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THE INTERMEDIATE FILAMENT CYTOSKELETON OF MACROPHAGES

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Abstract

This study characterizes two- and three-dimensional ultrastructure and surface topography of polymerized networks of intermediate filaments (IF) isolated from mouse peritoneal macrophages. Isolated IF bound to monoclonal anti-IF antibodies in enzyme-linked immunosorbent assays. Immunogold labeling of IF with specific antibodies revealed that epitopes are distributed along filaments particularly at junctions where filaments interconnect. Networks of IF, viewed by scanning electron microscopy, organized as ropelike groups of interconnecting filaments which swirl and encircle each other to form three-dimensional lattices containing ellipsoidal-, circular-, and vacuole-shaped cavities. Cavity diameters were similar in size to organelles and vacuoles; diameters were grouped as small (12-288 nm), medium (0.3-1.7 μm), and large (2-3 μm). The walls of the cavities appeared as beaded structures with alternating globular and linear regions. Linear regions were 14 nm. Repeat distances taken from the central axis of globular regions were 23-27 nm. The lattice organization of IF observed in vitro was similar to images seen in vivo in Triton-insoluble cytoskeletons immunofluorescently labeled with specific antibodies. In whole cells processed for TEM, swirling bundles of IF were found encircling membranous vacuoles. Based on the lattice architecture of IF, cavity dimensions, and IF location, we postulate that intermediate filaments may function in the mechanical and spatial distribution of vacuoles in the cell cytoplasm.

Key Words: Macrophages, intermediate filaments, SEM surface topography, immunogold labeling.

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Introduction

The cytomatrix is a heterogeneous network of interconnecting filaments and tubules. Major protein families include microtubules, intermediate filaments (IF), actin-rich microfilaments, clathrin baskets, and the microtrabeculae lattice.

Intermediate filaments are a unique family of cytomatrix proteins because they are insoluble in physiological buffers, have long half-lives, and do not undergo reversible assembly-disassembly cycles, like microtubules and microfilaments (Steinhert and Parry, 1985). Their precise role in cells is under active investigation. In muscle cells, IF have mechanical function (Lazarides, 1980; Lazarides and Granger, 1983). From biochemical, immunological, and molecular cloning experiments five classes of IF have been identified: desmin, vimentin, keratins, neurofilaments, and glial fibrillary acidic protein (Lazarides, 1982). Accessory proteins are structurally associated with IF and may serve to connect IF to each other, to organelles, and to other cytomatrix components (Wang et al., 1983; Wang, 1985).

Knowledge of IF structure and biochemistry comes predominantly from studies on keratins in sheep wool and epithelial cells, desmin in muscle cells, neurofilaments in neurons and vimentin in fibroblasts (Aebi et., 1983; Ahmadi et al., 1980; Starger et al., 1978; Gilbert, 1975; Whitman-Aynardi et al., 1984; Woods and Gruen, 1981; Zackroff and Goldman, 1979). Little information is available on the structure, distribution, and function of IF in macrophages. There is evidence that IF of macrophages can be labeled with anti-vimentin antibodies (Franke et al., 1978), and redistribute in response to phorbol myristate acetate (PMA) (Phaire-Washington et al., 1980b). Interestingly, IF redistribution is coupled to stimulated rates of pinocytosis and membrane spreading; and lysosomes are redistributed along IF and microtubule tracks within the cell (Phaire-Washington et al., 1980a). These seminal observations suggest a possible scaffolding role of IF in the mechanical coordination of vacuole, endosome, and organelle traffic during endocytosis and cellular spreading. These are virtually untapped areas worthy of further investigation. Studies might provide knowledge of specific function of IF in macrophages.

The aims of this study were to isolate IF in polymerized form from macrophages, characterize three-dimensional surface topography and architecture, and compare these views with IF organization and distribution in vivo. Our long term

interests are to elucidate IF structure, distribution, and mechanical functions in macrophages.

List of Abbreviations

AB	Antibody
ABTS	2,2 '-azime-di-3-ethyl-benzthiozoline 6-sulfonate;
ELISA	enzyme-linked immunosorbent assay;
IF	intermediate filaments;
PAB	phosphate buffered saline containing 0.1% sodium azide and 1.2% bovine serum albumin;
PBSa	0.6 M K^+, phosphate buffered saline containing 0.6 M KCl;
PIPES	piperazine-N,N'bis (2-ethanesulfonic acid);
PMA	4 beta-phorbol 12-myristate 13-acetate;
SEM	scanning electron microscopy;
TEM	transmission electron microscopy

Materials and Methods

Chemicals

Dimethylsulfoxide (DMSO), cytochalasin E (CE), colchicine, and phorbol myristate acetate (PMA), were purchased from Sigma Chemical Company, (St. Louis, MO). The CE was dissolved in 100% DMSO (2 mg/ml), colchicine was dissolved in water (1 mg/ml) and both frozen (-80°C) as stocks. Stocks were freshly diluted with culture medium prior to use and added to cells at a final concentration of 10^{-5}M. The PMA, stored as stock in 100% DMSO (1 mg/ml), was used at a final concentration of 10 ng/ml.

