrophages, is rapidly secreted, and is localized at cell membrane binding sites for gelatin-coated particles. In view of the known binding properties of fibronectin, it may serve as an endogenous opsonic factor promoting the binding of *Staphylococcus*, denatured collagen, fibrin, or other macromolecules to macrophages in the lower respiratory tract.

Fibronectin (FN) is a circulating plasma glycoprotein that enhances binding of macromolecules and particulate material to phagocytic cells of the reticuloendothelial system (25, 34). Plasma FN (cold-insoluble globulin) increases binding of gelatin (22) and fibrin (23) to rodent peritoneal macrophages in vitro. Binding of gelatin-coated particles by human blood monocytes (3) and uptake of gelatin-coated beads by rodent

peritoneal macrophages (11, 17) is also dependent on FN. In addition, plasma FN mediates binding of *Staphylococcus* to human polymorphonuclear leukocytes (43).

In the lung, alveolar macrophages (PAM) play a key role in the uptake of particulate debris and microorganisms from the terminal airways and alveoli (14). However, unlike blood monocytes and peritoneal macrophages, PAM residing in their

normal anatomic location in vivo lack access to high concentrations of plasma-derived opsonic proteins such as FN.[1] Thus, the synthesis of an endogenous opsonic factor by PAM is of potential importance to local clearance mechanisms and host defense in the lung. As a first approach to defining the role of FN in PAM, we studied its synthesis, secretion, and ultrastructural localization in both nonphagocytizing adherent PAM as well as during binding and phagocytizing of gelatin-coated and plain latex particles. Whereas reports of FN synthesis by other phagocytic cells have been published (1, 24, 47), we provide the first detailed biochemical and morphological analysis of endogenous FN synthesis by a phagocytic cell.

MATERIALS AND METHODS

PAM Culture

Human PAM were obtained from healthy smoking and nonsmoking volunteers under informed consent by subsegmental lavage of the right middle lobe (44). The recovered cells (average yield 81 × 10^6 cells from smokers, 15.1 × 10^6 cells from nonsmokers) were washed twice by centrifugation (500 g, 10 min) in Dulbecco's modified Eagle's medium (DMEM) and resuspended in DMEM at 1-2 × 10^6 cells per ml. Cell suspensions were plated in 35-mm tissue-culture dishes (Falcon #3001; Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) or 24 well tissue culture plates (Linbro #76-033-05; Linbro Division, Flow Laboratories, Inc., Hamden, Conn.) and, for histological staining, in Lab-Tek eight-chamber tissue culture slides (Lab-Tek Products, Div. Miles Laboratories, Inc., Naperville, Ill.), incubated for 1 h at 37°C in a 5% CO2 air atmosphere and rinsed twice to remove nonadherent cells. Adherent PAM were cultured in DMEM containing 10% fetal bovine serum (KC Biological, Inc., Lenexa, Kans.) depleted of FN by gelatin-Sepharose chromatography (32), 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml amphotericin-D. Culture medium was changed every 2 or 3 d. Viability of adherent cells was <95%, based upon trypan blue exclusion. Virtually all cells possessed Fc receptors, phagocytized latex beads, and were nonspecific esterase positive (48). No colonies of cells resembling fibroblasts were seen in our PAM cultures from normal volunteers (10).

Fibronectin Antiserum

Antiserum against pure human plasma FN (32) was raised in rabbits (titer 1:128,000 by radioimmunoassay). Anti-FN IgG was isolated by affinity purification on human plasma FN–Sepharose (49) and preimmune IgG by DEAE cellulose chromatography (12). Specificity of the affinity-purified anti-FN IgG was established as follows: (a) Immunodiffusion gave a single line of identity against human plasma or pure FN, and no precipitin reaction against FN-depleted plasma. (b) Immunoprecipitation of human plasma, cell lysates, or culture medium from metabolically labeled human lung fibroblast cultures (IMR-90) gave one polypeptide of molecular weight 440,000 (unreduced) or 220,000 (reduced) (see below). (c) Immunofluorescence and immunoperoxidase staining of cultured human lung fibroblasts revealed typical fibrillar extracellular staining (5, 19, 31, 49), and (d) Preincubation of the anti-FN IgG with pure human plasma FN abolished immunostaining and immunoprecipitation reactions noted above.

Preparation of Gelatin-coated Latex Beads

Gelatin (ICN Nutritional Biochemicals, Cleveland, Ohio) was washed with ice-cold saline and covalently bound to polystyrene beads (1.1 μm diameter, Sigma Chemical Co., St. Louis, Mo.) by published methods (11, 34). A 10% (wt/vol) suspension of beads (0.5 ml) was washed twice in 150 mM NaCl and harvested by centrifugation (12,000 g, 30 min). Solutions of 1-cyclohexyl-3(2-morpholinoethyl)-carbodiimide-meta-p-toluene sulfonate (45 μg in 0.1 ml of 150 mM NaCl; Sigma Chemical Co.) and gelatin (160 μg in 0.1 ml of 150 mM NaCl) were added to the beads and the mixture incubated for 30 min at room temperature with shaking. Nonreacted sites were blocked by the addition of 4 mg of gelatin. After an additional incubation for 30 min, the beads were washed five times with Dulbecco's phosphate-buffered saline (PBS), harvested by centrifugation, and finally resuspended in 4.5 ml of PBS. Plain latex beads were identically washed and collected.

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[1] Villiger, B., G. Heymach, T. Broekelmann, D. Kelley, and J. A. McDonald. Bronchoalveolar fibronectin in smokers and nonsmokers. Manuscript submitted for publication.

Metabolic Labeling and Immunoprecipitation

PAM, typically 2 × 10^6 cells/35-mm dish, were preincubated for 30 min with DMEM without cystine and methionine and rinsed with PBS. PAM were labeled with 1.0 ml of DMEM lacking unlabeled cystine and methionine and supplemented with 50 μg/ml of ovalbumin and 10 μCi each of [^35S]cystine and [^35S]methionine. After labeling, medium was removed, chilled to 0°C, and centrifuged (1,000 g, 20 min), and proteinase inhibitors (PI; 10 mM disodium ethylenediaminetetraacetate, 10 mM N-ethylmaleimide, 2.5 mM phenylmethylsulfonyl fluoride) were added to the supernate. Unless specified, all subsequent steps were carried out at 0°C to minimize proteolysis. The cell layer was rinsed with PBS and scraped into 1.0 ml of 7.5 mM Tris-HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 1.0% Tween-20, 50 μg/ml ovalbumin, pH 7.4 at 25°C containing PI (buffer A), and the cell suspension was freeze-thawed three times. Medium and cell lysates were centrifuged (15,000 g, 1 h), and the soluble supernates were processed immediately or frozen at −80°C. In some experiments, we used ^14C-labeled amino acid mixture (New England Nuclear, Boston, Mass.) in DMEM containing 1.0% of the usual amino acid content in place of [^35S]cystine and [^35S]methionine.

