

Cell-Substratum Interaction of Cultured Avian Osteoclasts Is Mediated by Specific Adhesion Structures

P. C. MARCHISIO, D. CIRILLO, L. NALDINI, M. V. PRIMAVERA,* A. TETI,* and A. ZAMBONIN-ZALLONE*

Institute of Histology and General Embryology, University of Torino Medical School, Torino, Italy; and

**Institute of Human Anatomy, University of Bari Medical School, Bari, Italy*

ABSTRACT The cell-substratum interaction was studied in cultures of osteoclasts isolated from the medullary bone of laying hens kept on low calcium diet. In fully spread osteoclasts, cell-substratum adhesion mostly occurred within a continuous paramarginal area that corresponded also to the location of a thick network of intermediate filaments of the vimentin type. In this area, regular rows of short protrusions contacting the substratum and often forming a cup-shaped adhesion area were observed in the electron microscope. These short protrusions showed a core of F-actin-containing material presumably organized as a network of microfilaments and surrounded by a rosette-like structure in which vinculin and α -actinin were found by immunofluorescence microscopy. Rosettes were superposable to dark circles in interference-reflection microscopy and thus represented circular forms of close cell-substratum contact. The core of ventral protrusions also contained, beside F-actin, fimbrin and α -actinin. Villin was absent. This form of cell-substratum contact occurring at the tip of a short ventral protrusion differed from other forms of cell-substratum contact and represented an osteoclast-specific adhesion device that might also be present in *in vivo* osteoclasts as well as in other normal and transformed cell types.

Osteoclasts are specialized cells involved in bone resorption. Even if many aspects of their function in bone physiology are not yet known, their morphology and ultrastructure are known in some detail (1, 15, 18). In particular, a ruffled border has been identified and described by both electron microscopy and microcinematography as a highly motile peripheral area probably involved primarily in bone resorption (17). An organelle-free area called "clear zone" or "sealing zone" (18) immediately adjacent to the ruffled border has been described. Schenk et al. (28) have suggested that the clear zone provides the osteoclast with a tight bone attachment area; King and Holtrop (21) have shown, in this same area, organized bundles of actin filaments ending within short processes contacting the bone surface. Further evidence that the clear zone is a specialized site of attachment has been provided also in different experimental conditions (27, 30).

Recently, a technique for isolating and culturing functional osteoclasts free of other cell types has been developed (36, 39); using this isolation procedure, the cytoskeleton of osteoclasts has been studied *in vitro* by means of immunofluorescence microscopy (37). A major finding was that in most osteoclasts, and especially in those highly flattened, multiple

actin-containing dots were located in the area that topographically and ultrastructurally corresponds to the clear zone observed *in vivo*. In this same area, dot-like points of close adhesion and a belt of intermediate filaments of the vimentin type were also located, while fibronectin was excluded from the whole attachment area (37). Having in mind the idea that the whole area corresponding to the clear zone could represent a specialized site of adhesion homologous to the attachment sites observed *in vivo* (21), the investigation has been extended to microfilament-associated proteins like vinculin (11), α -actinin (19, 24), and fimbrin (2, 5), which also are involved in adhesion structures of many cell types (4, 7, 8, 11–13, 34). It was found that indeed F-actin-containing dots correspond to vinculin, α -actinin, and fimbrin arranged with a different topographical pattern within a peculiar structure protruding from the ventral surface. This structure has never been previously described and represents an osteoclast-specific adhesion device.

MATERIALS AND METHODS

Isolation and Culture of Osteoclasts

Osteoclasts have been isolated from the medullary bone of calcium-deprived

laying hens and cultured in vitro as previously described (36). Briefly, medullary bone harvested from both femurs and tibiae was washed in two to four changes of sterile PBS and the bone fragments were gently scraped with needles to obtain an osteoclast-enriched cell suspension. The pooled suspensions were centrifuged at low speed for 5 min and the pellets resuspended in PBS. A nearly pure population of osteoclasts was obtained with 2–3-unit gravity sedimentations of 45 min of the cells suspended in PBS and layered in test tubes over a solution of 50% fetal bovine serum in PBS. The final pellet was resuspended in minimal essential medium, counted and the cells plated at a density of 1×10^5 in 35-mm tissue culture dishes containing round glass coverslips. Cells were maintained at 37.5°C in 95% air–5% CO₂ in minimal essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Unless otherwise specified, 5 d were allowed for complete spreading before fixation and other analytical procedures.

Antibodies

Vinculin antibodies were raised in rabbits immunized with homogeneous chicken gizzard vinculin and obtained through the courtesy of Dr. B. Geiger (Weizmann Institute, Rehovot, Israel). Vinculin antibodies, which have been extensively characterized (11–14), were used for immunofluorescence experiments at a dilution of 1:20 to 1:40. Two different batches of fimbrin and villin antibodies, raised by immunizing rabbits with either fimbrin or villin purified from chicken brush borders (2–5), were obtained from Dr. A. Bretscher (Cornell University) and Drs. M. Osborn and K. Weber, (Max Planck Institute for Biophysical Chemistry, Göttingen, FRG). Sera were used at 1:50 to 1:100 dilutions or, in the case of affinity-purified fractions (4, 5), at 50 µg/ml. A monoclonal mouse antibody against chicken gizzard α -actinin (code No. 353) was obtained from Amersham International (Amersham, UK) and used at 1:200 dilution. A monoclonal mouse antibody raised against hamster vimentin was found to recognize vimentin from most mammalian and nonmammalian cells (Prat, M., C. Corbascio, P. M. Comoglio, and P. C. Marchisio, manuscript in preparation); in the present study the vimentin antibody was used as ascitic fluid diluted 1:100. Affinity-purified fluorescein- or rhodamine-tagged goat anti-rabbit or goat anti-mouse IgGs were purchased from Kirkegaard and Perry, Laboratories, Inc. (Gaithersburg, MD) and used at 100 µg/ml.

Immunochemical Procedures

The presence of different cytoskeletal antigens in cultured osteoclasts was studied by immunoblotting (32) according to the modified procedure reported by Burnette (6). Cultures of osteoclasts free of contaminating cells were solubilized in 2% SDS containing 1% β -mercaptoethanol and run on 5–15% polyacrylamide gradient slab gel (23). The gel was then blotted onto Bio-Rad nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA) and transferred for 20 h with a constant current of 150 mA. The blots were then incubated in 1:150 dilutions of the appropriate antisera and further incubated in ¹²⁵I-labeled *Staphylococcus aureus* protein A. Blots were air dried and exposed for autoradiography at –80°C for 24 h.

Immunofluorescence Procedures

For immunofluorescence microscopy, osteoclasts, deliberately plated with some contaminating fibroblasts, were seeded on glass coverslips and used at different times until full spreading was attained. After rinsing once with PBS, coverslip-attached cells were treated with one of the following procedures:

(a) **FIXATION PLUS PERMEABILIZATION:** Coverslip-attached osteoclasts were fixed in PBS, pH 7.6, containing 2% sucrose and 3% formaldehyde (from paraformaldehyde) for 5 min at room temperature, rinsed, permeabilized in HEPES-Triton X-100 buffer (20 mM HEPES, pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl₂, and 0.5% Triton X-100) for 5 min at 0°C and finally rinsed in PBS.