Cell Culture

Thioglycolate-elicited macrophages were harvested from peritoneal cavities of New Castle's Swiss (NCS; Rockefeller University, New York) or Swiss-Webster (SW) mice (Dutchland Labs, Landsdale, PA) 4 days after injection of sterile (4%) thioglycolate solution. Cells were seeded onto 100 mm, 4-well Costar tissue culture plates (Flow Laboratories Inc., McLean, VA) at 10 x 10^6 cells per well. Cells were maintained for 2 h in Dulbecco's modified Eagle's medium, supplemented with 0.45% glucose, 20% Nu-Serum 100 IU/ml penicillin, 100 μg/ml of streptomycin, and buffered to pH 7.3 with $NaHCO_3$. Cells were cultured in a 5% CO_2-95% air atmosphere at 37°C. After 2 h, nonadherent cells were washed off and adherent monolayers were cultured for an additional 48 h.

Isolation of IF from Macrophages

Triton-extracted IF were isolated in polymerized form from 48 h macrophage cultures by virtue of their insolubility in 0.6 M KCl/PBSa/1% Triton-X-100 buffers as previously described (Starger et al., 1978) and modified by us. Modifications which increased IF yields included inducing cells to spread with PMA for 24 h and exposing spread cultures to CE and colchicine (10 μM, for 6-10 h) to depolymerize microfilaments and microtubules, respectively. After which cells were washed and IF isolated according to the method of Starger et al. (1978). These preparations were immunogold labeled for TEM or left unlabeled and processed for SEM.

SEM of Whole Cells

Cells were grown on 12 mm glass cover-slips (Bellco Plastics, Vineland, NJ), stimulated with PMA (10 ng/ml), fixed with 1% glutaraldehyde in 0.1 M cacodylate buffer, washed 3-5 times in buffer, dehydrated in ethyl alcohol and critical-point dried with liquid CO_2 (Tousimis, Model 810, Rockville MD; Anderson, 1951). Cells were sputter-coated with gold-palladium and examined on an International Scientific Instruments-DS-130 SEM (Santa Clara, CA), at accelerating voltages between 10 and 20 kV and tilt angles of 5-7°. Photographs were made with Polaroid type 55 P/N film, Kodak technical panatomic or panatomic X films.

SEM of Isolated IF

Isolated IF were pelleted by centrifugation, and washed with PBSa buffer. 10-15 μl of the suspension was adhered to Formvar-coated (0.25%) grids secondarily coated with poly-L-lysine (1 mg/ml). Protein on grids was fixed with 1-2% glutaraldehyde in a 0.1 M cacodylate buffer, washed, dehydrated, critical-point-dried, and rotary-shadowed with gold-palladium (Tousimis Shadowcaster evaporator). Protein was examined on a DS-130 at accelerating voltages between 10 and 25 kV. Control grids containing Formvar only, Formvar coated with poly-L-lysine (PLL), Formvar coated with carbon, or Formvar coated with PLL and carbon were examined.

Monoclonal Antibodies

Two types of monoclonal antibodies with IF binding specificity were used: anti-vimentin and anti-IF.

Monoclonal antibodies to vimentin were purchased (Miles Inc., Naperville, IL). Monoclonal antibodies to IF and clathrin cytoskeletal proteins were obtained by injecting Balb/c mice (Dutchland Laboratories, Landsdale, PA) with cloned hybridomas purchased from American Type Culture Collection (ATCC) (Rockville, MD). Hybridomas (TIB 131 and TIB 135) were grown for 2-6 weeks as ascites tumors. The TIB 131 hybridomas secrete monoclonal (IgG) antibodies which react with all classes of IF (Pruss et al., 1981). TIB 135 hybridomas secrete monoclonal (IgG1) antibodies which react with the 36-kilodalton (kd) light chain of bovine brain clathrin (ATCC 1985 catalog). Cell lines were propagated in Dulbecco's modified Eagle's medium supplemented with 0.45% glucose, 20% Nu-Serum, 1% each of L-glutamine, sodium pyruvate, oxaloacetate, insulin, hypoxanthine (Sigma Chemical Co., St. Louis, MO), thymidine (Sigma Chemical Co.), and penicillin and streptomycin (100 IU/ml and 100 μg/ml) (Flow Laboratories Inc. McLean, VA) and buffered to pH 7.3 with 7.5% $NaHCO_3$. Log-phase cells (6-10 million cells each) were injected intraperitoneally and subcutaneously into Balb/c mice primed with pristane (tetramethyl pentadecane; Sigma Chemical Co., St. Louis, MO). In 2-6 weeks ascites supernatants were collected and used at 1:5 or 1:10 dilution in indirect immunogold or immunofluorescent staining. Anti-vimentin antibodies were similarly diluted.

Immunogold Staining of Isolated IF for TEM Analysis

Grids containing IF were treated $NaBH_4$ (0.5 mg/ml) for 20 min at room temperature, washed 3 times with PBS buffer, treated with normal species-specific serum (20 min) and washed 3 times (10 min each) with 25 mM Tris buffer containing isotonic saline and 1% bovine serum albumin (TBS-BSA). After which 10 μl of diluted antibody was added (30 min, room temp.). Preparations were washed with TBS/BSA buffer 3 times for 10 min each, fixed in glutaraldehyde (0.1M), washed 2 times in TBS/BSA, with a final wash in water. Samples were dehydrated, critical point-dried, and examined on a Philips

201 TEM at 80 kV.

Enzyme-Linked Immunosorbent Assay (ELISA)