To immunoprecipitate labeled FN we added anti-FN IgG or preimmune IgG (15 μg/ml, final concentration) to medium and cell lysates, and incubated them for 30 min at 37°C and overnight at 4°C. Immunoglobulin-bound antigen was precipitated by adding 50 μl of a 10% suspension of formalin-fixed, heat-killed Cowan strain S. aureus (IgSORB^R; Enzyme Center, Boston, Mass.) and incubating at 37°C for 30 min. IgSORB^R was pelleted by centrifugation (10 min, 2,500 g) and washed three times with buffer A, and bound labeled proteins were eluted by heating in SDS gel sample buffer (32) for 5 min at 100°C. Analysis of labeled polypeptides was performed by SDS PAGE and autofluorography exactly as previously described (32). We used double antibody immunoprecipitation with goat anti-rabbit IgG in place of IgSORB^R (39). Both methods quantitatively removed exogenous labeled FN introduced into samples.

Labeled proteins were quantified by densitometric scanning of autofluorograms using a Zeineh soft-laser densitometer (LKB Instruments, Inc., Rockville, Md.) (26, 39). Incorporation of label into nondialyzable polypeptides was determined by adding SDS to a final concentration of 2% to medium and cell lysates, and PI to the same concentration as in buffer A. Samples were boiled for 5 min and dialyzed (Spectra/Por 2, putative molecular weight cutoff 12,000–14,000; Spectrum Medical Industries, Inc., Los Angeles, Calif.) exhaustively against 0.2% SDS. Incorporated counts per minute were determined by liquid scintillation spectrometry. Dialyzed samples were lyophilized, and labeled peptides were displayed by SDS PAGE autofluorography. Where indicated, labeled samples were precipitated and washed with 10% TCA and dissolved in hot SDS PAGE sample buffer for SDS PAGE (32).

Pulse-chase experiments were carried out in a manner similar to continuous labeling experiments, but the PAM cultures were incubated in cystine- and methionine-free DMEM for 45 min, and then labeled with 20 μCi/ml of each isotope for 30 min. After removal of labeling medium, cells were rinsed with DMEM and incubated in DMEM plus 50 μg/ml ovalbumin for the indicated period, and cell layers and medium were processed for immunoprecipitation as above.

Immunocytochemistry

F(ab')2 fragments of anti-FN IgG and preimmune IgG were obtained by pepsin digestion and Sephadex G-100 chromatography (13). F(ab')2 were conjugated to horseradish peroxidase (type VII; Sigma Chemical Co.) using a two-step method (40), and resulting F(ab')2-peroxidase conjugates isolated by Sephacryl S-300 chromatography. Molecular weights of F(ab')2 and conjugates were determined by SDS PAGE as above. F(ab')2 were fluoresceinated with dichlorotriazinylaminofluorescein (4) to a molar ratio of 2:1 fluorescein:F(ab')2.

Cells were fixed with periodate-lysine-paraformaldehyde (PLP) (33) for 2 h at 4°C, washed with PBS plus PI, incubated with F(ab')2-peroxidase conjugates for 30 min at 4°C, and washed with PBS plus PI for 1 h at 25°C. Bound conjugates were detected by incubation with 0.05% diaminobenzidine/0.01% H2O2/50 mM Tris-HCl, pH 7.4 for 10 min. Where indicated, cells were permeabilized with 0.05% Triton X-100 (electron microscopy) or ice-cold acetone (light microscopy) before incubation with conjugates.

Light microscopy was performed on cells mounted in 95% glycerol/5% PBS, using an Olympus BH microscope. Light micrographs were taken with Kodak Plus-X film, using the same light intensity and exposure for each experimental treatment to allow comparison between intact and permeabilized cells incubated with control or immune conjugates. Negatives were printed using identical conditions to allow direct comparison of different experimental treatments. Stained cells were processed for electron microscopy by washing in 0.1 M cacodylate buffer, postfixing in 1% OsO4 for 30 min, graded dehydration, embedding, and thin sectioning. Specimens were examined with a Philips EM 200 microscope.

Immunoelectron Microscope Visualization of Binding and Uptake of Plain and Gelatin-coated Latex Beads by PAM

PAM were cultured in 24-well culture dishes (4 × 10⁵ cells per well) as described above, washed three times with PBS and incubated with 0.5 ml of serum-free DMEM. Gelatin-coated or plain latex beads (20 μl of a 1% [wt/vol] suspension) were added and incubated for 1 h at 37°C. After incubation, culture medium was aspirated and the cells were washed six times with cold PBS to remove nonbound beads. Cells were then fixed with PLP fixative, incubated with anti-FN or preimmune F(ab')₂-peroxidase conjugates, and processed for immunoelectron microscopy as described above.

PAM FN Gelatin Binding

Medium from metabolically labeled PAM was treated with PI and incubated with gelatin-Sepharose Cl-4B or cyanogen bromide-activated and ethanolamine-blocked control beads (32) for 1 h at 25°C with constant tumbling, using a ratio of 2:1 vol/vol medium:beads. Beads were washed with buffer A lacking deoxycholate by repeated vortexing and sedimentation until the supernate was free of radioactivity. Bound protein was eluted with 8 M urea in 50 mM Tris-HCl, pH 7.4, and displayed by SDS PAGE and autofluorography.

RESULTS

Protein Synthesis by Human PAM In Vitro

Human PAM are readily maintained in vitro in serum containing medium for periods up to 54 d (7). However, although biochemical, functional, and histological observations have been performed on human PAM in vitro (see references 20 and 35 and references therein), little information about the effect of culture conditions upon protein synthesis and secretion are available. Dog and rabbit PAM synthesize and secrete metabolically labeled proteins in vitro, but the rate of protein synthesis decreased rapidly with labeling periods >1–2 h (28, 30). Moreover, PAM maintained in suspension appeared to be less active metabolically than adherent PAM (27). Because PAM are usually adherent in vivo (46), we studied protein synthesis and secretion by adherent PAM in vitro.

PAM were maintained for 5 d in DMEM supplemented with 10% fetal bovine serum depleted of FN (32), and total protein synthesis and secretion were determined by continuous labeling with [³⁵S]cystine and [³⁵S]methionine in serum-free medium. As shown in Fig. 1 A, protein synthesis and secretion was linear through 4 h. The qualitative pattern of cellular and secreted proteins as displayed by SDS PAGE and autofluorography was identical from 1 h through 4 h of labeling (Fig. 1 B). However, after 24 h of labeling in the absence of serum, polypeptides of >90,000 mol wt were absent, suggesting significant proteolysis (not shown). Based upon these results, we maintained PAM in DMEM plus fetal bovine serum depleted of FN for the experiments discussed below, and limited continuous labeling experiments to 4 h or less.