(b) **PERMEABILIZATION PLUS FIXATION:** Coverslip-attached cells were first permeabilized in HEPES-Triton X-100 buffer for 30 s at 0°C, rapidly rinsed in PBS containing both sucrose and formaldehyde, fixed in the same buffer for 5 min at room temperature, and finally rinsed in PBS.

Coverslips were incubated with primary antibodies for 45 min at 37°C, thoroughly rinsed in PBS, and further incubated in the appropriate fluorochrome-labeled second antibody. For the simultaneous detection of F-actin (35), 10 µg/ml fluorescein-labeled phalloidin (F-PHD,¹ a kind gift of T. Wieland, Max Planck Institute for Experimental Medicine, Heidelberg, FRG) was

¹ *Abbreviation used in this paper:* F-PHD, fluorescein-labeled phalloidin; IRM, interference-reflection microscopy; TEM, transmission electron microscopy.

added in the incubation step with the rhodamine-tagged secondary antibody. Controls were routinely carried out by replacing the primary antibody with preimmune rabbit or mouse IgGs. Stained preparations were mounted in PBS-glycerol and viewed with the appropriate filters in a Leitz Dialux microscope equipped with Zeiss planapochromat 63×/1.4 (oil) lens or in Leitz Diavert inverted microscope equipped for both epifluorescence and interference-reflection microscopy (IRM). Fluorescence photographs were taken with Kodak Tri-X films rated at 1250 ISO and developed in Gradual ST20 developer (Chimifoto Ornano, Milano, Italy); IRM pictures were recorded on Agfa Ortho 25 films developed in Agfa Neutol NE.

Electron Microscopy

For transmission electron microscopy (TEM), cells were fixed in modified Karnovsky fixative (20), dehydrated in dihydroxypropyl-metacrylate, and embedded in Epon directly in the culture dish. After polymerization, the blocks were snapped free from plastic by a short dip in liquid nitrogen. Sections, cut with a LKB Ultratome IV and stained with uranyl acetate and lead citrate, were viewed in a Philips 400 electron microscope.

RESULTS

Fluorescence Microscopy

Osteoclasts isolated from the medullary bone of calcium-deprived laying hens and plated in vitro undergo spreading (36) and appear as large flattened multinucleated cells (Fig. 1A); in the paramarginal area, mostly around the whole cell, phase-dark dots may be observed as a rather regularly arranged belt (Fig. 1A, arrows). When stained with F-PHD, which selectively binds to F-actin (35), this paramarginal area shows thin microfilament bundles and intercalated intensely fluorescent dots (Fig. 1B), which correspond to similar dots seen in phase contrast. This same area contains also a thick network of intermediate filaments of the vimentin type arranged as a continuous paramarginal belt (Fig. 1C). This area is then particularly rich in cytoskeletal filaments and may be expected to represent a reinforced ring playing a role in the peripheral adhesion of osteoclasts in vitro. Whether this ring of filaments may be relevant also for the mechanism of adhesion to bone matrix in vivo remains to be studied.

To gain information about the mechanism of adhesion of osteoclasts, the localization of some structural proteins involved in the organization of adhesion structures of cultured cells, i.e., vinculin (11, 12), α -actinin (8, 34), and fimbrin (5), has been investigated. Vinculin antibodies bind to a 130,000-mol-wt protein extracted from osteoclasts (Fig. 2, lane b) and selectively immunostain rosette-shaped areas that form a ring around each actin-containing dot. With double-label fluorescence a clearcut correspondence of F-actin dots and vinculin circles may be shown (Fig. 3, A and B); this suggests the existence of a compound structure which, observed along its axis, is formed by a core of F-actin and a shell of vinculin. Fimbrin is also found in extracts of isolated osteoclasts (Fig. 2, lane c) and its location, studied by immunostaining with specific antibodies, appears to correspond exactly to that of F-actin dots but it is never found along peripheral microfilament bundles (Fig. 3, C and D). Such overlapping of dots is so precise as to cast doubts on a possible contaminating antiactin activity of the specific antiserum (obtained from Dr. A. Bretscher); this possibility was ruled out, however, by (i) the identical pattern obtained with an affinity-purified fimbrin antibody (obtained from Drs. M. Osborn and K. Weber), by (ii) the absence of any staining of stress fibers in co-cultured fibroblasts, by (iii) the staining of microvilli and peripheral lamellipodia obtained in the same cells by either antibody (not shown), and by (iv) the lack of any 43,000-mol-wt detectable band in fimbrin immunoblots (Fig. 2, lane c). At

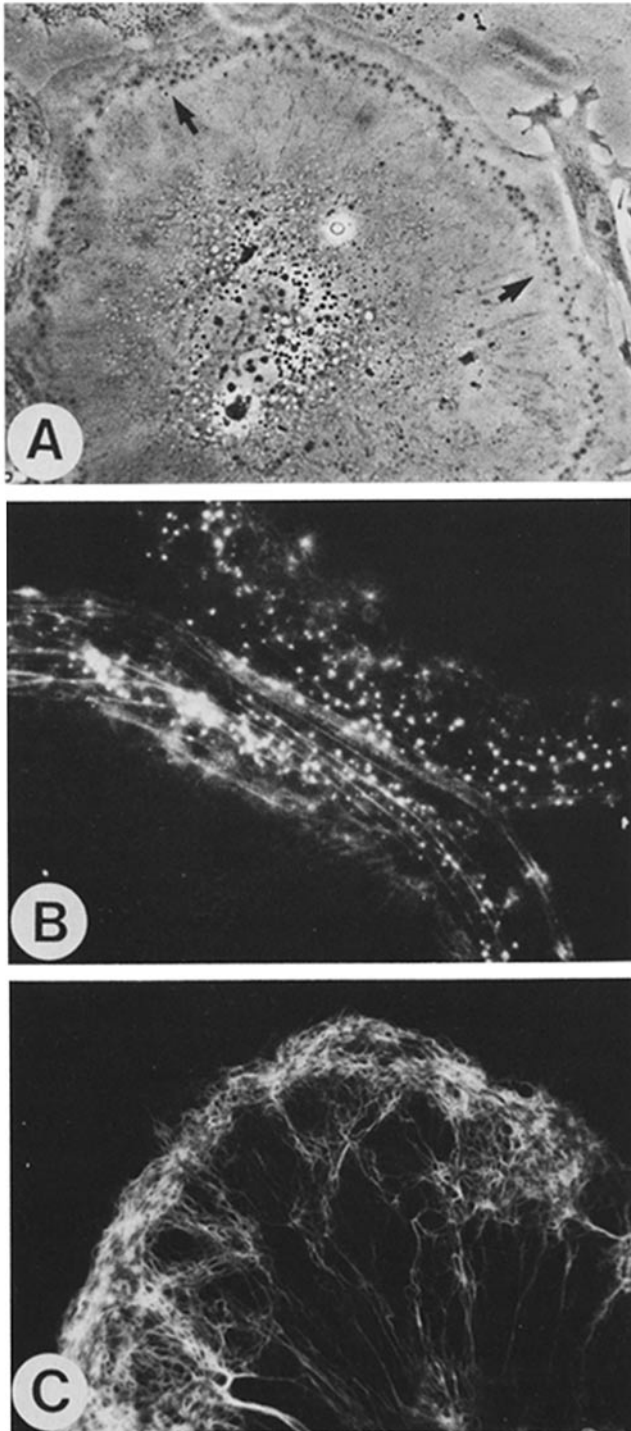


FIGURE 1 Phase-contrast and fluorescence microscopy pictures of cultured hen osteoclasts. A spread osteoclast (A) shows the nuclear mass and a row of phase-dark dots (black arrows) distributed along the whole paramarginal area. A co-cultured fibroblast is visible on the right side. With F-PHD staining for F-actin (B) fluorescent dots as well as thin microfilament bundles appear in the paramarginal area. Immunofluorescence staining with a monoclonal vimentin antibody shows bundles of radially arranged intermediate filaments merging into a dense network corresponding to the same paramarginal area (C). (A and C) $\times 300$. (B) $\times 500$.

higher magnification and under favorable conditions of observation, spokes radiating from the F-actin (Fig. 3E) and fimbrin (Fig. 3F) dots are visible; under the same conditions, the vinculin shell appears to be formed by circularly arranged

discrete subunits probably complementary to core spokes (Fig. 3G).