Isolated IF were centrifuged (15 min at 15,000 rpm). The pellet was resuspended to 250 µl in PBS buffer containing 0.1 mM phenylmethyl sulfonyl chloride (PMSF), EDTA, and 1 mg/ml tosyl arginine methyl ester (TAME). Resuspended pellet (600µg/ml) of protein) was placed in wells of a microtitration enzyme immunoabsorbent assay (EIA) plate (Flow Labs, McClean, VA) and allowed to dry. Adhered protein was washed three times with a solution of PBS containing 0.05% Tween-80, pH 8.0. After which monoclonal anti-vimentin or anti-IF ascites fluid, diluted 1:1000 with PAB (PBS, 0.1% sodium azide, 1.2% bovine serum albumin) was added and plates incubated (1 h) at room temperature. Anti-clathrin monoclonal antibodies from TIB-135 ascites fluid (1:1000) were added to appropriate wells as negative controls. Antigen-antibody complexes were labeled with affinity-purified, peroxidase-conjugated goat anti-mouse IgG (1 h), washed, and subsequently treated with peroxidase substrate solution consisting of equal volumes of 2,2' azimo-di-(3-ethyl-benthiazoline-6-sulfonate) and hydrogen peroxide. Following 1 h, absorbances were read at 414 nm on a Titretek Multiscan (Flow Labs, McClean, VA). All studies were performed in triplicate.

TEM of Whole Cells

One ml of cells ($2x10^6$/ml) were allowed to adhere to surfaces of 35-mm 6-cluster Costar tissue culture wells. After 48 h in culture, cells were treated with PMA (10 ng/ml; 2-24 h), fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 30 min, washed, and post-fixed in 40 mM osmium tetroxide for 30 min. Post-fixed cells were stained with 0.5% uranyl acetate, washed, and dehydrated in increasing concentrations of ethyl alcohol (50-100%) for 10 min each. Monolayers were overlaid with 1 ml of Mix-Fix containing a solution of 1:1 of 100% ethyl alcohol and Epon 812 embedding mixture for 24 h (room temperature). Mix-Fix treatment facilitates EPON penetration. Plates were dried upside down (1-4 h), and overlaid with 400 µl; of 100% Epon mixture and cured for 24 h at 65°C. To section in-situ embedded cells, plates were broken, plastic gently removed, the block was sawed into 3 mm pieces and glued (cell side up) onto the tip of a Beem capsule (E. Fullam Co., Schenectady, NY) containing hardened Epon only. The block was trimmed to 1 mm square and ultrathin sections (50 to 60 nm) were made and placed on Formvar-coated (0.25%) copper grids, stained with uranyl acetate (0.5%), lead citrate, and viewed. Photographs were taken with Kodak 4484 film.

Immunofluorescent Microscopy

Cells, stimulated to spread with PMA (10 ng/ml), were washed twice in stabilizing buffer, pH 6.9, containing 0.1 M Pipes (Sigma Chemical Co, St. Louis, MO), 10 mM EGTA (Sigma Chemical Co., St. Louis, MO) 0.1mM $MgCl_2$, and 4 M glycerol. Cells were exposed to 0.2% Triton X-100 (Sigma) in stabilizing buffer (4 min at 37°C). Triton-insoluble cytoskeletons were fixed for 4 min in absolute methanol (-20°C), air-dried, and sequentially incubated (45-60 min each) with 100 µl of a 1:10 dilution of appropriate monoclonal antibody (1st AB) and FITC-labeled goat anti-mouse antibody (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). After rinsing with phosphate buffered saline, coverslips were mounted, cell side down, with 90% glycerol/10% PBS, supplemented with 1 mM phenylenediamine (to prevent rapid quenching), and viewed on a Zeiss IM 405 with epifluorescent optics.

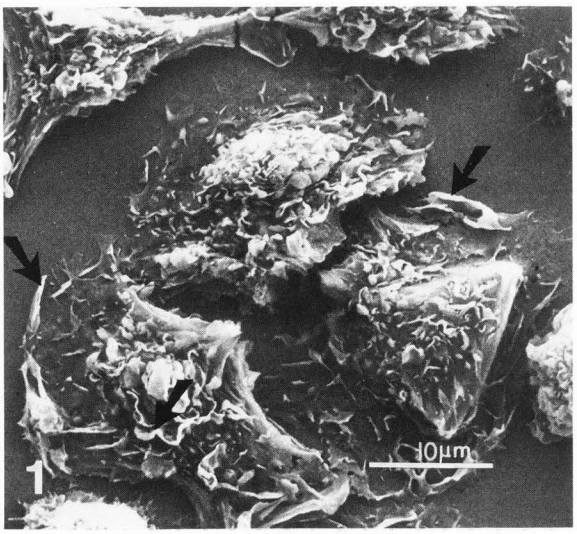
Morphometry

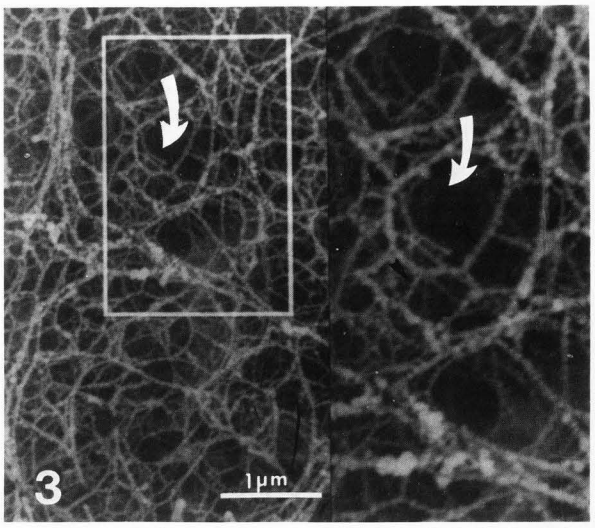
Morphometric measurements of lattice walls and cavity diameters were conducted on 275 x 350 mm photographs made from SEM and TEM negatives. In SEM, micron bars computer-digitized on the negative, were correspondingly enlarged on the print and used as a scale to determine final sizes of structures. In TEM, dimensions were calculated by determining the magnifications of the negative and final print, and developing a micron scale. Appropriate conversions were made with an ultrastructure-size calculator (E. Fullam, Inc., Schenectady, NY). At least 100-150 cavities were measured. When cavities were ellipsoidal, measurements were made along their long axes.