Human blood monocytes maintained in vitro for several days exhibit changes in the pattern of secreted proteins (38). To determine whether human PAM exhibit similar changes in vitro, we examined the pattern of secreted proteins from PAM maintained in vitro and labeled on days 1, 3, 5, and 7. As shown in Fig. 2, the qualitative pattern of high molecular weight polypeptides as displayed by SDS PAGE and autofluorography did not change significantly during culture for 7 d. New polypeptides of <68,000 mol wt did appear by day 3, reflecting either proteolysis or synthesis of new polypeptides.
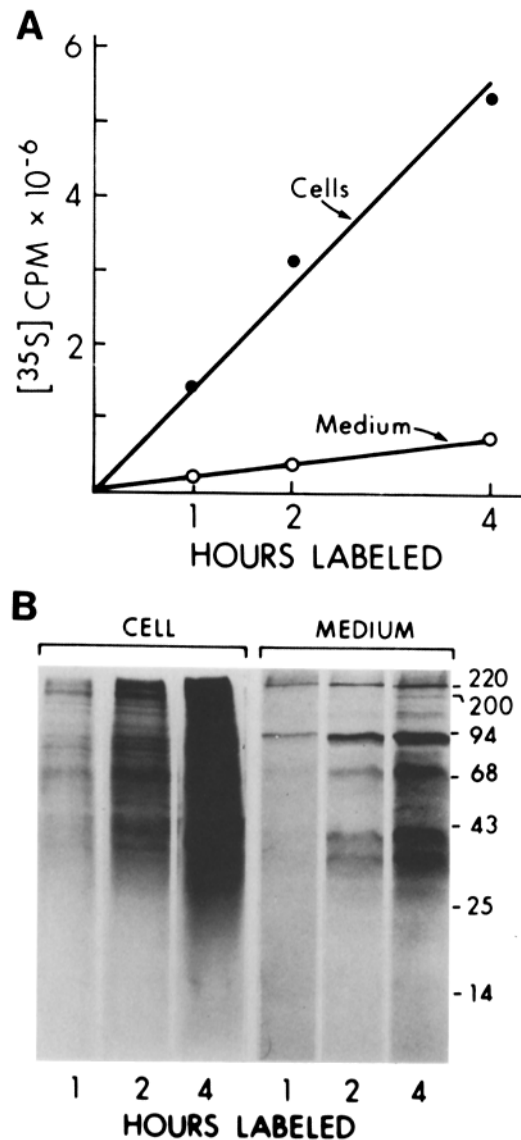

FIGURE 1 (A) Time-course of [³⁵S]cystine and [³⁵S]methionine incorporation into nondialyzable cell and medium proteins by PAM. Cells were maintained for 7 d in DMEM supplemented with 5% FN depleted fetal bovine serum as described in Materials and Methods, and continuously labeled in the absence of serum for the indicated period. Cellular protein synthesis and secretion is linear through 4 h. Panels on B show the pattern of newly synthesized cellular and secreted proteins displayed by SDS 10% PAGE and autofluorography. Molecular weight standards (in kdaltons) include fibronectin (220), myosin (200), phosphorylase (94), serum albumin (68), ovalbumin (43), chymotrypsinogen (25), and lysozyme (14).

FN Synthesis by PAM

Fig. 3 displays the total cell and medium proteins from a 4-h continuous labeling experiment and the corresponding immunoprecipitates with anti-FN IgG. One labeled polypeptide of 220,000 mol wt (reduced) is present in both cell and medium immunoprecipitates. Immunoprecipitation of metabolically labeled medium from human lung fibroblasts (IMR-90) gave similar results. Human PAM FN comigrated with fibroblast FN on SDS PAGE and, although not shown in this gel, slightly slower than plasma FN, as previously shown for fibroblast cellular FN (50).

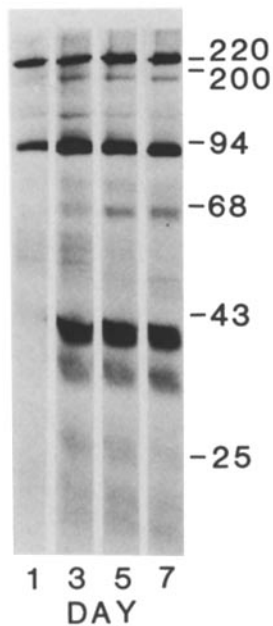Specificity of immunoprecipitation was tested by incubating

FIGURE 2 Effect of maintenance in vitro upon PAM polypeptide secretion. PAM were maintained as described (Materials and Methods) and continuously labeled for 4 h on days 1, 3, 5, and 7. Secreted proteins were displayed by SDS 10% PAGE and autofluorography. Note that several high molecular weight polypeptides are continuously secreted through 7 d in vitro. Molecular weight standards (in kdaltons) are the same as shown in Fig. 1.
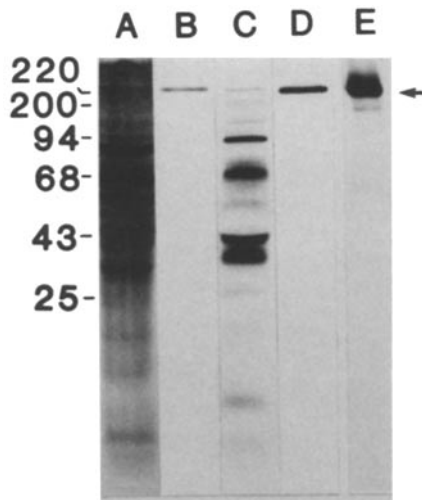

FIGURE 3 Immunoprecipitation of metabolically labeled PAM cell lysates and conditioned medium with anti-FN-IgG. PAM were labeled with [35S]cystine and [35S]methionine (Materials and Methods). Total labeled polypeptides and corresponding anti-FN-IgG immunoprecipitates from cell lysate (A and B) and conditioned medium (C and D) were displayed by SDS 10% PAGE autofluorography. Lane E is an immunoprecipitate of metabolically labeled human lung fibroblast medium. Autofluorograms of the FN immunoprecipitates shown in B and D were exposed five times longer than the starting cell lysate and medium samples to enhance intensity of the FN band (arrow).

metabolically labeled PAM medium with anti-FN IgG, preimmune IgG, or IgSORB[R] alone. Anti-FN IgG precipitated only one labeled polypeptide of 220,000 mol wt. Preimmune IgG and IgSORB[R] alone precipitated trace amounts of the same polypeptide, but no other labeled polypeptides were seen. This may represent limited solubility of PAM FN or slight binding

by the heat-killed *S. aureus* (43). Double antibody immunoprecipitation with preimmune IgG gave no labeled polypeptides. Smaller fragments were seen occasionally in immunoprecipitates of PAM culture medium or cell lysates labeled without serum, probably reflecting proteolysis.