Double-label fluorescence with a monoclonal α -actinin antibody shows the presence of α -actinin in correspondence of F-actin dots (Fig. 4, A–D). When pictures are taken at the same focal plane the localization of α -actinin is slightly broader and less sharply defined than that of actin (Fig. 4, A and B); occasionally, the center of α -actinin dots appear less intensely stained than the periphery (Fig. 4, A and B, e.g., at arrowheads). When pictures of the same field are taken at a slightly raised focal plane, the overlying microfilament bundles appear finely dotted suggesting that α -actinin is also associated with microfilament bundles with the typical periodicity described in other cell types (e.g., 24, 34) (Fig. 4, C and D). The periodical association of α -actinin with both radial and circumferential microfilament bundles is even better shown in Fig. 4E. In co-cultured fibroblasts α -actinin is found also in streak-like adhesion plaques (Fig. 4F). Villin, a protein present with fimbrin in brush border microvilli (2–5), is absent in these structures and cannot be immunologically identified in osteoclast extract blots.

To show whether these structures are related to adhesion systems, osteoclasts, immunostained for vinculin, were analyzed by IRM, which has been previously shown to provide information on the distance between the substratum and the ventral membrane of *in vitro* cells (9, 22, 34). At low power it is clear that the paramarginal belt hosting the rows of dots represents also an IRM-dark area and thus is a zone of

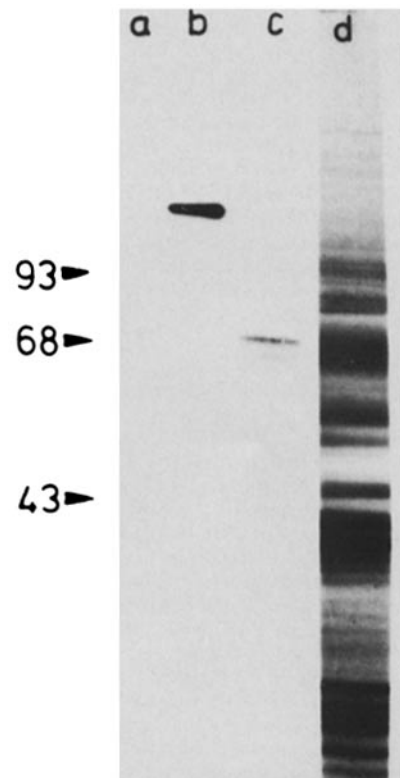


FIGURE 2 Autoradiographs of nitrocellulose immunoblots of SDS-polyacrylamide gel-separated osteoclast extracts (6, 36). (Lane a) preimmune rabbit serum; (lane b) vinculin antibody (1:150); (lane c) fimbrin antibody (1:150); (lane d) Coomassie Blue staining of the whole osteoclast extract. The immunoblots were developed with ^{125}I -labeled *S. aureus* protein A. The minor band in lane c probably represents a proteolytic fragment of fimbrin.