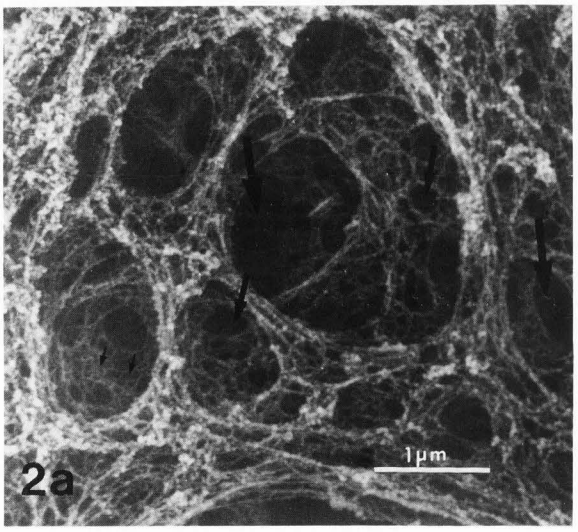
Results

This study compares two- and 3-D architecture of IF isolated in polymerized form from macrophages. The in vitro organization is compared with its organization seen in-situ in whole cells and in Triton-insoluble cytoskeletons. Protein was isolated from extensively spread cells which contained actin-rich ruffles along membrane peripheries and dorsal surfaces (arrows in Figure 1). In SEM images, IF organized as rope-like networks of filaments which anastomosed, interlaced, and encircled each other to form swirling lattice-like networks consisting of small (12-88 nm dia.), medium (0.3-1.7 µm dia.) and large (2-3 µm dia.) cavities (small, medium and large arrows, respectively, in Figures 2a, b). The cavities were circular-, ellipsoidal- and vacuole- shaped. Lattice walls appeared as smooth-surfaced, beaded structures (Figure 3). At higher magnifications, the beaded structures consisted of alternating globular and linear regions (closed and open arrows, respectively in Figure 4a.). Linear segments (open arrows in Figure 4a), were approximately 14 nm in length and occurred at intervals between globular regions. Similar bulges and constrictions (closed and open arrows respectively) were observed in negatively stained samples (Figure 4b). Repeat distances of globular regions measured 27 nm in SEM (Figure 4a) and 23 nm in TEM (Figure 4b) studies. The ropelike architecture of IF were also apparent (Figure 4b). Repeat distances were measured from the central axis of each globular region.

Isolated protein was examined for TEM (Figure 5a). The lattice arrangement of interconnecting groups of filaments is apparent. Immunogold labeling of protein with monoclonal anti-IF antibodies and gold conjugated (10 nm) goat anti-mouse IgG (GAM-10) revealed that epitopes recognized by the antibodies are distributed along filaments particularly at junctions where filaments interconnect, cross-over, and anastomose (dingbat arrow in Figure 5a). The lattice architecture observed in vitro resembled the organization of IF in situ, as seen in Triton-insoluble cytoskeletons immunofluorescently labeled with anti-IF antibodies (Compare Figures 5a, b). Single filaments are seen (hatched arrows in Figures 5a and 5b); but filament bundles predominate (closed arrows in Figures 5a and 5b).







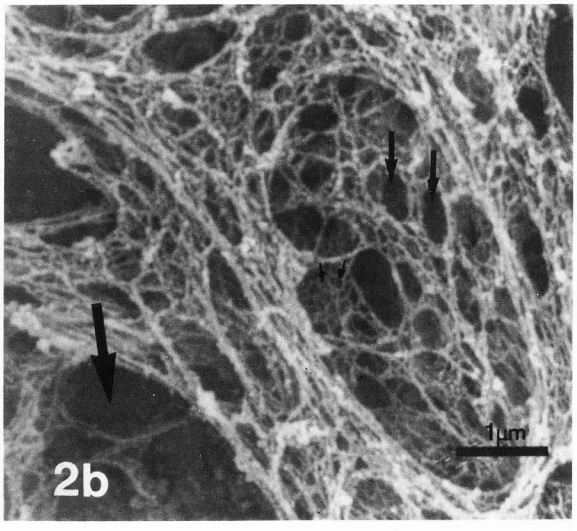


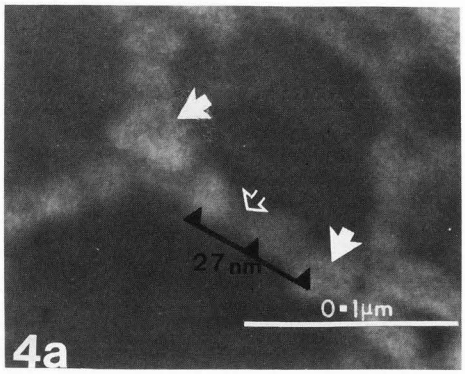
Figure 1. SEM of macrophages induced to spread with phorbol myristate acetate (PMA). Spread populations contain numerous dorsal and peripheral ruffles (arrows). Intermediate filaments were isolated from these PMA-stimulated cultures.

