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STUDIES ON INTERCELLULAR INVASION IN VITRO USING RABBIT PERITONEAL NEUTROPHIL GRANULOCYTES (PMNS)

I. Role of Contact Inhibition of Locomotion

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ABSTRACT

Intercellular invasion is the active migration of cells of one type into the interiors of tissues composed of cells of dissimilar cell types. Contact paralysis of locomotion is the cessation of forward extension of the pseudopods of a cell as a result of its collision with another cell. One hypothesis to account for intercellular invasion proposes that a necessary condition for a cell type to be invasive to a given host tissue is that it lack contact paralysis of locomotion during collision with cells of that host tissue. The hypothesis has been tested using rabbit peritoneal neutrophil granulocytes (PMNs) as the invasive cell type and chick embryo fibroblasts as the host tissue. In organ culture, PMNs rapidly invade aggregates of fibroblasts. The behavior of the pseudopods of PMNs during collision with fibroblasts was analyzed for contact paralysis by a study of time-lapse films of cells in mixed monolayer culture. In monolayer culture, PMNs show little sign of paralysis of the pseudopods upon collision with fibroblasts and thus conform in their behavior to that predicted by the hypothesis.

The cells of most tissue types tend to remain within the confines of the parent tissues for the lifetime of the adult organism. Such cells can be said to be noninvasive. However, most of these cell types retain the ability to locomote by means of the activities of pseudopods, as is shown by the motile behavior of cells placed in tissue culture (for review, see Abercrombie, 1970) and the active locomotion of cells engaged in wound healing (Lash, 1955). In contrast, certain other cell types show invasive behavior, by which is meant that these cells can migrate into the interiors of tissues and organs composed of dissimilar cell types. This behavior is exhibited by malignant cells (for reviews, see Abercrombie and Ambrose, 1962; Leighton, 1967), by the various motile blood cells of vertebrates (Clark et al., 1936; Allison et al., 1955; Cliff, 1966) and invertebrates (Andrew, 1965), by mammalian trophoblast (Boyd and Hamilton, 1952; Enders, 1971; Enders and Schlafke, 1972), and by certain ameboid protozoan parasites (Culbertson, 1971; Neal, 1960; Eaton et al., 1970; Proctor and Gregory, 1972).

Of particular interest to the study of the mechanisms of intercellular invasion is an elucidation of the factors which are important, on the one hand, in preventing most cell types from engaging in invasive behavior and, on the other hand, in causing certain cell types to be invasive. Invasion appears largely to be the result of active motility of

the invading cells. This has best been shown by observation of white blood cells (Clark et al., 1936; Allison et al., 1955; Grant et al., 1962; Graham et al., 1965; Cliff, 1966) and carcinoma cells (Wood et al., 1968) in vivo in the tadpole tail fin and the rabbit ear chamber where the motile activities of invading cells can be seen directly, and by fine structural observaton of pseudopodia of invading cells (malignant cells: Luibel et al., 1960; Ashworth et al., 1961; Fisher and Fisher, 1961; Nilsson, 1962; Mao et al., 1966; Tarin, 1967; Riley and Seal, 1968; Sugár, 1968; Seal et al., 1969; Woods and Smith, 1969; Butterworth, 1970; Ozzello and Sanpitak, 1970; Babai and Tremblay, 1972; white blood cells: Marchesi and Florey, 1960; Florey and Grant, 1961; and vertebrate trophoblast: Enders and Schlafke, 1972). This involvement of active cellular locomotion in invasion suggests that invasion may result from a defect in control mechanisms that prevent potentially motile, noninvasive tissue cells from actually moving from their parent tissues.

One control mechanism of potential importance in this regard is contact inhibition of locomotion, described for cells in tissue culture by Abercrombie and co-workers (reviewed in Abercrombie, 1970; see also Armstrong and Armstrong, 1973, for references). When cultured in contact with a solid adhesive substratum, many cell types organize as a monolayer. When moving cells of this monolayer collide, movement in the direction of the contact tends to slow or come to a halt. In particular, contacting cells show a profound reluctance to migrate onto the dorsal surfaces[1] of neighboring cells. The cessation of forward movement upon contact is generally accompanied by a cessation of the activity of the locomotory processes of the cell (Abercrombie and Ambrose, 1958; Trinkaus et al., 1971 *a*, *b*; Bell, 1972; Vesely and Weiss, 1973; Armstrong and Armstrong, 1973). Thus, in monolayer culture, most cells do not use other cells as substrata on which to locomote. The phenomena described above have been included under the term "contact inhibition of movement" (defined by Abercrombie [1970] as "the directional restriction of displacement on contact"). Martz and Steinberg (1973) have distinguished between contact inhibition of speed, of overlapping, and of ruffling activity[2] (where ruffling is defined as the surface activity of the cell processes involved in motility). Abercrombie has distinguished between type I and type II contact inhibition of movement: in type I inhibition both an inhibition of overlapping and contact paralysis are observed, whereas in type II inhibition extensive overlapping does not occur although there is no inhibition of pseudopodial activity. Presumably, in solid tissues in vivo, where cells are in contact on all sides, contact inhibition of movement would serve to restrain the movement of cells (but see Armstrong and Armstrong, 1973; Elsdale and Bard, 1972; Wiseman and Steinberg, 1973). Once an adhesive surface depleted of cells (either a wound surface or the surface provided in tissue culture) is present, cell movement would be able to commence along the margins where cells are not in contact with other cells.