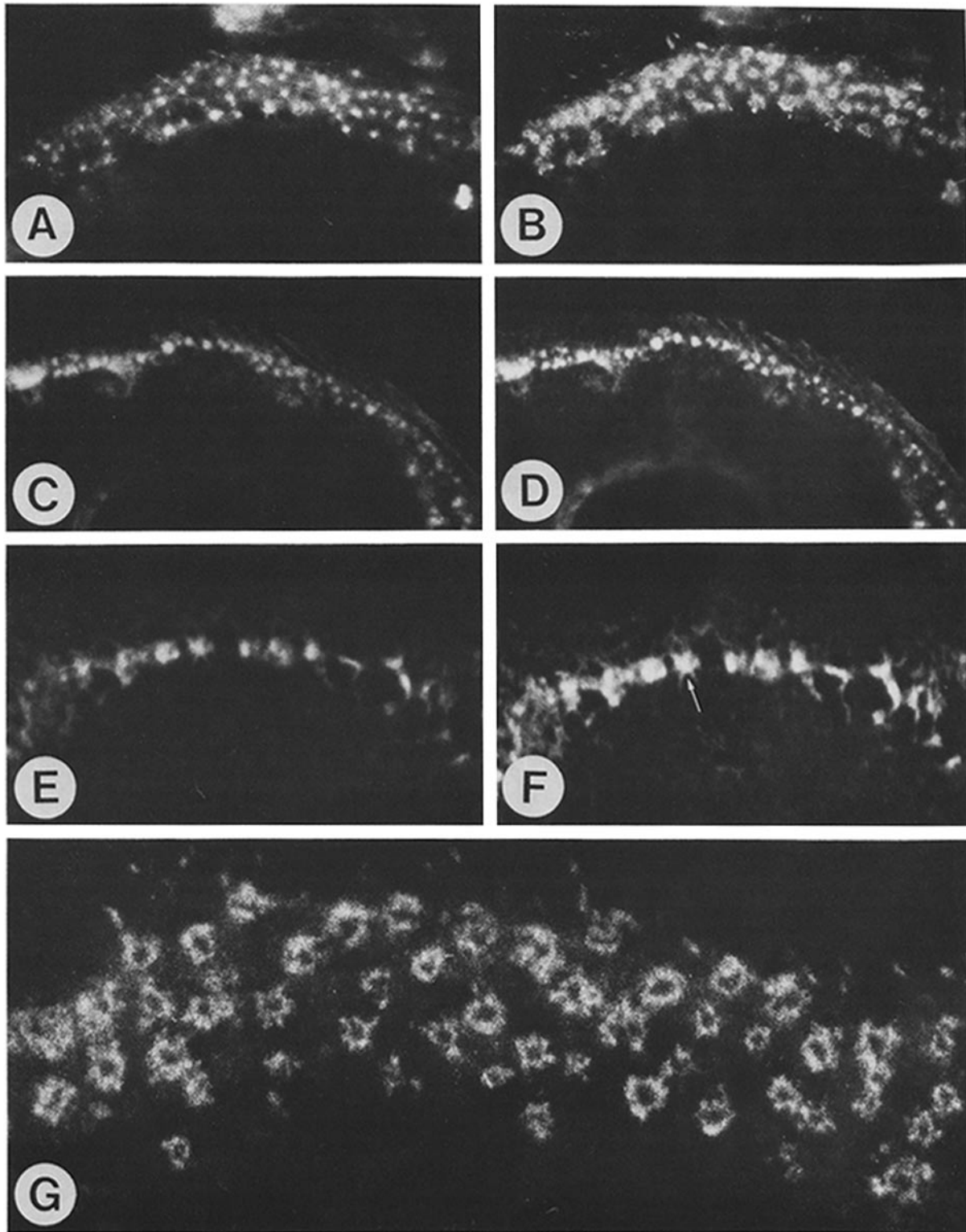


FIGURE 3 Double-stain fluorescence pictures of the paramarginal area of hen osteoclasts. F-PHD staining for F-actin (A, C, and E) shows dot-like structures corresponding to the center of rosettes decorated with vinculin antibodies (B) and are coincident with nearly identical dot-like structures decorated with fimbrin antibodies (D and F). At higher magnification the F-actin- and fimbrin-containing centers appear to be provided of radially oriented spokes (F, arrow) while the vinculin rosettes are apparently formed by discrete subunits (G). (A–D) $\times 500$. (E and F) $\times 1,300$. (G) $\times 2,100$.

considerably closer contact than other parts of the osteoclast attachment side (Fig. 5, A and B). At higher magnification, vinculin-containing rosettes mostly overlap with IRM dark circles, indicating they are very close or even in focal contact

(Fig. 5, C and D). The core of the structure obviously corresponds to the IRM-clear spot. This indicates that the ventral membrane is somewhat removed from the substratum in the center of the structure and adheres all around the core prob-

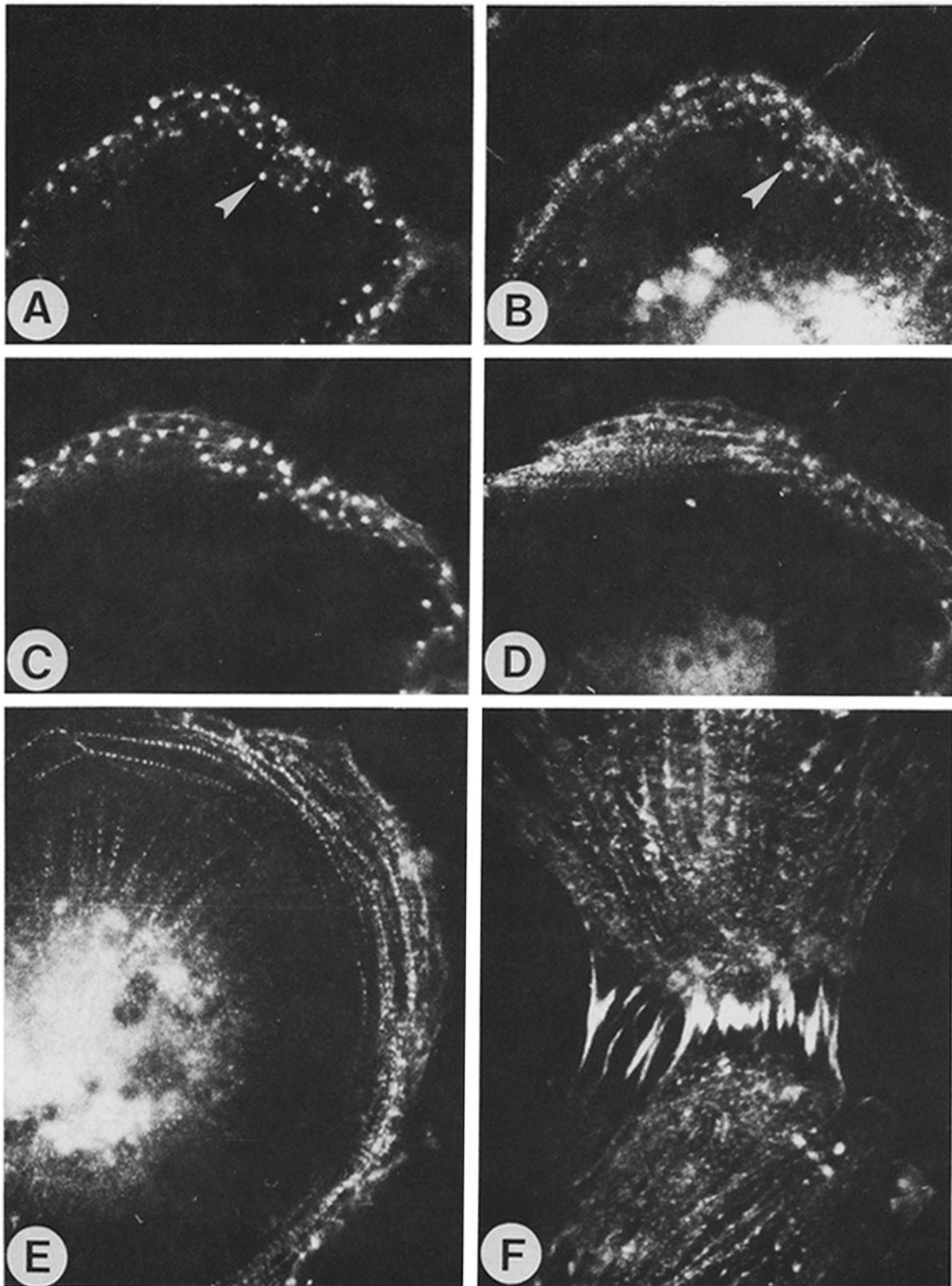


FIGURE 4 Immunofluorescence localization of α -actinin in hen osteoclasts and fibroblasts. F-PHD staining for F-actin (A) shows dot-like structures corresponding to somewhat less defined dots decorated with a monoclonal α -actinin antibody (B); such dots occasionally show a less densely stained center (e.g., at arrowhead). When the focal plane is slightly raised over the substratum, dots appear out of focus and overlying microfilament bundles appear with the typical periodicity of α -actinin (D). The corresponding F-PHD staining is shown in C. The periodicity of α -actinin association with microfilament bundles is shown in an osteoclast (E) and in co-cultured fibroblasts (F). In the latter α -actinin is obviously associated also with adhesion plaques. The brightly fluorescent spots at cell centers (B, D, and E) represent yellow-orange autofluorescence in the rhodamine wavelength. (A–D) \times 500. (E) \times 750. (F) \times 1,300.

ably with a cup-like pattern. Co-cultured fibroblasts observed in the same coverslip show vinculin speckles (Fig. 5 E) corresponding to the typical IRM pattern of focal contacts (Fig. 5 F).

The distribution of vinculin and its stability to detergent extraction has also been studied in conditions in which fixation was preceded by a mild extraction with a Triton X-100-

containing buffer (30 s at 0°C). In these conditions, the regular arrangement of vinculin rosettes is partially damaged (Fig. 5 G), while the association of vinculin with "traditional" adhesion plaques of fibroblasts is not substantially altered (Fig. 5 H).