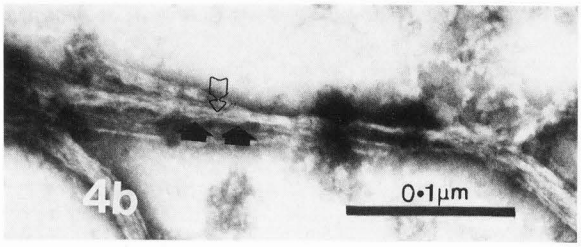
Figure 2 (a, b). SEM of intermediate filaments (IF) isolated in polymerized form from macrophages to highlight three-dimensional lattice networks. Lattice cavities are of adequate size to spatially coordinate organelle and membrane traffic. Seen are small (12-288 nm dia.), medium (0.3-1.7 µm dia.), and large (2-3 µm dia.) cavities as indicated by small, medium, and large arrows respectively. Cavities may be sites where vacuoles are positioned in vivo.

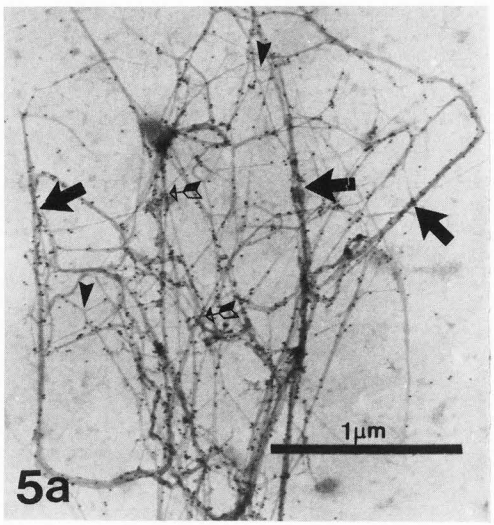
Figure 3. SEM of isolated IF. In view are three-dimensional lattice networks of medium-sized cavities (indicated by arrows). The split image on the right is 5X the magnification of the image on the left. Note the "beadedness" of the cavity walls (arrowheads).

Binding specificity of isolated IF was confirmed in ELISA assays with monoclonal anti-IF antibodies produced from TIB 131 hybridomas (American Type Culture Collection, Rockville, MD). Optical densities were 0.699 using a 414 nm filter. Negative controls included wells coated with substrate or enzyme only, or native IF and anti-clathrin antibodies; or monoclonal anti-IF antibodies and an unrelated protein such as bovine serum albumin. Negative controls demonstrate 0-2.2% relative binding (Table 1).

TEM analysis of the cytoplasm of macrophage monolayers embedded in situ showed swirling filament bundles (open arrows in Figure 6) encircling membrane-bound vacuoles. A representative vacuole (v) is shown in Figure 6. Close association of IF bundles with microfilament meshworks (mf) are evident.







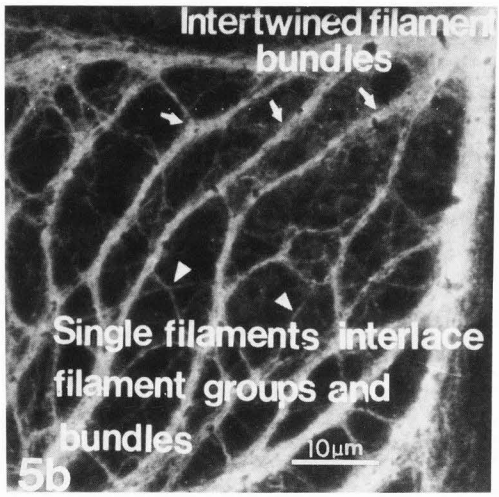


Figure 4 (a, b). SEM showing the "beadedness" of the cavity walls is shown in Figure 4a. Lattice walls are composed of globular structures reminiscent of "beads on a string". Linear regions, approximately 14 nm in length (open arrow), occur at intervals between globular regions (closed arrows). Repeat distances taken from the central axis of globular regions, are 27 nm. Figure 4b is an image of negatively stained IF, viewed by TEM. Similar globular (closed arrows) and linear regions (open arrows) are observed. In TEM repeat distances measured 23 nm. Note the ropelike organization of filament groups.

Figure 5 (a, b). In vitro (a) and in vivo (b) images of IF. Isolated IF were immunogold labeled with monoclonal anti-IF antibodies and affinity-purified gold-conjugated goat anti-mouse IgG. Epitopes are distributed along filament lengths (closed arrows), particularly at junctions where filament groups anastomose and interconnect (dingbat arrows in (a)). Triton-insoluble cytoskeletons immunofluorescently labeled in vivo with monoclonal anti-IF antibodies are shown in (b). Compare and note the appearance of single filaments (arrowheads in (a) and (b)) and intertwined filament bundles (closed arrows in (a) and (b)). Swirling, interlacing, anastomosing lattice networks are common to both images.

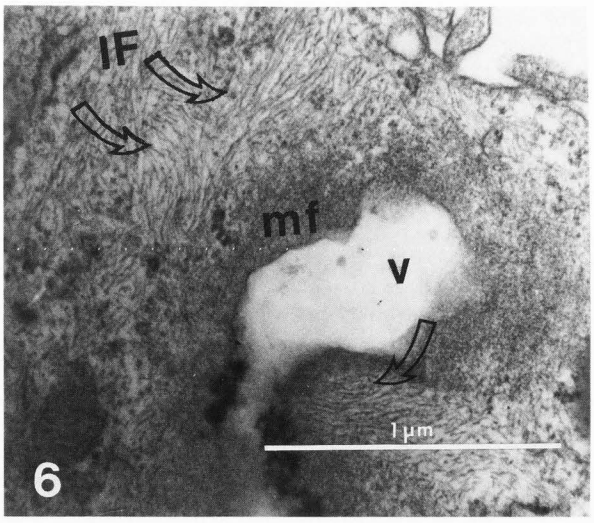


Figure 6. TEM of the macrophage cytoplasm. Swirling interwoven bundles of IF (arrows) are seen encircling a vacuole (v). A microfilament meshwork (mf) also encircles the vacuole and microfilament-IF associations are evident.