Of interest to the problem of mechanisms of intercellular invasion is a possible relationship between invasive ability and an absence of contact paralysis. Abercrombie (1958, 1961 *a*, *b*, 1967 *a*, *b*, 1970), Abercrombie and Ambrose (1958), and Vesely and Weiss (1973) observed that a variety of sarcomas lack contact inhibition of pseudopodial activity under conditions where normal fibroblasts show contact paralysis. These observations suggest that a necessary condition for a cell to be invasive to a given host tissue is that it lack contact paralysis on collision with cells of that host tissue. An obvious test of the hypothesis is to examine the behavior of the variety of invasive cells to ascertain whether they all do indeed lack inhibition of pseudopodial activity upon collision with host tissue cells. The present paper reports a test of the hypothesis for invasive rabbit peritoneal neutrophil granulocytes (PMNs), with chick embryo fibroblasts serving as the host tissue.

MATERIALS AND METHODS

Culture of Fibroblasts

Tissues from 7–10 day chick embryos were dissociated with 0.1% trypsin (Difco Laboratories, Detroit, Mich., 1:250) as described previously (Armstrong, 1970, 1971). Dissociated cells were plated onto plastic tissue culture dishes for growth to confluent monolayers or onto cover

[1] The surface of the cell in monolayer culture facing the culture medium is referred to as the dorsal surface, and the surface contacting the substratum as the ventral surface.

[2] In the report that follows, the terms "contact paralysis" (Wolpert and Gingell, 1968) and "contact inhibition of pseudopodial activity" will be substituted for "contact inhibition of ruffling."

slips for filming and for overlap analyses. Culture medium was M199 + 10% calf serum (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid-[HEPES]-buffered) or Dulbecco-modified Eagle's medium + 10% calf serum (bicarbonate-buffered, 5% CO_2-air gas phase). Fibroblasts from 8–10-day chick embryo heart ventricle and gizzard were used most extensively; fibroblasts from 10-day mesonephros and lung were used for a few experiments. Where necessary, nonfibroblastic cells were removed after 1–2 h in culture by pouring off the unattached cells and adding fresh medium to the culture dishes. Since fibroblasts attach more rapidly than most nonfibroblastic cells, relatively pure populations of fibroblasts can be obtained in this manner (see Polinger, 1970). The glass cover slips were cleaned before use by ultrasonication in hot detergent solution followed by exhaustive washing in glass-distilled water and a wash in 95% ethyl alcohol. Alcohol was removed by wiping with soft absorbent paper. The cover slips were steam autoclaved.

Rabbit Peritoneal Neutrophil Granulocytes (PMNs)

PMNs were obtained from the exudate produced after intraperitoneal injection of warm normal saline into primed adult New Zealand white rabbits (Hirsch, 1956). When sterile saline (250–400 ml per injection) was used, oyster glycogen (Sigma Chemical Co., St. Louis, Mo.) was added to a concentration of 0.1%. Glycogen was omitted when nonsterile saline (800–1,000 ml per injection) was injected. The saline was collected after 3–4 h or after 12–14 h. PMNs were further treated as described in Lackie (1974). In some cases, PMNs were stored in saline exudate at 4°C for 1 or 2 days before use. Such cells appeared to be as motile in monolayer culture and as invasive in organ culture as freshly collected PMNs.

Organ Culture

Solid aggregates of fibroblasts were prepared by dividing confluent monolayers grown in 90-mm diam plastic petri dishes into 1-cm squares by scoring with a rubber policeman. The squares of tissue were gently scraped from the dish with the rubber policeman and were placed in siliconized 25-ml Erlenmeyer flasks in 3 ml culture medium. The flasks were rotated on a gyratory shaker at 100 rpm at 37°C. During culture overnight, the fragments rounded up into compact spheres of tissue. Moderately cohesive aggregates of PMNs were prepared by centrifuging a washed cell suspension and cutting the pellet into cubes approximately 1–2 mm on a side.

Invasion of PMNs into fibroblast aggregates was studied in hanging-drop organ cultures (technique of Armstrong and Niederman, 1972; Niderman and Armstrong, 1972; Armstrong and Armstrong, 1973). Each drop contained a single fibroblast aggregate and a single PMN aggregate. Prepared initially as standing drops, the two aggregates come into close contact at the bottom of the hanging drop when the culture is inverted. Cultures were maintained for 5–10 h at 37°C in moist chambers. Aggregates were fixed in 10% neutral-buffered Formol-saline, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin or Giemsa stain. PMNs could easily be distinguished from fibroblasts in sectioned preparations by the characteristic morphology of the nucleus and the eosinophilia of the cytoplasm.

Time Lapse Microcinematography

Fibroblasts and PMNs were filmed on inverted cover slips. At low magnifications, Sykes-Moore chambers (Bellco Glass, Inc., Vineland, N. J.) were used in conjunction with a long working-distance phase-contrast condenser. For filming at high magnifications, chambers were constructed by sealing cover slips to both sides of a 1.6-cm diam hole cut through a 0.75-mm thickness microscope slide. These permitted the use of a 1.3 NA phase-contrast and Nomarski differential interference-contrast condenser. Wild time-lapse equipment (Wild Heerbrugg Instruments, Inc., Farmingdale, N.Y.) was used with Zeiss WL and Photomicroscope III microscopes and a Zeiss RA microscope modified for interference-reflection microscopy (Curtis, 1964).[3] Most of the filming utilized a Planapochromat 40/1.0 phase-contrast objective or a Planapochromat 100/1.25 objective with Nomarski differential interference contrast optics. The interference reflection microscope was used with the Planapochromat 40/1.0 objective. Some filming was also performed with lower power objectives. Filming speeds of 15, 30, or 60 frames per minute were used. Culture chambers were maintained at 37°C with air curtain incubators. Since PMNs appear to be sensitive to strong illumination, a dark shutter was used to shield the chambers between exposures.