To define the time course of the *in vitro* appearance of these adhesion structures, the location of vinculin rosettes was

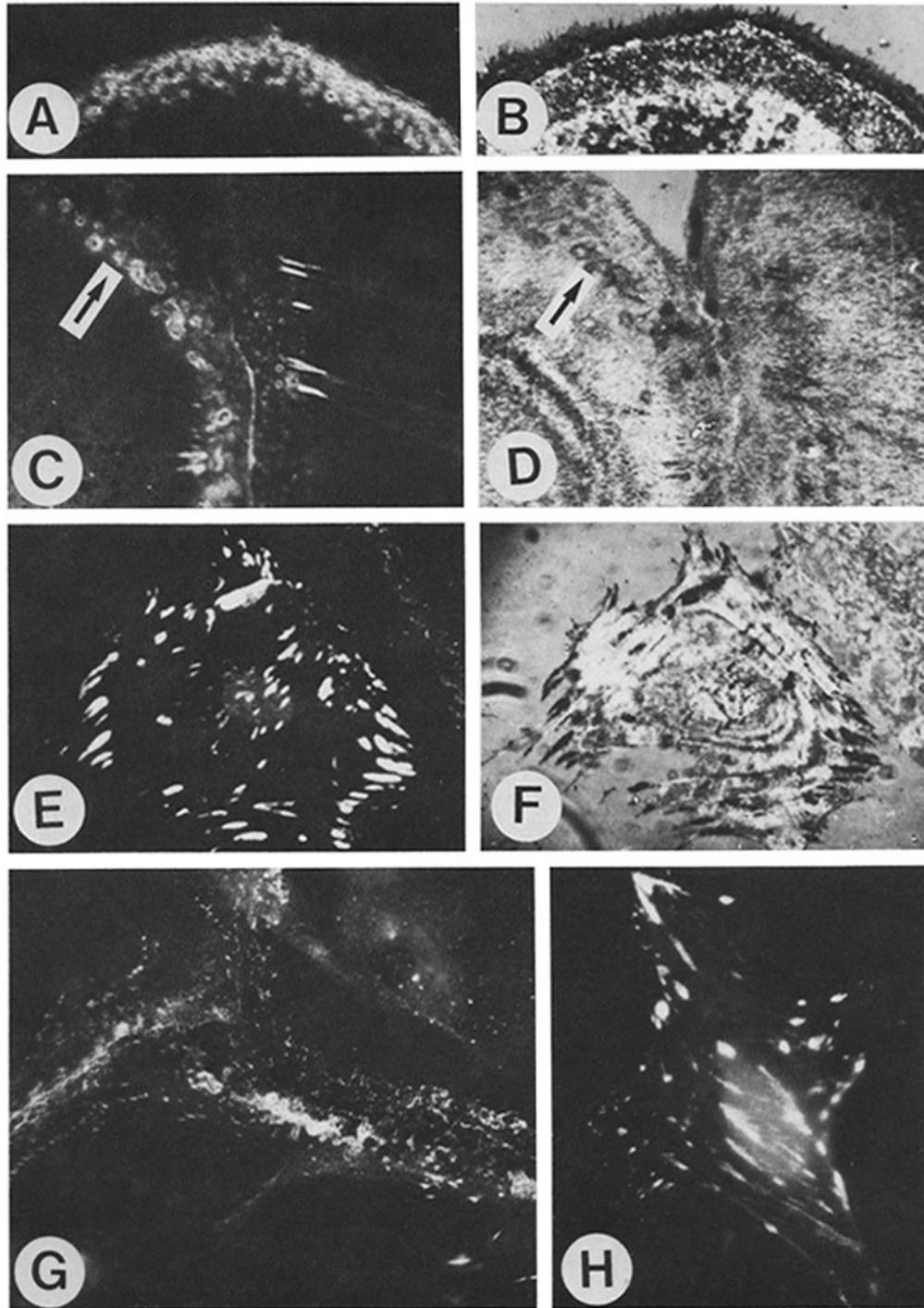


FIGURE 5 Immunofluorescence decoration (A, C, and G) and IRM patterns (B and D) of isolated hen osteoclasts show that vinculin rosettes and speckles correspond to IRM dark areas. At low magnification, the paramarginal band containing circular structures (A) is very dark in IRM (B), indicating that spots of focal contact are concentrated in this band. Most rosettes correspond to dark circles in IRM (C and D, arrows): this indicates that the vinculin-containing circle is also the site where the closest contact occurs. Adhesion plaques in the form of vinculin speckles are also present in fully spread osteoclasts (C and D) and in co-cultured fibroblasts (E and F). The association of vinculin to rosettes in osteoclasts is relatively less resistant to mild detergent treatment (G) than in fibroblasts (H). (A-H) $\times 500$.

followed at different times after plating. A short time after osteoclast attachment, vinculin rosettes predominantly appear in clusters located beneath the cell center and close to the ventral membrane (Fig. 6A). Osteoclasts appear, at this stage, as phase-refringent, nearly spherical cells. Adhesion structures are then located in an area corresponding to the position of the nuclear mass. Upon initial flattening (day 1–2), along with the formation of a typical microtubule array (37), vinculin rosettes spread within the whole ventral surface (Fig. 6B). At somewhat later spreading times (day 3–4) the structures become partially and irregularly confined to the paramarginal area (Fig. 6C). Finally, when full spreading is reached (day 5) and osteoclasts reach a diameter of up to 200–300 μm , vinculin rosettes are exclusively confined in the paramarginal area, usually as continuous rows; occasionally, vinculin speckles similar to those present in adhesion plaques of the fibroblast type are also present (Fig. 6D; see also the co-cultured fibroblast showing speckle-type adhesion plaques).

Electron Microscopy

Spread osteoclasts have been observed in TEM in fine sections cut either parallel or perpendicular to the substratum (Fig. 7).

The general ultrastructural features of osteoclasts *in vitro* do not significantly differ from those described *in vivo* cells (e.g., 15, 17, 21). Cells are rich in mitochondria, lysosomes, and free ribosomes, as expected, while the cluster of nuclei is usually found close to the cell center. The outer cytoplasmic ring immediately adjacent to the peripheral rim is devoid of organelles except for some microtubules and other filamentous structures. In sections cut parallel to the substratum, structures, corresponding in size and location to the adhesion structures described by immunofluorescence microscopy, appear as darker areas formed by an electron-dense nonhomogeneous material that may represent the section of a thick and irregular network of microfilaments (Fig. 7, A and B).

In parallel sections cut very close to the plane of the substratum only the tip of the adhesion structure appears as a dark round foot containing amorphous material and a relatively lighter central area (Fig. 7C). If the section plane is slightly higher and includes the surrounding cytoplasm the structure appears as a dark ring of material surrounding a small and more electron-lucent area (Fig. 7D).