Table 1. ISOLATED INTERMEDIATE FILAMENTS BIND MONOCLONAL ANTI-IF ANTIBODIES, AS DETERMINED BY ELISA

Relative binding of IF to monoclonal antibodies is compared with antibody binding to unrelated proteins. EIA microtitration plates were coated with IF and incubated with monoclonal anti-IF (sample #3) or anti-clathrin antibodies (sample #4). Binding specificities were determined in ELISA assays using affinity-purified peroxidase-conjugated goat anti-mouse antibodies. All studies were performed in triplicate. Note the 100% relative binding of isolated protein to monoclonal anti-IF; and 2.2% relative binding of protein to anti-clathrin antibodies.

No.	Sample	Optical Density Reading (414 nm)	Relative percent Binding
1.	Substrate only	0.0	0
2.	Peroxidase only	0.0	0
3.	IF, Anti-IF	0.699	100
4.	IF, Anti-clathrin	0.022	2.2
5.	Bovine serum albumin, Anti-IF	0.011	1.1

Discussion

There exists an enormous amount of evidence to support the concept of intermediate filament structural proteins as a multigene family (Fuchs et al., 1981). The five IF classes and their corresponding structural proteins demonstrate cell-type specific expression. An interesting observation: in vivo some cells have more than one IF class (Zackroff et al., 1984); and structural proteins of different IF classes can form heteropolymers in vitro (Steven et al., 1983). In epithelial cells expression parallels pathways of embryonic differentiation; yet the functional significance of different IF classes in non-epithelial cells has not been delineated.

Biochemical characterization of IF from sequence data, in combination with EM have shown that IF consist of structurally conserved central alpha-helical domains of some 310 residues, flanked by non-alpha-helical terminal domains that are hypervariable both in sequence and length. The central domain gives rise to the coiled-coil models of filament structure (Geisler et al., 1985; Woods, 1983; Woods and Gruen, 1981; Woods and Inglis, 1984).

In studies described in this paper IF were isolated in polymerized form from macrophages by virtue of insolubility in 0.6 M KCl/1% Triton-X-100 buffer and resistance to depolymerization in colchicine and cytochalasin E. As such, the preparations are unlikely to be contaminated by microtubules and microfilaments but may contain intermediate filament-associated proteins and structural proteins other than vimentin. IF were examined using SEM. SEM does not provide the fine detail seen with TEM (Ip et al., 1985; Milam and Erickson, 1982; Woods and Inglis, 1984) or scanning transmission EM (STEM) (Steven et al., 1983; Eichner et al., 1985); yet the technique provides important 3-D information on surface topography. Three-dimensional data reported here provide images of polymerized IF as intertwined ropes of filament groups which organize as 3-D lattice networks. Lattices consist of small, medium, and large cavities which are circular-, ellipsoidal- and vacuole- shaped. Such cavity dimensions can spatially compartmentalize organelle, vacuole, endosome, and vesicle traffic.

At higher magnifications lattice walls are composed of bead-like substructures consisting of alternating globular and linear segments. Similar bulges and constrictions are seen on the surface of vimentin homopolymers reassembled in vitro (Ip et al., 1985). Our morphological observations are consistent a supramolecular model of individual intermediate filaments coiled and intertwined as ropelike groups which may be held together by cross-bridges. The bridging may occur along the 14 nm linear segment. The 23-27 nm repeat we observe may represent common core regions where coiled-coil segments of adjacent filaments and protofibrils are in register.

Ultrastructural images are available on the early stages of IF assembly in vitro. Metal-shadowed images of single filaments show a lateral 21 nm repeat structure (Henderson et al., 1982; Milam and Erickson, 1982). The significance of the repeat structure is still under investigation, but may be indicative of the organization and arrangement of IF protofilaments. Reassembled vimentin-IF, adsorbed to mica, quick-frozen, and freeze-etched show alternating bulges and constrictions every 20-22 nm. In quick-frozen, deep-etch views of mammalian axons, minimum spaces between successive cross-bridges of filament groups is also 21 nm. As such, the 21 nm repeat may represent cross-bridges of filament between laterally organized filament groups (Ip et al., 1985). A model of IF as a hollow tube built on a framework of half unit-staggered tetramers has been proposed to explain the 21 nm repeat (Geisler et al., 1985). Other studies which analyze electron micrographs of partially unraveled filaments discuss hypotheses involving additional protofibrils and therefore arrive at ropelike models (Aebi et al., 1983; Eichner et al., 1985). From observations reported in this paper we suggest that globular 23-27 nm repeat regions of the lattice walls represent surface topography of common coiled-coil regions of filaments twisted in ropelike arrangements. The linear regions of 14 nm may serve as cross-bridging points.

IF function is more subtle than that of microtubules and microfilaments. IF coil around the nucleus in fibroblasts microinjected with different IF antibodies, but locomotion, cell division, and morphology remained unaltered. In other cells, roles of IF have been postulated as mechanical integrators of cytoplasmic space (Lazarides, 1980; Lazarides and Granger, 1983). Our measurements of lattice cavities provide morphometric support for the hypothesis that IF may be the mechanical coordinators of the spatial distribution of vacuoles. Diameters approximate dimensions whereby organelles, vesicles, endosomes and various membrane compartments could be spatially integrated within lattice cavities. Our TEM images of the macrophage cytoplasm show bundles of IF encircling vacuole membranes.