Film Analysis

A key aspect of the present study is the quantitative evaluation of contact behavior of PMNs with fibroblasts from time-lapse films. To accomplish this, every collision between a PMN and a fibroblast on the films analyzed was tabulated regarding the position of the fibroblast contacted, the length of time in contact, and the result of the contact in terms of degree of overlapping or underlapping, and the behavior of the pseudopodial apparatus of both PMN and fibroblast. Detailed descriptive notes were also made. An L.-W. stop-action projector (L.-W. Photo, Inc., Van Nuys, Calif.) was used for these studies. Great care was taken in the analysis: every cell was assigned an identity number, and note was made of the beginning and ending frame for each contact. This al-

[3] M. Abercrombie and G. A. Dunn. Manuscript in preparation.

lowed easy relocation of every contact event. The time-lapse microcinematographic studies were supplemented by careful and detailed studies made directly on living cultures.

Overlap Analysis

The extent of overlapping of cells in mixed monolayer cultures containing both PMNs and fibroblasts was performed and analyzed as suggested by (Abercrombie et al., 1957). Fibroblasts were plated onto glass cover slips at 300–600 cells/mm² and were allowed to attach and spread for 1 day. At these densities, more than half of the area of the substratum is free of cells. PMNs were then added at 1,900 cells/mm². After 3–5 h, the cultures were fixed, stained in hematoxylin, and mounted as whole mounts. The duration of staining was adjusted so that PMN nuclei were darkly stained but fibroblast nuclei were lightly stained (7 min in Mayer's hematoxylin), allowing certain identification of the two cell types. The number of nuclear overlaps was determined using a microscope fitted with a Planapochromat 40/1.0 objective with phase-contrast optics and a 10 × 10 eyepiece grid. In excess of 1,000 cells were counted for each culture. The observed number of nuclear overlaps (O) was compared to the number of overlaps that would be expected (E) if the cells (nuclei) were distributed at random by the overlap index (I):

$$I = \frac{O}{E} \times 100\%.$$

Values of I of less than 100% indicate an inhibition of overlapping. For fibroblast-fibroblast nuclear overlaps, $E_{F\text{-}F}$ was calculated from the formula:

$$E_{F\text{-}F} = \frac{P}{nC}(N_F),$$

where P is the number of times an intersection of the eyepiece grid falls within the perimeter of a fibroblast nucleus, N_F is the number of fibroblasts seen, n is the number of fields counted, and C is the number of grid intersections (100). The expected number of PMN-fibroblast overlaps ($E_{P\text{-}F}$) was calculated from the same formula with the substitution of N_P (the number of PMNs seen) for N_F. The significance of the difference of the overlap index from 100% was tested by the chi-square test.

PMN Adhesion to Collecting Monolayers

Chick heart fibroblasts were grown as monolayer cultures on 13-mm glass cover slips until fully confluent, and were then placed in Linbro tissue culture trays, with one cover slip per well. Control cover slips were placed in adjacent wells, and 1 ml of PMN suspension in M199 + 10% calf serum (HEPES-buffered) was added to each well. The tray was incubated for 30 min at 37°C then the cover slips were removed, washed by dipping twice through a Hanks-HEPES/air interface and fixed in neutral-buffered Formol-saline. The cover slips were then stained with Giemsa's (10 min), cleared, and mounted. With an eyepiece grid, 10 separate fields were counted, the area of grid used being chosen to give a total count of approximately 1,000 PMNs. Only areas of confluent fibroblasts were counted, and the PMNs could readily be distinguished from fibroblasts.

RESULTS AND OBSERVATIONS

Invasive Potential of Rabbit Peritoneal Neutrophils in Organ Culture

The invasive abilities of rabbit PMNs to host tissues composed of chick embryo fibroblasts were determined in hanging-drop organ culture. Under these conditions, PMNs invade aggregates of fibroblasts derived from all organs tested (heart, gizzard, lung, and mesonephros). Invasion is rapid and frequently extensive (Fig. 1). These observations suggest that rabbit peritoneal PMNs are truly invasive to host tissues composed of chick embryo fibroblasts under conditions of culture as similar as possible to those used for the production of time-lapse films.

Morphology and Locomotion of Fibroblasts and PMNs in Monolayer Culture

Under the conditions of monolayer culture used in the present study, chick embryo fibroblasts are flattened on the glass substratum. As has been described previously (Abercrombie et al., 1970 *a*, *b*), the pseudopods (termed lamellipods) involved in locomotion are broad and much flattened. Locomotion is correlated with expansion of the lamellipod over the substratum. Lamellipods show local ridgelike thickenings (or ruffles) of the surface exposed to the culture medium. The ruffles are initiated at the margin of the pseudopod and fold inward to merge with more proximal portions of the leading lamella (Abercrombie and Ambrose, 1958; Abercrombie, 1961 *b*; Ingram, 1969; Harris, 1973 *a*).