In sections perpendicular to the substratum thin bundles of microfilaments parallel to the outer membrane seem to come in touch with such electron-dense material (Fig. 7F, e.g., at arrowheads). In sections cut across the middle of blunt pro-

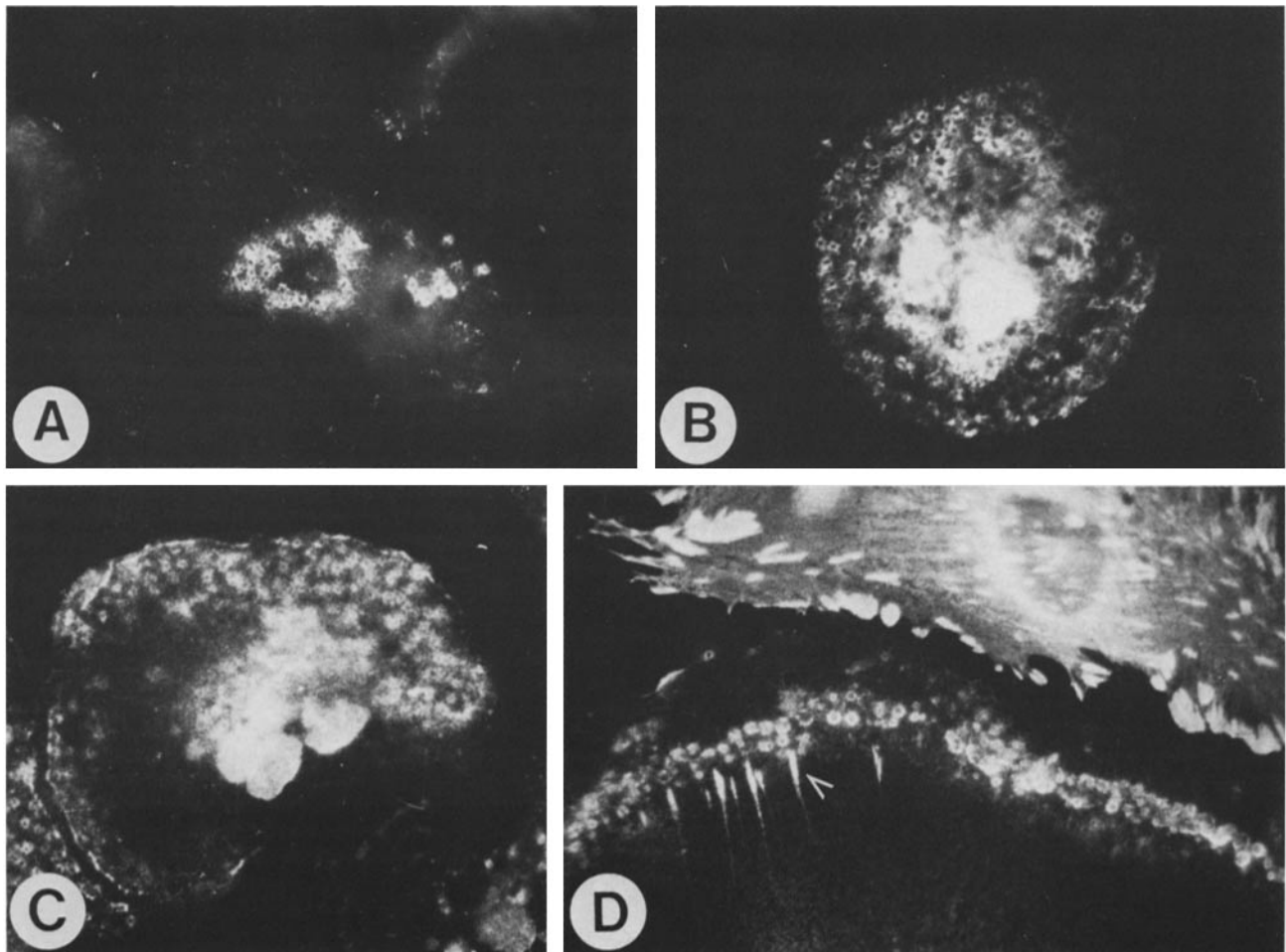


FIGURE 6 Immunofluorescence detection of vinculin in cultured hen osteoclasts during spreading. 6–12 h after seeding, when osteoclasts are still rounded and phase-refringent, vinculin rosettes appear in small clusters lying beneath the nuclear mass (A). Upon initial flattening (day 1–2) vinculin rosettes appear scattered rather homogeneously in the ventral membrane (B) and later on (day 3–4) migrate to form a paramarginal band (C). When full spreading is reached (from day 5 on), vinculin rosettes are confined in a discrete paramarginal area and some streak-like adhesion plaques appear (D, arrowhead). The common appearance of vinculin speckles may be observed in a co-cultured large fibroblast (D, upper half of the picture). (A–D) $\times 500$.

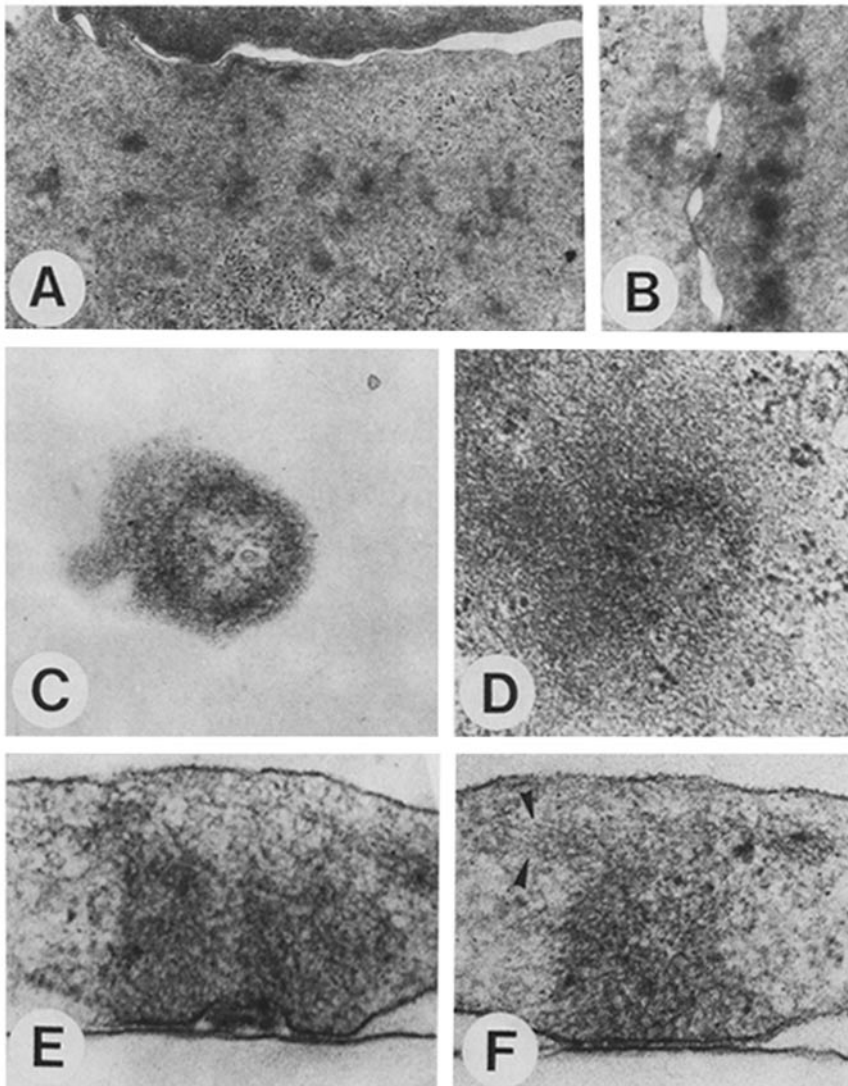


FIGURE 7 Electron micrographs of cultured osteoclast sections cut parallel (A–D) and perpendicular (E and F) to the adhesion substratum. Electron-dense material that may represent the section of F-actin filamentous networks are observed in the paramarginal area (A and B). Sections cut across the protrusion close (C) or removed (D) from the adhesion substratum show a circular arrangement of electron-dense structures. The pattern of such structures directed toward the site of adhesion may be also observed in perpendicular sections of the adhesion microvilli (E and F); parallel bundles of microfilaments seem to contact and probably also partially merge into the electron-dense meshwork found within the protrusion (e.g., F, at arrowheads). A cup-shaped pattern of adhesion is observed in E: some amorphous material is apparently contained in the corresponding cavity. (A) $\times 10,000$. (B) $\times 16,000$. (C and D) $\times 47,500$. (E and F) $\times 75,000$.

trusions the central area appears to be removed from the substratum forming a cup-like chamber often occupied by electron-dense amorphous material (Fig. 7E).