Macrophages are actively motile cells that constitutively internalize, translocate, shuttle, and recycle membrane compartments (Steinman et al., 1976, 1983). Based on the morphological organization of isolated IF in vitro, similarities to the

organization of IF in-situ, we suggest that critical roles of IF in macrophages are to compartmentalize and mechanically integrate vacuole membrane traffic. Whether this proposed role is active or passive is unknown at this time.

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References

Aebi U, Fowler WE, Rew P, Sun TT (1983). The fibrillar substructure of keratin filaments unravelled. J. Cell Biol. 97:1131-1143.

Ahmadi B, Boston NM, Dobb MG, Speakman PT (1980). Possible four-chain repeating unit in the microfibril of wool. In: Fibrous Proteins: Scientific, Industrial and Medical Aspects. Vol. 2. DAD Parry, LK Creamer (eds.) Academic Press, Inc., New York, pp 161-166.

Anderson TF (1951). Techniques for the preservation of 3-D structure in preparing specimens for the electron microscope. Trans. NY Acad. Sci. 13:130-134.

Eichner R, Rew P, Engel A, Aebi U (1985). Human Epidermal Keratin Filaments: Studies on their Structure and Assembly. Annals NY Acad. Sci. 455:381-402.

Franke WW, Schmid E, Osborn M, Weber K (1978). Different intermediate-sized filaments distinguished by immunofluorescence microscopy. Proc. Natl. Acad. Sci. USA 75:5034-5038.

Fuchs EV, Coppock SM, Green H, Cleveland DW (1981). Two distinct classes of keratin genes and their evolutionary significance. Cell 27:75-84.

Geisler N, Kaufmann E, Weber K (1985). Antiparallel orientation of the two doublestranded coiled-coils in the tetrameric protofilament unit of intermediate filaments. J. Mol. Biol. 182:173-177.

Gilbert DS (1975). Axoplasm architecture and physical properties as seen in the Myxicola giant axon. J. Physiol. (London) 253:257-301.

Henderson D, Geisler N, Weber K (1982). A periodic ultrastructure in intermediate filaments. J. Mol. Biol. 155:173-176.

Ip W, Heuser J, Pang S, Hartzer M, Robson R (1985). Subunit structure of desmin and vimentin protofilaments and how they assemble into intermediate filaments. Annals NY Acad. Sci. 455:185-199.

Lazarides E (1980). Intermediate filaments as mechanical integrators of cellular space. Nature (London) 283:256.

Lazarides E (1982). Intermediate filaments: a chemically heterogeneous, developmentally regulated class of proteins. Ann. Rev. Biochem. 51:219-250.

Lazarides E, Granger B (1983). Transcytoplasmic integration in avian erythrocytes and striated muscle. The role of intermediate filaments. In: Modern Cell Biology, Volume 2, JR McIntosh (ed.), Alan R. Liss Inc., New York, 143-162.

Milam L, Erickson HP (1982). Visualization of a 21 nm axial periodicity in shadowed keratin filaments and neurofilaments. J. Cell Biol. 94:592-596.

Phaire-Washington L, Wang E, Silverstein SC (1980a). Phorbol myristate acetate (PMA) stimulates membrane spreading and pinocytosis in mouse peritoneal macrophages. J. Cell Biol. 86:634-640.

Phaire-Washington L, Silverstein SC, Wang E (1980b). PMA stimulates microtubule and 10 nm filament extension and lysosome redistribution in mouse macrophages. J. Cell Biol. 86:641-655.

Pruss R, Mirsky R, Raff M, Thorpe R, Anderson B (1981). A monoclonal antibody recognizes a determinant on common as well as class specific intermediate filament subunit. In: Cold Spring Harbor Reports in the Neurosciences: Monoclonal Antibodies to Neuronal Antigen, Vol., 2, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 125-132.

Starger JM, Brown WE, Goldman AE, Goldman RD (1978). Biochemical and immunological analysis of rapidly purified 10 nm filaments from baby hamster kidney (BHK-21) cells. J. Cell Biol. 78:93-109.

Steinhert P, Parry D (1985). Intermediate filaments. In: Annual Review of Cell Biology, G Palade, B Alberts, J Spudich (eds.). Ann. Rev. Inc., Palo Alto, CA, vol 1:41-65.

Steinman R, Brodie SE, Cohn ZA (1976). Membrane flow during pinocytosis. A stereologic analysis. J. Cell Biol. 68:665.

Steinman R, Mellman I, Muller W, Cohn Z (1983). Endocytosis and the recycling of plasma membrane. J. Cell Biol. 96:1-27.

Steven AC, Hainfield JF, Trus BI, Wall JS, Steinert PM (1983). The distribution of mass in heteropolymer intermediate filaments assembled in vitro: STEM analysis of vimentin/desmin and bovine epidermal keratin. J. Biol. Chem. 258:8323-8329.

Wang E, Cairncross JG, Yung WKA, Garber EA, Liem RKH (1983). An intermediate filament-associated protein, p50, recognized by monoclonal antibodies. J. Cell Biol. 97:1507-1514.

Wang E, (1985). Intermediate filament associated proteins. Annals of the NY Acad. Sci. 455:32-56.

Whitman-Aynardi M, Steinert P, Goldman R (1984). Human epithelial cell intermediate filaments: isolation, purification, and characterization. J. Cell Biol. 98:1407-1421.