The PMNs obtained from sterile peritoneal exudates are morphologically uniform in monolayer culture. The cells are refractile and little flattened on the substratum (Figs. 2, 3). The cells extend and retract pseudopodia with great vigor and move rapidly across the substratum. Even when moving freely over the substratum, PMNs show frequent change in the direction of movement. The following account is based on real-time

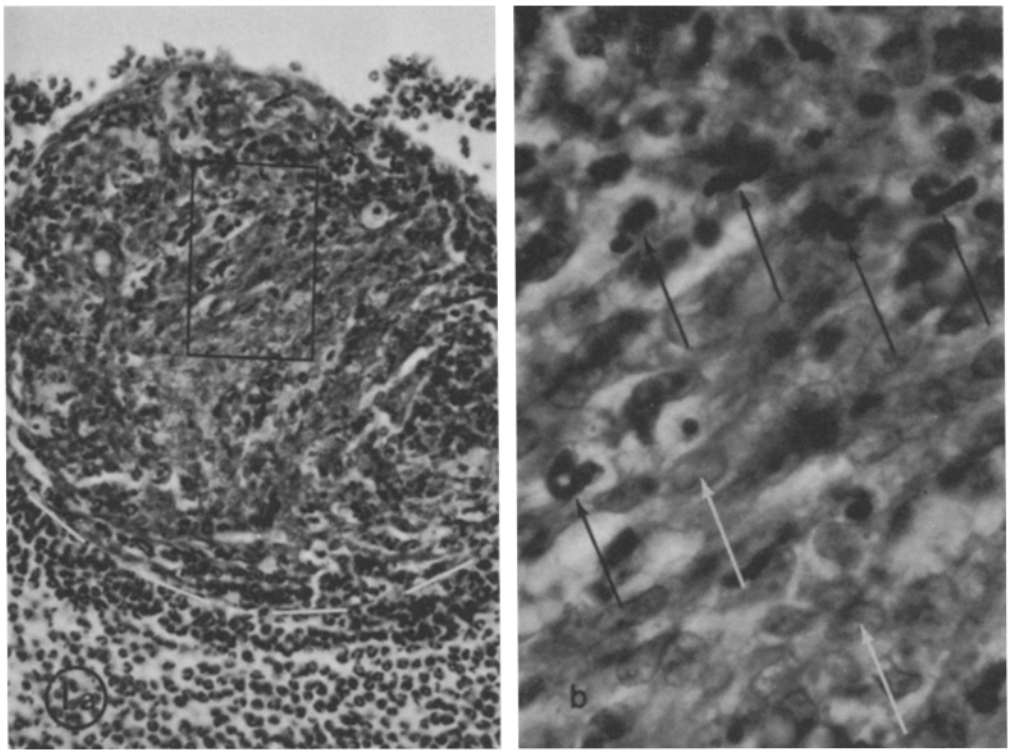


FIGURE 1 Invasion of an aggregate of chick embryo gizzard fibroblasts by rabbit peritoneal PMNs in organ culture. The fibroblast aggregate was prepared by allowing a fragment of a confluent monolayer of gizzard fibroblasts to round up into a solid tissue mass during overnight culture in suspension culture. The fibroblast aggregate and the PMNs were then cocultured in a hanging drop of medium for 8.5 h before fixation and processing. PMNs can be identified by the darkly staining pleiomorphic nuclei (and in the original slides by the eosinophilic cytoplasm) (dark arrows). Fibroblast nuclei (white arrows) are pale and oval with finely granular chromatin. Fig. 1 *b* encompasses the area delineated by the black rectangle in the center of the fibroblast aggregate depicted in Fig. 1 *a* at a higher magnification. Even in the center of the aggregate (*b*) there is a considerable number of PMNs; at the periphery of the fibroblast aggregate (delineated by the dashed line in [*a*]), PMNs which have invaded the aggregate outnumber the fibroblasts.

and time-lapse cinematographic observations of cell movement by use of Nomarski optics with a Planachromat 100/1.25 objective and on the examination of glutaraldehyde-fixed and critical point-dried cells with the scanning electron microscope.

Two sorts of cell movement of PMNs have been observed in monolayer culture: (*a*) lamellipodial movement (Fig. 2) in which the cell extends flattened lamellar processes over the substrate. The processes usually have an even or finely serrated margin during extension and are hyaline and lack visible cell organelles. Extension is rapid, taking 5–15 s. An extended process may or may not serve as a basis for forward movement of the cell. If it does serve as a basis for forward movement, the process first thickens proximally while remaining hyaline and is then invaded by cell organelles which stream rapidly into the process, moving the cell forward. This manner of movement is similar to that described by Ramsey (1972) for human peripheral blood neutrophils; (*b*) looping movement where in addition to the lamellar processes extended in contact with the substratum, the cells continually protrude and retract processes of various shapes and sizes from their exposed surface (Fig. 3). Some of these are hyaline and organelle-free and may be lamellar or cylindrical in shape, whereas others appear to involve the main body of the cell with a full complement of cell organelles. Although most of these processes never contact the substratum, occasionally one does.