Metabolic labeling with cystine and methionine can introduce bias because of different amounts of these residues in various proteins. Therefore, to determine the relative proportion of FN to other polypeptides secreted by PAM, we labeled with ^{14}C–amino acid mixture. In three experiments with PAM from different volunteers, FN constituted up to 30% of the total newly synthesized and secreted nondialyzable polypeptides from PAM, demonstrating that FN is the major secreted product of freshly harvested human PAM in vitro. We found no differences between qualitative patterns of protein synthesis by PAM adherent to plastic or maintained in suspension.

Subunit Composition and Gelatin Binding of PAM FN

Immunoprecipitates of metabolically labeled PAM medium were analyzed on SDS 5% PAGE with and without reduction (Fig. 4). Without reduction, the major polypeptide was of 440,000 mol wt, whereas reduced samples migrated with a molecular weight of 220,000 as previously shown. These results demonstrate that PAM FN is dimeric, composed of two similar-sized monomers of ~220,000 mol wt. FN characteristically binds tightly to gelatin (denatured collagen), and this property probably underlies its opsonization of gelatin for binding to macrophages (3, 11, 17, 22). As shown in Fig. 4, only a 220,000 mol wt polypeptide (reduced) from conditioned medium bound to gelatin-Sepharose, whereas no polypeptide binding to control-Sepharose was noted. Taken together, these experiments demonstrate that PAM FN shares similar subunit organization and gelatin-binding activity with other described vertebrate FNs (37).

Time-course of FN Secretion by PAM

Continuous labeling experiments (Fig. 1) showed that ~10% of newly synthesized, nondialyzable polypeptides were secreted into medium. However, much more FN was found in immunoprecipitates from medium than from cell lysates (Fig. 3 B and D), suggesting that most newly synthesized FN was secreted. After pulse labeling, PAM rapidly secreted a variety of labeled polypeptides including FN into medium (Fig. 5 A). The time-course of secretion was similar for all visualized polypeptides. In immunoprecipitates of cell lysates and medium (Fig. 5 B), FN first appeared in medium between 30 min and 1 h, whereas cellular FN decreased progressively with time. Densitometric scanning of the immunoprecipitates (Fig. 5 C) revealed that ~50% of the total FN was secreted by 1 h, and >80% appeared in medium by 8 h. This experiment was complicated by a small amount of FN proteolysis, as shown by lower molecular weight fragments in the immunoprecipitates. However, the pattern of FN secretion into medium was identical to that in Fig. 5 C if we plotted (*a*) total immunoprecipitated counts, (*b*) density of all immunoprecipitated bands, or (*c*) density of the 220,000 mol wt band in medium vs. time. Although fibroblast cell-associated or matrix FN requires denaturing agents such as urea or SDS for solubilization, this was not the case for PAM FN, as we could account for all of the newly synthesized FN solubilized by hot SDS in immunoprecipitates of cells plus medium. These results show that almost

HOURS AFTER PULSE

B HOURS AFTER PULSE

C
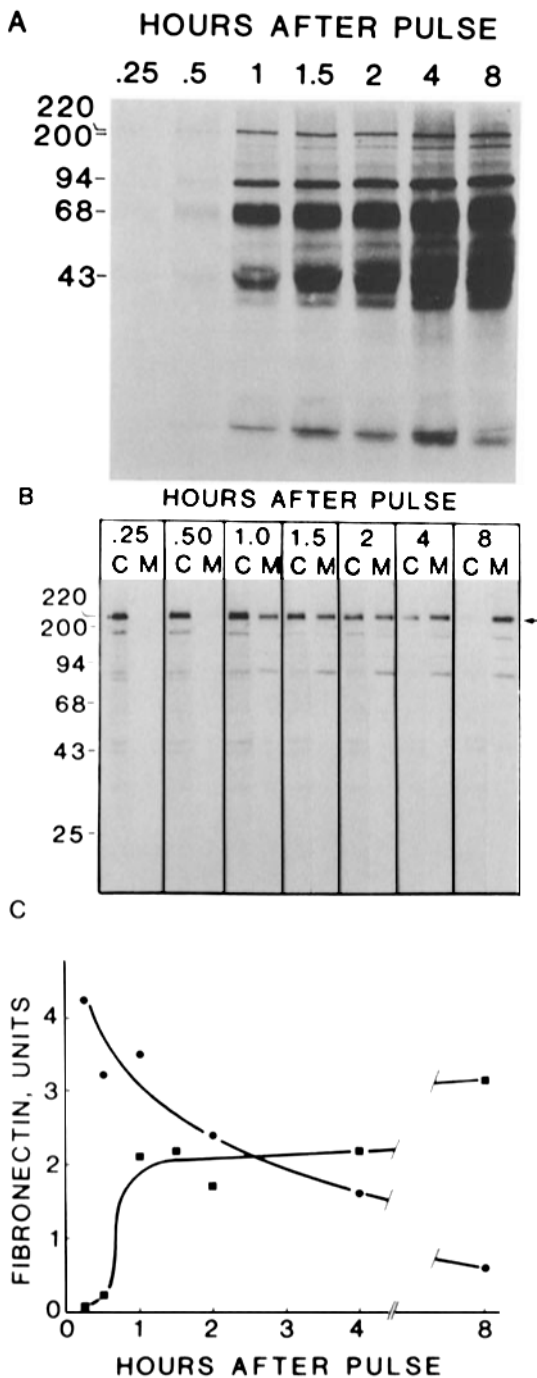
FIGURE 4 Secretion of newly synthesized polypeptides and FN by PAM. PAM (7 d after harvesting) were pulse labeled (Materials and Methods), and the secreted polypeptides were displayed by SDS 10% PAGE autofluorography at the indicated period after labeling (A). Kinetics of secretion were similar for all visualized polypeptides. B shows the results of immunoprecipitating cell lysates (C) and medium (M) at the indicated intervals after pulse labeling. FN (indicated by the arrow) first appeared in medium between 30 min and 1 h after labeling, whereas cell-associated FN decreased progressively with time. C displays the results of densitometric scanning of the 220,000 mol wt bands from immunoprecipitates of cell lysates (●) and medium (■) shown in B. Immunoreactive FN was rapidly released into medium, 80% of the total appearing after an 8-h chase.

all (80%) newly synthesized FN is destined for secretion, in contrast to fibroblasts, where >60% remains cell associated up to 10 h after synthesis (2, 41).

Immunohistochemical Localization of PAM FN

Human PAM maintained in vitro displayed morphology typical for alveolar macrophages (7, 20, 29, 44). Freshly obtained PAM spread rapidly on glass or plastic substrates in serum-free medium by 1 h, and remained tightly adherent. PAM maintained in DMEM plus FN-depleted fetal bovine serum were typically either elongated, bipolar cells exhibiting lamellipodia at the ends of the longest axis of the cell, or were rounded with circumferential lamellipodia (Fig. 6).