Woods EF, Gruen LC (1981). Structural studies on the microfibrillar proteins of wool: characterization of the alpha-helix-rich particle produced by chymotryptic digestion. Aust. J. Biol. Sci. 34:515-526. (alpha)

Woods EF (1983). The number of polypeptide chains in the rod domain of bovine epidermal keratin. Biochem. Int. 7:769-774.

Woods EF, Inglis AS (1984). Organization of the coiled-coils in the wool microfibril. Int. J. Biol. Macromol. 6:277-283.

Zackroff RV, Goldman RD (1979). In vitro assembly of intermediate filaments from baby hamster kidney (BHK-21) cells. Proc. Natl. Acad. Sci. USA 76:6226-6230.

Zackroff RV, Goldman AE, Jones JCR, Steinert PM, Goldman RD (1984). The isolation and characterization of keratin-like proteins from cultured fibroblasts. J. Cell Biol. 98:1231-1237.

Discussion with Reviewers

R.O. Kelley: How do you know that ruffles in macrophages activated under these conditions are actin-rich?
Authors: Ruffles, activated under these conditions, are structurally damaged by the F-actin severing drugs: cytochalasins A, B, C, D, or E. In controls, numerous longitudinal and curved ruffles are distributed along membrane dorsal surfaces and peripheries. Following cytochalasin exposure (5-60 min at 10^{-5}M), ruffles flatten on cell surfaces, membrane surfaces become smooth and eventually ruffles disappear. Differential damage to ruffles observed by SEM correspond to affinities of cytochalasins to bind and fragment actin filaments in vitro. The microtubule depolymerizing drugs: colchicine, vinblastine, vincristine, and colcemid do not induce removal of ruffles from cell surfaces (Phaire-Washington et al., manuscript submitted). Because differential cytochalasin damage correlates well with binding affinities of the drugs for F-actin, we conclude that ruffles are actin-rich.

R.O. Kelley: The diameters of individual filaments are confusing. Observed diameter is 10 $\pm$ 1.8 nm coated by a 10 nm layer of gold-palladium. Is the filament then only 1.8 nm diameter?
Authors: Filament diameters were measured on coated samples prepared for SEM analysis and uncoated samples prepared for TEM analysis. Measurements from SEM micrographs take into account the 10 nm coat of gold-palladium, i.e., final values of 10 + 1.8 nm represent subtraction of the 10 nm coat. Single filaments measured 10 nm in diameter on uncoated samples. Additional evidence for diameters were provided by immunogold labeling with 10 nm gold particles. When single filaments were seen, a gold particle occupied the full width of the filament (hatched arrow in Figure 5a).

R.O. Kelley: Are "lattice cavities" real or simply artifacts of shrinkage during specimen preparation?
Authors: They may be, but we don't think so. Comparative morphological data suggest to us that the lattice cavities are real. Lattices occur in TEM and SEM images of isolated filaments, and in immunofluorescent images of IF in situ. These methods utilize different preparatory procedures; lattices are visualized in all. Cavity dimensions can spatially accommodate varying-sized vacuoles. Lattice cavities may be the sites where vacuoles are positioned in vivo. Indeed, in TEM studies of cells embedded in situ, we observe filament bundles encircling vacuoles in the cell cytoplasm.

R.O. Kelley: What is the "beadedness" suggesting about the structure of IF?
Authors: The "beadedness" may represent the surface topography of compacted coiled-coil regions common to adjacent filament groups.

Reviewer IV: What is the repeat distance in Figure 4?
Authors: The repeat distances of globular regions range between 23-27 nm. Similar bulges and constrictions are seen in negatively-stained preparations published by others (Ip et al., 1985). Reasons for the minor differences in the length of the repeat distances (21 nm observed by Milam and Erickson 1982; 20-22 nm observed by Ip et al., 1985; and 23-27 nm observed in these studies) are not apparent at this point.

Reviewer IV: Please relate this work to previously published assembly studies of IF which normally lead to single IF of 10 nm diameters.
nyAuthors: Our studies examine organization of IF which are isolated from cells in polymerized form, not purified soluble subunits of IF which are reassembled in vitro. Our morphological data are 2-D and 3-D images of isolated IF which are already polymerized. Preparations were isolated by virtue of insolubility in 0.6 M KCl/1% Triton-X-100 and resistance to depolymerization after colchicine and cytochalasin exposure (6-10 h at 10^{-5} M). Although single filaments are seen, the predominant organization is ropes of intertwined bundles of filaments which assume a higher level of organization into structured lattice networks. We feel this may represent IF architecture in-situ where IF-accessory proteins, which cross-bridge filaments to each other, are intact. Previously reported assembly studies, which show single filaments, are conducted on purified IF subunit proteins and lack IF accessory proteins. Thus, single filaments are predominately seen.

Reviewer IV: One would expect in macrophages that the authors are dealing with vimentin. Why don't they say so?
Authors: The biochemistry of macrophage-IF is not known at present. Although our preparations can be immunogold and immunofluorescently stained with anti-vimentin antibodies, we are cautious and do not assume that IF of macrophages are composed of vimentin homopolymers only. IF can exist as homopolymers or heteropolymers of various structural proteins in vitro and in vivo (Steven et al, 1983; Zackroff et al., 1984). Although our preparations react with anti-vimentin antibodies, on polyacrylamide gel electrophoresis, our samples migrate as 4-7 bands, ranging in molecular weight from 14-90 kilodaltons (Phaire-Washington et al. manuscript in preparation). More than one IF class-specific structural protein has been identified in a single cell type (Zackroff et al., 1984). This may also be the case with macrophages.