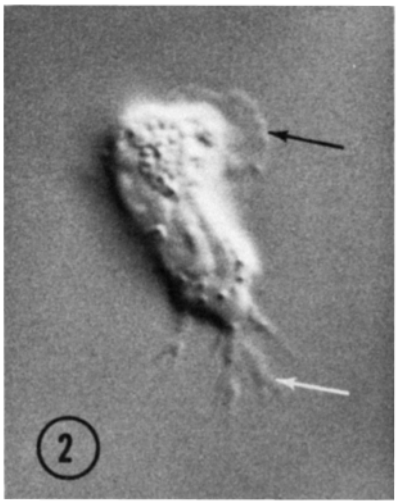
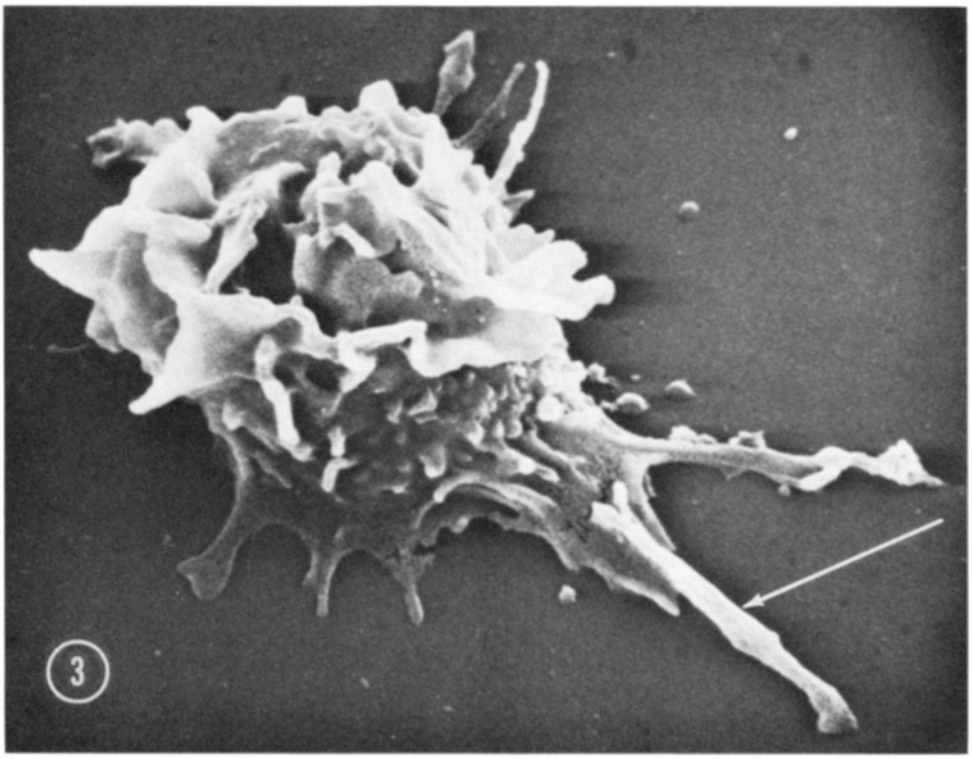


FIGURE 2 Nomarski differential interference contrast photograph of a rabbit peritoneal PMN (collected at 4 h from a sterile exudate) in monolayer culture. The microscope is focused on the glass-culture medium interface. The PMN has protruded a lamellipod (dark arrow). At the trailing end of the cell, the uropod with its retraction fibers is evident (white arrow).

FIGURE 3 Scanning electron micrograph of the dorsal surface of a critical-point dried rabbit peritoneal PMN in monolayer culture showing the elaborate array of lamellar processes produced at this surface. Notice also the retraction fibers (white arrow).



After such contacts, the area of contact broadens and granule flow in a forward direction may occur. During this form of locomotion, initial contact with the substratum may occur along ridges parallel to the direction of movement (Fig. 4) or may occur at a site separate from the pre-existing zone of contact. In either case, broadening of the new area of contact is rapid.

The two modes of locomotion described above occur in the same cell (Fig. 4 *h*). In both cases, forward flow of cytoplasmic granules is a prominent feature. In rapidly and continuously moving cells, granule flow in continuous. In less active cells, granule streaming is sporadic, occurring principally during phases of forward movement of the cell as a whole. During other times, cytoplasmic granules show small-scale Brownian movement, indicating a fluid consistency to the endoplasm.

Regardless of the mode of movement, moving

cells show a tapered trailing process (Figs. 2, 3) which is probably similar to the uropod seen in cultured lymphocytes (Trowell, 1958). As the cell moves, retraction fibers are continually being drawn out from the uropod to residual points of attachment (to the substratum, other PMNs, or fibroblasts) made previously along the path of movement (Figs. 2, 3). Retraction fibers as long as 3.5 cell diameters have been observed. As the retraction fibers become stretched by the movement of the cell they either detach from the substratum and retract into the cell, or they contract, pulling the cell free of the substratum. In the latter case, in inverted chambers the cell may dangle from the substratum supported by the retraction fiber and either fall off completely or eventually recontact the substratum and respread. While dangling in the fluid culture medium, the cells continue to produce both cylindrical and lamellar pseudopods with unabated vigor, indicating that contact with an adhesive substratum is not required for pseudopodial extension. Granule streaming is sometimes noted in dangling cells.

In sum, in monolayer culture, the rabbit peritoneal PMN is a highly active, rapidly moving cell. Both the rounded morphology and the readiness