Attempted immunostaining of PAM with anti-FN IgG using either the double antibody immunofluorescence or peroxidase-antiperoxidase technique was not successful because marked nonspecific surface staining with preimmune IgG. Probably, this was a result of surface Fc receptors, as noted in similar studies with blood monocytes (1). In contrast, there was no nonspecific staining of intact or permeabilized PAM when preimmune F(ab')₂-peroxidase conjugates were employed (Fig. 6A and B). Staining of intact, nonpermeabilized PAM with anti-FN-F(ab')₂-peroxidase conjugates revealed only an occasional lightly stained cell (Fig. 6C and D). Treating the fixed PAM with dilute Triton X-100 or acetone before staining to allow penetration of the conjugate resulted in marked granular perinuclear and diffuse cytoplasmic staining of virtually all cells (Fig. 6E and F). Identical patterns of FN distribution were seen in PAM stained immediately after plating or after 7 d in vitro. Blocking the immune conjugate with purified plasma FN abolished staining (not shown). A similar staining pattern was seen with fluoresceinated anti-FN-F(ab')₂ conjugates, only rare cells showing surface staining, while intracellular staining was granular and perinuclear (not shown).

Using electron microscopy to visualize immunoreactive FN at the subcellular level, osmiophilic peroxidase reaction product was localized to endoplasmic reticulum and to cisternae of the Golgi apparatus (Fig. 7A and B). Focal cell surface staining was frequently seen where cell membranes were closely ap-
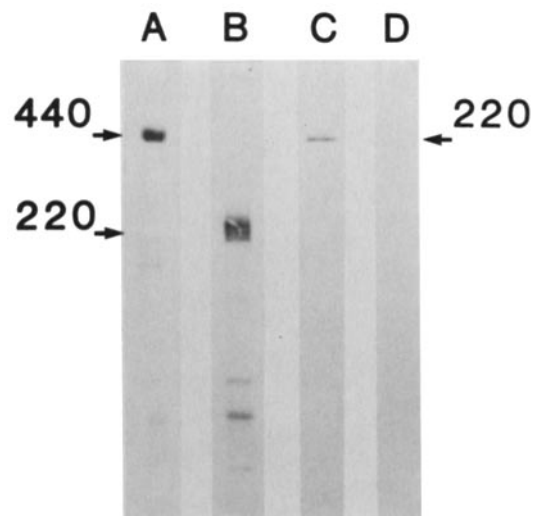
FIGURE 5 Subunit organization and gelatin binding activity of PAM FN. Immunoprecipitates of conditioned medium (see Fig. 3) from radiolabeled PAM were run unreduced (A) and reduced (B) on parallel slots of the same SDS 5% PAGE and labeled polypeptides displayed by autofluorography. Slot C displays one labeled polypeptide of 220,000 mol wt from similar conditioned medium that bound to gelatin-Sepharose, while slot D shows the absence of binding to control-Sepharose (See Materials and Methods). Slots C and D are from a separate SDS 10% PAGE autofluorogram.