DISCUSSION

Osteoclasts are large multinucleated cells originated from the fusion of monocytes (16, 31, 38). They adhere to and actively resorb bone matrix by means of lysosomal mechanisms (1, 15, 18). At the resorbing front, the membrane of osteoclasts is richly folded and forms a ruffled border. Immediately behind an area of adhesion marks a boundary between the peripheral and central portions of the bone facing area (1, 15, 18).

The availability of a simple and reliable procedure for isolating and culturing an enriched fraction of osteoclasts free of other cell types (36) has allowed a preliminary study of the cytoskeletal and adhesive properties of these cells. In that report, actin-containing dots were described in the paramarginal area of isolated osteoclasts (37). In the present investigation we have found that such dots represent the overview of F-actin-containing structures occupying short protrusions of the ventral surface and most likely responsible for its adhesion to the substratum. The main reason for investing these peculiar processes with an adhesive role is essentially

their content of structural proteins that have been previously localized in the membrane-microfilament interaction at adhesion sites. These are two relatively minor proteins, like fimbrin and α -actinin, which are not strictly specific for such sites (2–5, 8, 19, 24, 34), and a major component such as vinculin (11), which is specifically found at adhesion plaques in several cell types (10–14) and also at other sites of microfilament-membrane connection (14, 25).

A further strong reason to propose an adhesive role of such short protrusions is their extremely close contact with the substratum shown by IRM and TEM. Unlike adhesion plaques found in other cell types (see, e.g., Fig. 5E), vinculin is organized in these protrusions as a rosette-like structure surrounding a core of F-actin-containing material and clearly corresponding to a dark circle in IRM. Alpha-actinin, although in a looser fashion, has a similar distribution. Even if the molecular architecture of the microfilament-membrane connection is not yet well known in these and in other cells, the data of the present paper suggests that vinculin and α -actinin may interact both with the membrane and the F-actin core (Fig. 8). The latter seems directly connected to the membrane without any detectable vinculin link but this does not exclude that the core connection may be mediated by some unknown component (Fig. 8).

As suggested by IRM and TEM pictures, osteoclast adhe-

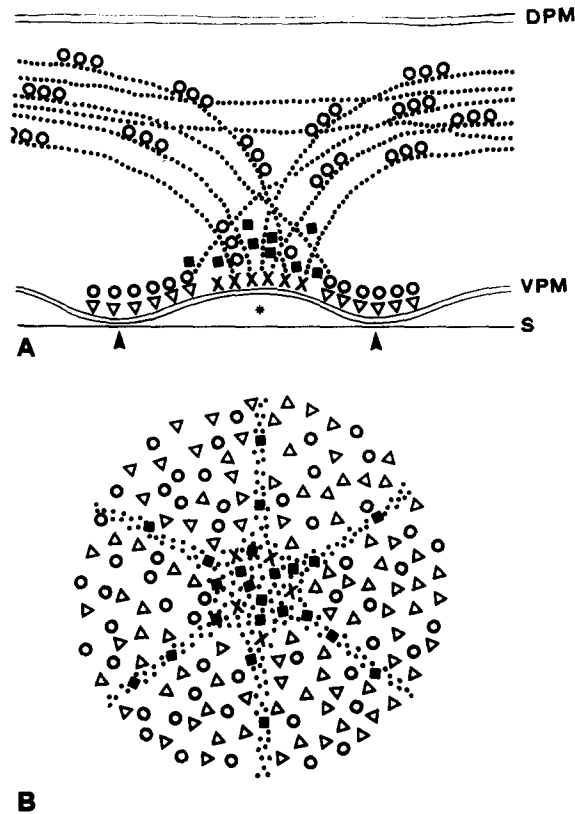


FIGURE 8 Highly schematic representation of the molecular ultrastructure of osteoclast adhesion structures in a perpendicular (A) and top view (B). The relative topography of molecules is deduced from immunofluorescence data. DPM, VPM, and S designate dorsal plasma membrane, ventral plasma membrane, and substratum, respectively. Δ , vinculin; \blacksquare , fimbrin; \circ , α -actinin; X, unknown molecule(s) mediating direct microfilament-membrane connection. F-actin microfilaments are represented by dotted lines. (A) Circumferential microfilaments converge into a network which is connected to the slightly raised nonadherent portion of the membrane (*). Such connection is not apparent mediated by vinculin but may depend on unknown components (x). Vinculin and α -actinin are restricted to the adherent ring of the structure (see also IRM picture, Fig. 5C). Fimbrin is associated only with the core network. The overview scheme (B) suggests that vinculin and α -actinin are presumably organized in a rosette formed by discrete clusters whereas fimbrin and actin occupy the core as well as its radial spokes. See text for further details.

sion occurs primarily in correspondence of ventral rosettes. The stability and the possible functions of these adhesion structures are just a matter of speculation. The F-actin core may be the basis of some form of motility and be in dynamic continuity with the overlying network of cytoplasmic microfilaments. The whole network may then control both adhesion and the crawling migration of osteoclasts both in vitro and in vivo.

To our knowledge, the presence of protrusions of the ventral surface in nontransformed in vitro cells has not yet been described (33). Their involvement in cell-substratum adhesion phenomena seems to be a novel finding in nontransformed cells. Some similarity exists though between these adhesive protrusions and those described in some transformed cell types (33). Another point of similarity between transformed cells and in vitro osteoclasts is represented by the peculiar rosette-like distribution of vinculin described in several types

of Rous sarcoma virus-transformed cells (10, 26, 29) even if their association with protrusions was not explicitly pointed out. Then, we propose that this structure really represents a peculiar adhesion device typical of osteoclasts; but we do not exclude that similar or identical adhesion structures may exist in other normal or transformed cells.

This structure is responsible for the osteoclast adhesion to an artificial substratum in the absence of fibronectin that has previously been shown to be selectively excluded from the osteoclast attachment area in vitro (37). It cannot be ruled out that some other component of the extracellular matrix in vitro or some unknown physico-chemical mechanism could be responsible for the formation of substratum attachment points. It cannot yet be excluded that these peculiar adhesion structures may be directly or indirectly involved in the removal of matrix components from the substratum.

Even if evidence is so far missing, it is conceivable that a similar mechanism of adhesion occurs also in osteoclasts attached to bone matrix in vivo. Previous studies have shown that microfilament bundles directed toward the bone attachment area within short processes sprouting from the osteoclast clear zone do exist in living bone (21). Studies are in the progress to show the nature and the biochemical anatomy of such processes both in the bone matrix and in explanted bone fragments exposed to isolated osteoclasts.