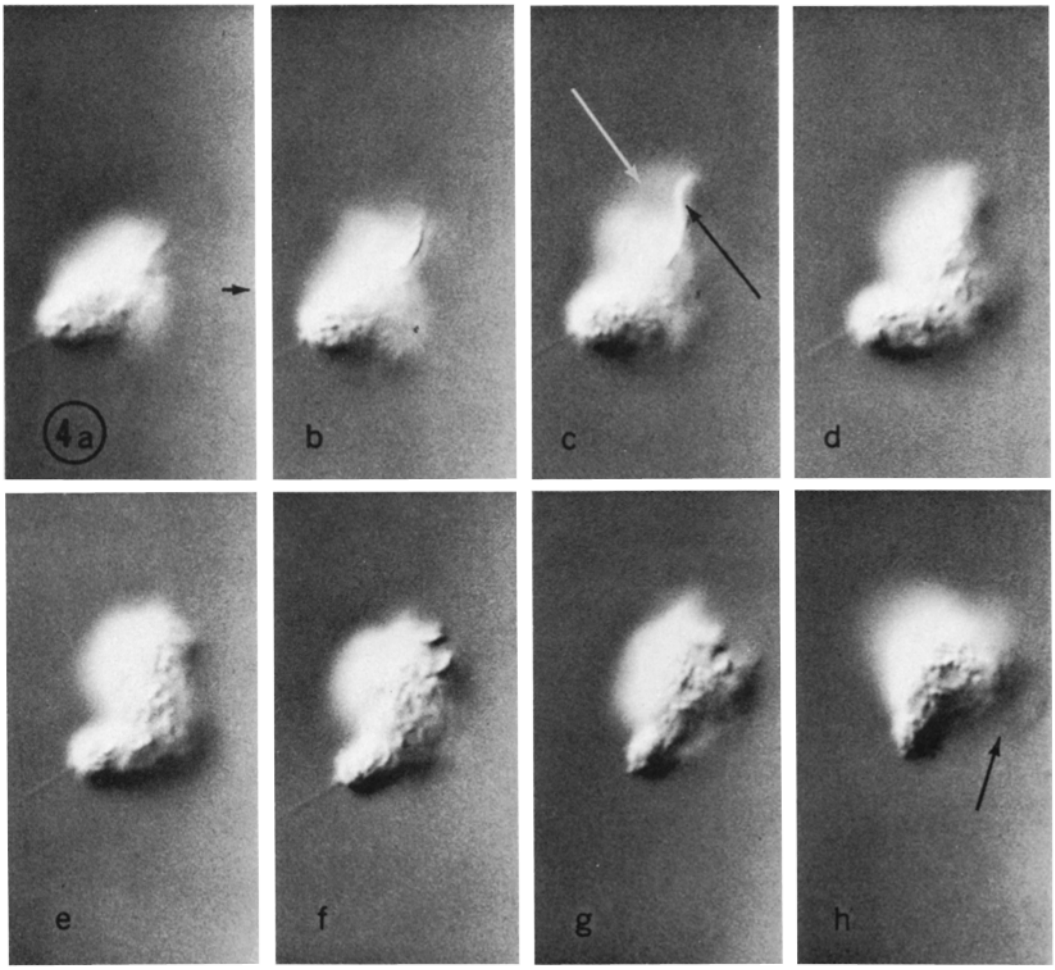


FIGURE 4 Successive pictures made at 10–15-s intervals of a PMN in monolayer culture showing "looping" movement. The plane of focus is at the culture medium-glass interface. In Fig. 4 *b,c*, the PMN protrudes a pseudopod in a plane above the substratum (evident as an out-of-focus pale area [white arrows]) which approaches and contacts the substratum along a narrow ridge (dark arrow). In Fig. 4 *d,e*, the area of contact of the pseudopodium broadens and the cell moves forward as the cytoplasmic contents flow into this region (*e–g*). In Fig. 4 *g*, the PMN is protruding a lamellar pseudopod (arrow) of the same kind as shown in Fig. 2. All pictures are in register (notice the small dimple of the cover slip in all pictures, arrow, Fig. 4 *a*).

with which PMNs are dislodged from the substratum support the impression that they adhere less strongly to the substratum than do fibroblasts. Further support is gained from examination of cells with the interference-reflection microscope under conditions which allow a determination of the extent and degree of close contact between cell and substratum (Curtis, 1964; Lochner and Izzard, 1973).[3] Although PMNs may show moderate areas of gray interference colors indicative of moderately close approach to the substratum (Fig. 5*a*, *b*), the dark gray or black patches ("feet") sometimes observed with fibroblasts and thought to represent regions of very close approach (Lochner and Izzard, 1973)[3] have not been observed. In rapidly moving cells, the gray area is located beneath extending lamellipods, the cell body, the uropod, and at the ends of retraction fibers. The gray area beneath the cell body has scalloped and indented margins and fluctuates very rapidly in extent and contour (changing within fractions of a second). The extent of the gray area may shrink rapidly to a small point, after which the cell usually detaches. Both lamellipodial and looping movement have been observed with the interference-reflection microscope.

The PMNs produced from 12–14-h exudates induced by injection of nonsterile saline were morphologically heterogeneous. Most are identical in appearance and similar in behavior to the rounded cells collected from sterile exudates, but others show varying degrees of flattening on the substratum (Fig. 6 *a*). In the interference-reflection microscope, flattened cells did not show dark gray feet but did show broader areas of light gray interference than did rounded PMNs. Ruffling activity may be observed in flattened cells (Fig. 6 *b*). During translocation, the streaming of cytoplasmic granules observed in rounded cells is present also in flattened cells. In the report that follows, PMNs collected from sterile exudates will be designated as "sterile PMNs" to distinguish them from "nonsterile PMNs" collected from exudates produced by injection of nonsterile saline.[4]

[4] The basis for the differences in morphology and behavior of PMNs collected by the two different procedures is not understood by us and may, perhaps, be independent on the presence or absence of bacteria in the injected saline.