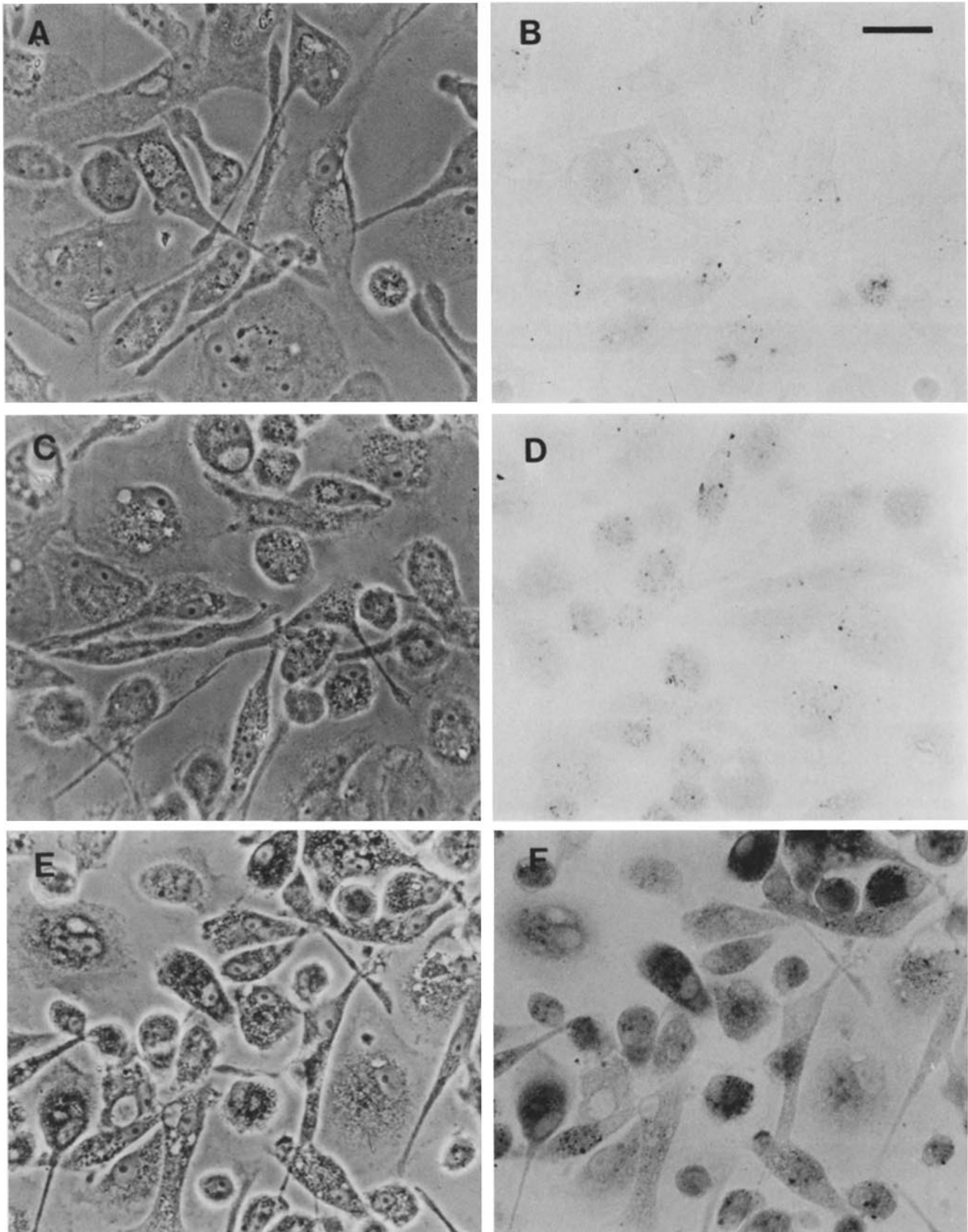
FIGURE 6 Cellular localization of immunoreactive PAM FN. PAM from a nonsmoking volunteer were plated in multichamber culture slides (Materials and Methods) and stained with preimmune F(ab')$_2$-peroxidase conjugates after permeabilizing the cells with acetone (A, phase contrast; B, bright field). Note the absence of significant staining. Staining of nonpermeabilized PAM with anti-FN-F(ab')$_2$ resulted in virtually no detectable extracellular staining (C, phase contrast; D, bright field), whereas permeabilized PAM stained with the same conjugate resulted in granular perinuclear and diffuse cytoplasmic staining of virtually all cells (E, phase contrast; F, bright field). The differences in staining intensity between different cells are probably related to the degree of spreading and hence distribution of intracellular staining. Staining was blocked by preincubation of the anti-FN-F(ab')$_2$ with pure plasma FN (not shown). Bar, 50 µm.
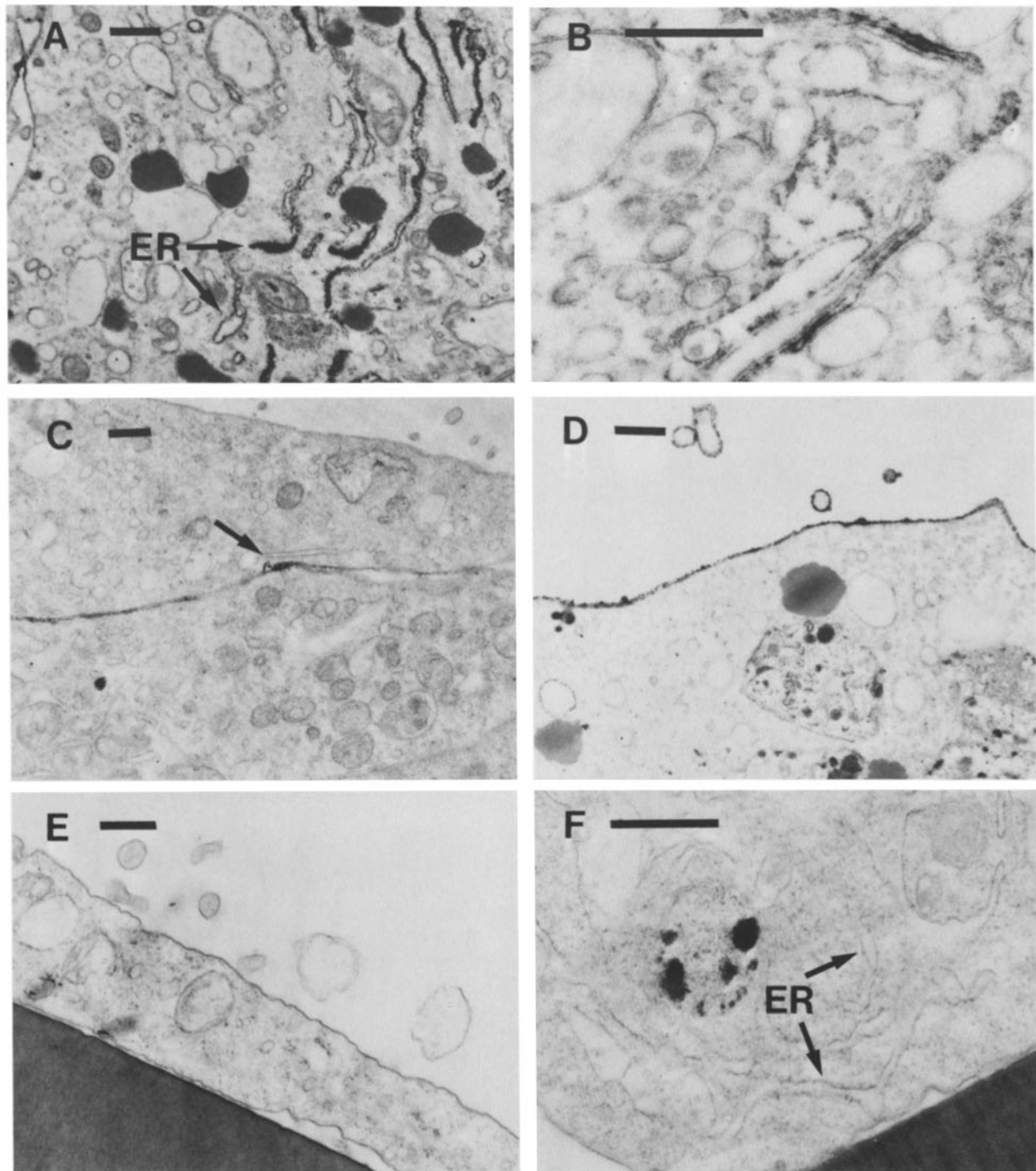
FIGURE 7 Immunoelectron microscopic localization of PAM FN. *A* shows osmophilic reaction product localized to endoplasmic reticulum (*ER*), whereas *B* shows staining in the Golgi apparatus. *C* displays focal staining of the cell membrane at a region where two PAM are closely apposed, but absence of staining at other areas of the cell membrane. *D* displays diffuse cell membrane staining, only rarely seen in PAM (<1% of cells). *E* is a section tangential to the substrate displaying the absence of immunoreactive FN where the PAM is adherent to the tissue culture plate. *F* shows the absence of staining when preimmune F(ab')₂-peroxidase conjugates were substituted for anti-FN conjugates. Similar results were obtained when anti-FN conjugates were blocked with purified FN before staining. Bars, 0.5 μm.