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REFERENCES

- Bonucci, E. 1981. New knowledge on the origin, function and fate of osteoclasts. *Clin. Orthop.* 158:252-269.
- Bretscher, A. 1981. Fimbrin is a cytoskeletal protein that cross-links F-actin in vitro. *Proc. Natl. Acad. Sci. USA.* 78:6849-6853.
- Bretscher, A., and K. Weber. 1978. Localization of actin and microfilament-associated proteins in the microvilli and terminal web of intestinal brush border by immunofluorescence microscopy. *J. Cell Biol.* 79:839-845.
- Bretscher, A., and K. Weber. 1979. Villin: the major microfilament-associated protein of the intestinal microvillus. *Proc. Natl. Acad. Sci. USA.* 76:2321-2325.
- Bretscher, A., and K. Weber. 1980. Fimbrin, a new microfilament-associated protein present in microvilli and other cell surface structures. *J. Cell Biol.* 86:335-340.
- Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195-203.
- Burridge, K., and J. R. Feramisco. 1980. Microinjection and localization of a 130K protein in living fibroblasts: a relationship to actin and fibronectin. *Cell.* 19:587-595.
- Chen, W.-T., and S. J. Singer. 1982. Immunoelectron microscopic studies of the sites of cell-substratum and cell-cell contacts in cultured fibroblasts. *J. Cell Biol.* 95:205-222.
- Curtis, A. S. G. 1964. The mechanism of adhesion of cells to glass. A study by interference reflection microscopy. *J. Cell Biol.* 20:199-215.
- David-Pfeuty, T., and S. J. Singer. 1980. Altered distribution of the cytoskeletal proteins vinculin and α -actinin in cultured fibroblasts transformed by Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA.* 77:6687-6691.
- Geiger, B. 1979. A 130K protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. *Cell.* 18:193-205.
- Geiger, B. 1982. Involvement of vinculin in contact-induced cytoskeletal interactions. *Cold Spring Harbor Symp. Quant. Biol.* 46:671-682.
- Geiger, B., A. H. Dutton, K. T. Tokuyasu, and S. J. Singer. 1981. Immunoelectron microscopic studies of membrane-microfilament interactions: the distribution of α -actinin, tropomyosin, and vinculin in intestinal epithelial brush border and chicken gizzard smooth muscle cells. *J. Cell Biol.* 91:614-628.
- Geiger, B., K. T. Tokuyasu, A. H. Dutton, and S. J. Singer. 1980. Vinculin, an intracellular protein localized at specialized sites where microfilament bundles terminate at cell membranes. *Proc. Natl. Acad. Sci. USA.* 77:4127-4131.

15. Gothlin, G., and J. L. E. Ericsson. 1976. The osteoclast: review of ultrastructure, origin and structure-function relationship. *Clin. Orthop.* 120:201-228.
16. Hall, B. K. 1975. The origin and fate of osteoclasts. *Anat. Rec.* 183:1-12.
17. Hancox, N. M., and B. Boothroyd. 1961. Motion picture and electron microscopic study on the embryonic avian osteoclasts. *J. Biophys. Biochem. Cytol.* 11:651-661.
18. Holtrop, M. E., and G. J. King. 1977. The ultrastructure of osteoclast and its functional implications. *Clin. Orthop.* 123:177-196.
19. Jochkusch, B. M., and G. Isenberg. 1981. Interaction of α -actinin and vinculin with actin: opposite effects on filament network formation. *Proc. Natl. Acad. Sci. USA.* 78:3005-3010.
20. Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.* 27(2, Pt. 2):137A-138A. (Abstr.)
21. King, G. J., and M. E. Holtrop. 1975. Actin-like filaments in bone cells as demonstrated by binding to heavy meromyosin. *J. Cell Biol.* 66:445-451.
22. Izzard, C. S., and L. R. Lochner. 1976. Cell-to-substrate contacts in living fibroblasts: an interference reflexion study with an evaluation of the technique. *J. Cell Sci.* 21:129-159.
23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
24. Lazarides, E., and K. Burridge. 1975. α -Actinin: immunofluorescence localization of a muscle structural protein in non-muscle cells. *Cell.* 6:289-300.
25. Pardo, J. V., J. D'Angelo Siliciano, and S. W. Craig. 1983. Vinculin is a component of an extensive network of myofibril-sarcolemma attachment regions in cardiac muscle fibers. *J. Cell Biol.* 97:1081-1088.
26. Rohrschneider, L., and M. J. Rosok. 1983. Transformation parameters and *pp60src* localization in cells infected with partial transformation mutants of Rous sarcoma virus. *Mol. Cell. Biol.* 3:731-746.
27. Ryder, M. I., S. D. Jenkins, and J. E. Horton. 1981. The adherence to bone by cytoplasmic elements of osteoclasts. *J. Dent. Res.* 60:1349-1355.
28. Schenk, R. K., D. Spiro, and J. Wiener. 1967. Cartilage resorption in the tibial epiphyseal plate of growing rats. *J. Cell Biol.* 34:275-291.
29. Shriver, K., and L. Rohrschneider. 1981. Organization of *pp60src* and selected cytoskeletal proteins within adhesion plaques and junctions of Rous sarcoma virus-transformed rat cells. *J. Cell Biol.* 89:525-535.
30. Suda, T., N. G. Testa, T. D. Allen, D. Onions, and O. Jarrett. 1983. Effect of hydrocortisone on osteoclasts generated in cat bone marrow cultures. *Calcif. Tissue Int.* 35:82-86.
31. Tinkler, S. M. B., J. E. Linder, D. M. Williams, and N. W. Johnson. 1981. Formation of osteoclasts from blood monocytes during 1α -OH Vit.D-stimulated bone resorption in mice. *J. Anat. (Lond.)*. 133:57-70.
32. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
33. Vasiliev, J. M., and I. M. Gelfand. 1977. Mechanisms of morphogenesis in cell cultures. *Int. Rev. Cytol.* 50:159-274.
34. Wehland, J., M. Osborn, and K. Weber. 1979. Cell-to-substratum contacts in living cells. A direct correlation between interference-reflexion and indirect immunofluorescence microscopy using antibodies against actin and α -actinin. *J. Cell Sci.* 37:257-273.
35. Wulf, E., A. Deboben, F. A. Bautz, H. Faulstich, and T. Wieland. 1979. Fluorescent phalloxin, a tool for the visualization of cellular actin. *Proc. Natl. Acad. Sci. USA.* 76:4498-4502.
36. Zamboni-Zallone, A., A. Teti, and M. V. Primavera. 1982. Isolated osteoclasts in primary culture: first observations on structure and survival in culture media. *Anat. Embryol.* 165:405-413.
37. Zamboni-Zallone, A., A. Teti, M. V. Primavera, L. Naldini, and P. C. Marchisio. 1983. Osteoclasts and monocytes have similar cytoskeletal structures and adhesion property in vitro. *J. Anat. (Lond.)*. 137:57-70.
38. Zamboni-Zallone, A., A. Teti, and M. V. Primavera. 1984. Monocytes from circulating blood fuse in vitro with purified osteoclasts in primary culture. *J. Cell Sci.* 66:335-342.
39. Zamboni-Zallone, A., A. Teti, and M. V. Primavera. 1984. Resorption of vital or devitalized bone by isolated osteoclasts in vitro: the role of lining cells. *Cell Tissue Res.* 235:561-564.