Contact Inhibition of Pseudopodial Activity after Collisions between Sterile PMNs and Fibroblasts in Monolayer Culture

Contact paralysis as exhibited by fibroblasts involves a local cessation of expansion of the leading lamella (e.g., the pseudopod) within a few minutes of collision (Figs. 7, 8). This is often followed by a local retraction of the inhibited lamellipod (Fig. 8) (Abercrombie and Ambrose, 1958; Trinkaus et al., 1971 *a*, *b*; Bell, 1972; Vesely and Weiss, 1973; Armstrong and Armstrong, 1973). The behavior of fibroblasts after collision with other fibroblasts conforms to that of type I contact inhibition as defined in the introductory paragraphs. The hypothesis under investigation requires that invasive cell types should not show this manner of behavior upon collision with host cells. The data presented in Table I and Figs. 9, 10, and 11 describe our observations on this score. In sum, the rabbit peritoneal PMN *does not* show contact paralysis upon collision with fibroblasts. A majority of collisions resulted in extensive or moderate overlapping of the exposed surface of the fibroblast. In the most extreme cases, the PMN pulled free of the substratum and moved completely onto the exposed surface of the fibroblast (Fig. 9). More frequently, overlapping of the margin of the fibroblast was less extensive but pseudopodial activity of the PMN along the margin of overlap continued unabated. Sometimes, the entire body of the granulocyte moved onto the fibroblast with contact to the glass being maintained via a single stretched retraction fiber (Fig. 10). Renewed contact with the glass occurred concomitantly with contraction of the retraction fiber.

The overlapping of fibroblasts by PMNs is of two types: (*a*) the overlapping pseudopods are protruded from the dorsal surface of the PMN into the medium without extensive contact with the dorsal surface of the fibroblast; and (*b*) during overlapping, the PMN is in contact with the dorsal surface of the fibroblast. Type *a* overlapping is not of interest to the present study since no opportunity for contact paralysis is afforded (there being little or no contact between the overlapping pseudopod and the fibroblast). Both kinds of overlapping were observed during direct observation of overlapping PMNs by use of high numerical aperture objectives and critical focusing of the

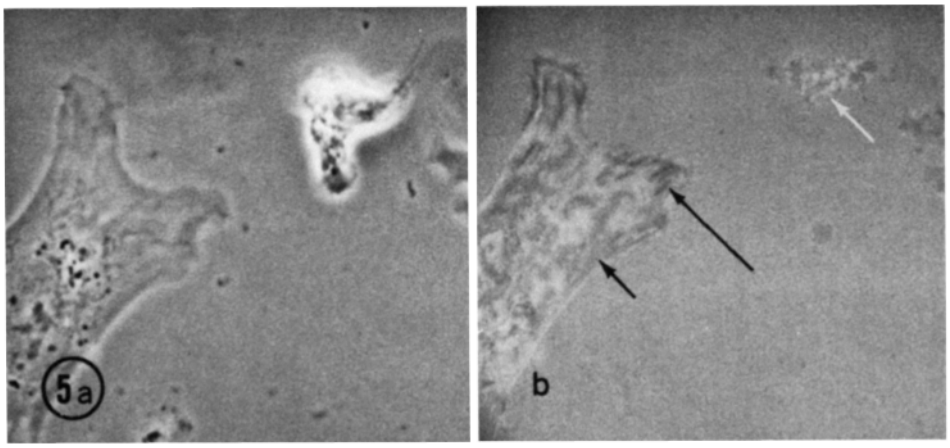


FIGURE 5 Phase-contrast (Fig. 5 *a*) and interference-reflection (Fig. 5 *b*) photographs of a heart fibroblast and a PMN made a few seconds apart. In the interference-reflection photograph, the fibroblast shows areas of gray interference (short arrow) and of black interference ("feet," long arrow). The PMN (white arrow) shows a highly irregular area of gray interference but no black feet. The areas showing gray and black interference are thought to be regions of moderately close and close approach to the substratum, respectively.