posed (Fig. 7 *C*). However, rare cells (<1%) exhibited areas of diffuse cell surface staining (Fig. 7 *D*). Sections of PAM cultures tangential to the substratum revealed no extracellular fibrillar structures, nor was any staining suggestive of FN-mediating PAM substratum interaction noted (Fig. 7 *E*). Cell surface staining appearing to represent cell membrane–associated FN in a fibrillar distribution was seen in human lung fibroblasts

with the immunoperoxidase technique (Fig. 8 *A* and *B*).

The positive intracellular staining for FN of all cells in the population is important, because the clonal proliferation of fibroblasts in PAM cultures obtained from patients with lung fibrosis has been observed. However, fibroblast proliferation occurs very rarely in PAM obtained from normal volunteers (10). The uniform staining of PAM for intracellular FN shows
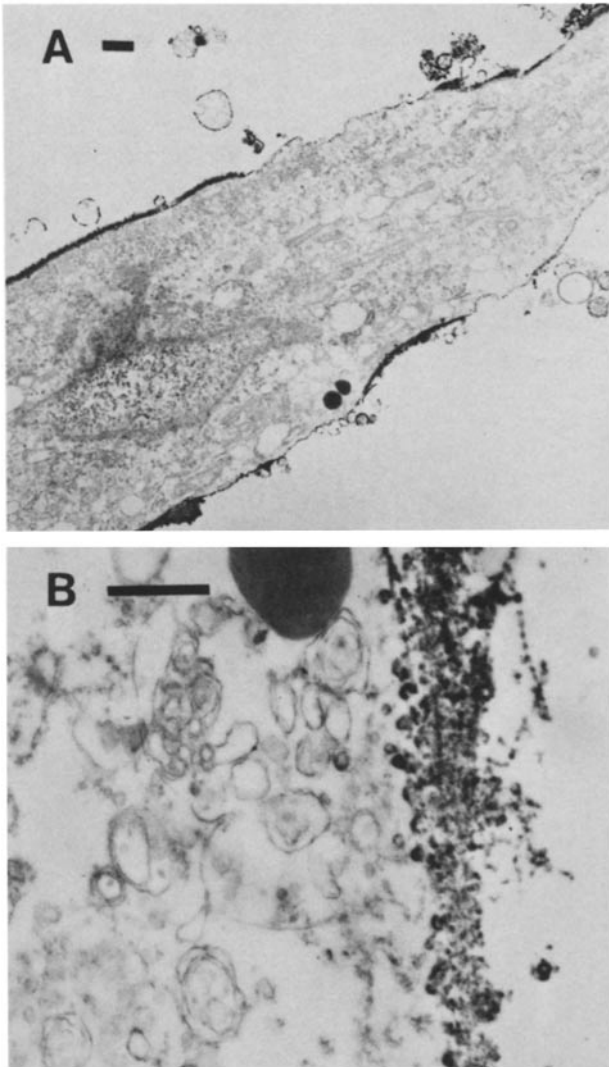
FIGURE 8   Immunoelectron microscopic localization of human lung fibroblast (IMR-90) FN. A is a low-power view of a cell cut parallel to the surface of the tissue culture plate and stained with anti-FN-F(ab')₂-peroxidase conjugate. Note the extensive staining associated with the cell membrane. B is a high-power view of the same cell, demonstrating fibrillar extracellular FN deposits associated with the cell membrane. Bars, 1 μm.

that fibroblast contamination of PAM cultures was not responsible for FN synthesis and secretion.

Ultrastructural Localization of FN during Binding of Gelatin-coated Latex Particles

The absence of generalized cell surface FN from PAM contrasted with our findings in fibroblasts, which demonstrated that the ultrastructural methods used could detect extracellular FN. Moreover, the lack of cell surface FN in PAM indicated that FN could not be a diffuse membrane-associated receptor for macromolecule binding.

To begin to clarify the involvement of endogenous FN in macrophage binding of gelatin-latex particles, we studied FN localization during binding of plain latex and gelatin-coated latex particles by PAM. Gelatin-coated latex particles incubated with PAM in serum-free medium were uniformly stained for FN (Fig. 9A, C, and D). Interestingly, not only were the gelatin-coated particles coated with immunoreactive FN as

expected, but their binding sites on the cell membrane were also stained for FN (Fig. 9A, C, and D). The cell membrane staining was focal, occurring only at binding sites, but staining did extend some distance from the actual site of cell membrane/gelatin-coated bead binding. After uptake by PAM, FN staining of gelatin-coated latex particles was not visualized (Fig. 9A), either because of failure of the immune conjugate to penetrate the cells, or because of proteolysis after formation of phagolysosomes. Despite the similar binding and uptake of plain latex particles by PAM, neither anti-FN nor preimmune peroxidase conjugates gave any staining of plain latex beads or their cellular binding sites (Fig. 9B).

DISCUSSION

Several conclusions may be drawn from our biochemical studies. First, human PAM in vitro synthesized and secreted FN, a dimeric glycoprotein with important biological activities, as well as other unidentified polypeptides, for at least 1 wk. In PAM studied just after harvesting, FN was the major newly synthesized and secreted polypeptide. Second, our experimental conditions allowed linear protein synthesis and secretion by PAM for at least 4 h in serumfree medium, in contrast to earlier investigations of protein synthesis and secretion by nonhuman PAM (27, 28, 30). Third, human PAM FN is structurally, immunologically, and biologically similar to other described vertebrate FNs (36, 37). Because of the difficulty of obtaining sufficient quantities of PAM to chemically isolate FN, we cannot comment upon those characteristics that distinguish cellular from plasma FN (50). Fourth, FN is primarily a soluble secretory product of human PAM in vitro. Thus, PAM differ from mesenchymal cells such as fibroblasts that also synthesize and export FN, but organize an insoluble extracellular matrix containing FN, collagens, and glycosaminoglycans (6, 18).

Morphological analysis of PAM FN by immunohistochemical techniques confirmed and extended our biochemical findings. First, virtually all PAM stained positively for intracellular FN. Thus, FN synthesis is a general property of PAM obtained from the terminal respiratory tract of normal individuals, rather than the product of a particular subset of the recovered cell population. Second, the intracellular localization of FN at the electron microscopy level correlated well with biochemical analysis, as staining of endoplasmic reticulum and Golgi apparatus was compatible with synthesis and secretion of FN. The general absence of extracellular FN at sites of cell-tissue culture plastic attachment and from the cell membrane was compatible with pulse-chase analysis of the fate of newly synthesized PAM FN. Similarly, iodination of the cell surface of mouse peritoneal macrophages and human blood monocytes failed to reveal cell membrane–associated FN (1, 42).

Taken together, these observations suggested that the primary role of PAM FN was extracellular. As a first step to investigate this question, we visualized FN during binding and uptake of plain and gelatin-coated latex beads by PAM. PAM bound and internalized both plain latex and gelatin-coated latex beads in serum-free medium not supplemented with FN. Despite the qualitatively identical fate of both particles, FN was visualized only on gelatin-latex beads and their cell membrane binding sites. Thus, particle binding and ingestion per se does not appear to involve endogenous FN. This, coupled with the absence of generalized cell membrane FN, demonstrates that FN is not a cell membrane component promoting nonspecific binding. Studies upon the effect of anti-FN Fab' and exogenous FN on plain and gelatin-coated latex particle bind-
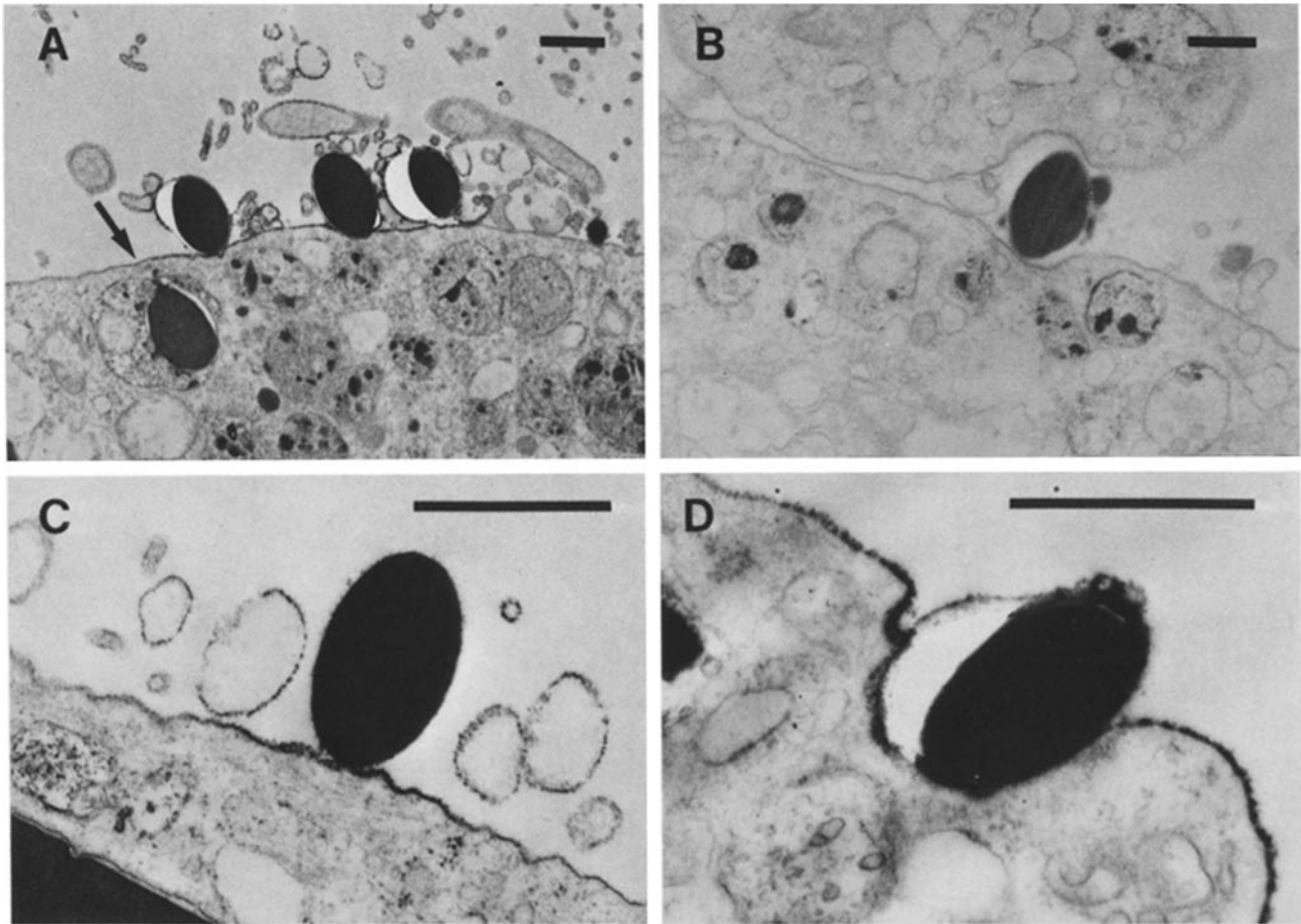
FIGURE 9 Immunoelectron microscopic localization of FN during binding and uptake of gelatin-coated and plain latex beads by PAM. *A* shows FN staining on extracellular gelatin-coated beads and their binding sites on the cell membrane. Note that the focal staining on the cell membrane extends some distance from the actual site of cell-bead interaction, but does not occur at other area of the cell surface. The arrow indicates a particle in a phagocytic vacuole. *B* shows the absence of staining on plain latex beads and their binding sites. Similar results were obtained when gelatin-coated beads were stained with preimmune-F(ab')$_2$-peroxidase conjugates. *C* and *D* provide a high-power view of FN localization on gelatin-coated beads and cell membrane during binding (*C*) and internalization (*D*). Bars, 1 μm.

ing and uptake by PAM support this view.[2]

Studies with fibroblast cell strains (15, 16), as well as blood monocytes (3) and peritoneal macrophages (17), suggest that cell membrane receptors for FN or FN-gelatin complexes exist. The localization of endogenous FN to gelatin-latex beads and their cell membrane binding sites in PAM by immunoelectron microscopy provides additional evidence for this hypothesis. However, the presence of binding sites for FN-gelatin complexes does not appear to completely explain the presence of FN on the PAM cell surface close to where the FN-gelatin-latex beads are bound. It may be that FN-gelatin is transferred to the cell membrane during binding, or that receptors for FN are elicited or concentrated adjacent to cell membrane binding sites for FN-gelatin-latex beads.

One critical remaining question is the physiological role of PAM FN. Unlike blood monocytes (3) and elicited guinea pig peritoneal macrophages (9), PAM do not appear to utilize FN as an attachment factor.[2] It seems clear that FN promotes

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[2] Villiger, B., D. Kelley, T. Broekelmann, and J. A. McDonald. Effect of exogenous fibronectin on attachment, spreading and uptake of gelatin-coated beads in human alveolar macrophages. Manuscript in preparation.

binding of denatured collagen and fibrin by other phagocytic cells (22, 23). Moreover, it appears likely that PAM FN serves as a nonimmune opsonin for the binding of *S. aureus*. Two lines of evidence support this notion. First, Hof and co-workers (21) have demonstrated that human PAM bound and internalized *S. aureus* in serum-free medium containing only trace amounts of serum albumin, and that immunoglobulin and complement played no role in this binding. Second, human polymorphonuclear leukocytes utilize exogenous FN and plasma transglutaminase (coagulation factor XIIIa) to bind *S. aureus* (37). Because we have found that inclusion of small amounts of proteins such as ovalbumin are necessary to prevent degradation of secreted PAM FN in serum-free medium, it appears likely that the uptake of *S. aureus* observed by Hof et al. resulted at least in part from endogenous FN secretion by PAM, and that albumin merely protected secreted FN from degradation.

Our studies serve to emphasize the importance of studying differing phagocytic cell types under appropriate conditions. Most studies of FN-promoted binding of macromolecules to phagocytic cells have appropriately used exogenous FN (3, 11, 17, 22, 23, 34), although macrophages derived from blood monocytes (1), peritoneal macrophages (24), polymorphonuclear leukocytes (47), and PAM synthesize and secrete FN.

Certainly for the PAM, studies of endogenous FN appear to be more relevant than those using high concentrations of plasma FN as an opsonic factor, because this cell type is not exposed to high FN concentrations in vivo.[1] The reason for the apparent differences between human PAM, rodent peritoneal macrophages, and human blood monocytes in the FN requirement for particle binding and cell spreading is unclear but possibly related to the stimulated state of elicited peritoneal macrophages or to the less-differentiated state of monocytes. Blood monocytes may not synthesize FN until differentiation into macrophagelike cells in vitro occurs (1), and the requirements for adherence, spreading, and particle or macromolecule binding may differ with the differentiated state of monocyte-macrophages. Direct comparison of different phagocytic cell types under our experimental conditions will be necessary to verify these apparent differences.

Finally, studies of FN interaction with cells and macromolecules such as fibrin-fibrinogen complexes have been hampered by large amounts of extracellular insoluble FN in fibroblastic cell cultures (8, 45). PAM, which lack significant quantities of extracellular insoluble FN, may prove of value in studying FN–cell membrane interaction and elucidating the molecular mechanisms underlying FN-mediated opsonization of macromolecules.

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