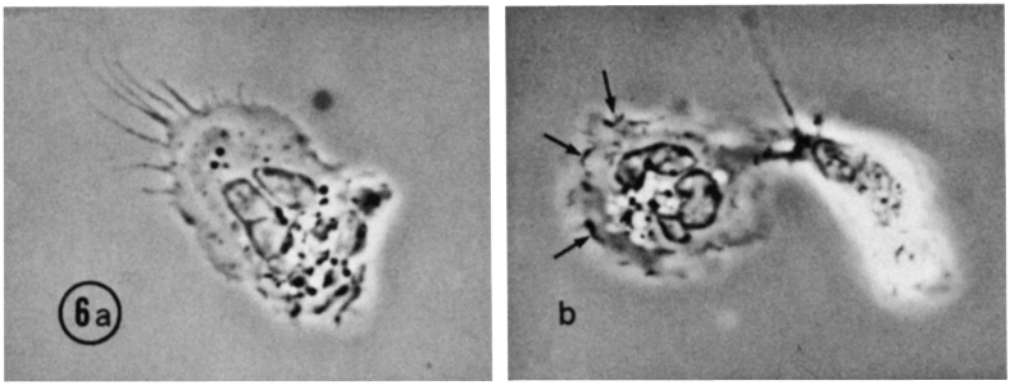
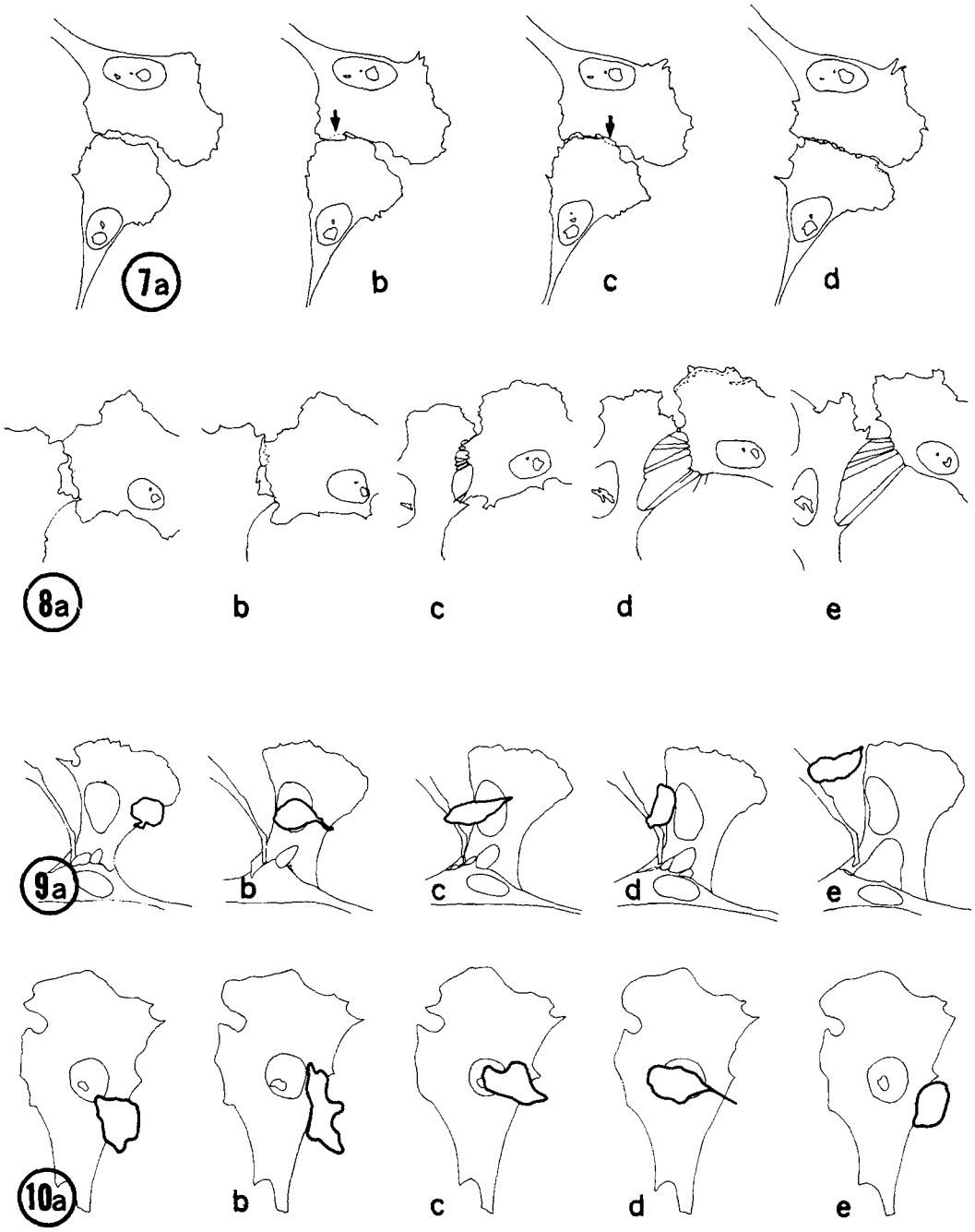


FIGURE 6 Phase-contrast pictures of highly flattened PMNs from nonsterile exudates. In Fig. 6 *a*, the plane of focus is at the glass-culture medium interface. In Fig. 6 *b*, the plane of focus is slightly above the glass-medium interface in order to show better the rufflings (arrows) of the leading lamella of the PMN. The highly refractile cell in 6 *b* is a nonflattened PMN present in the same culture.

microscope. In cases where the PMN moves largely or wholly onto the dorsal surface of the fibroblast (Figs. 9, 10), one can be certain that type *b* overlapping has occurred. Definitive type *b* overlapping of the lamellipods of fibroblasts by PMN lamellipods has been filmed with a 100/1.3 Planachromat objective with Nomarski optics. In the sequence of overlapping depicted in Fig. 11, a PMN can be seen to be protruding a hyaline lamellipod, in a plane of focus of the microscope at the dorsal surface of the fibroblast, indicating that this is a case of type *b* overlapping.

Although overlapping of the PMN onto the fibroblast was the usual result of collision, underlapping also occurred. Underlapping was easily distinguished from overlapping. During overlapping, PMNs retained their rounded refractile appearance (Fig. 11) and continued the protrusion of pseudopods from their upper surfaces. The fibroblasts were not deformed during contact. On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d
e

several occasions, granulocytes wholly on the free surface of fibroblasts were observed to detach and fall off the substratum in the inverted cultures (which would be impossible were the PMN between fibroblast and substrate). During underlapping, the PMNs became flattened, often to a degree that the nucleus became visible, and more hyaline (Fig. 12). Both in living cultures and in fixed cultures examined directly with a high numerical aperture objective, spread hyaline PMNs associated with fibroblasts were observed, by careful focusing, invariably to lie ventral to the fibroblast (i.e., between fibroblast and substrate). Occasionally, the fibroblast being underlapped was visibly lifted from the substratum, occasionally retracting contacts with the substratum or other fibroblasts as a result of being underlapped. In several cases, PMNs first overlapped and then underlapped a given fibroblast and, in other cases, overlapping and underlapping of a fibroblast by a PMN occurred simultaneously in different parts of the PMN (Fig. 12 *a*). PMNs placed in contact with fibroblast cultures sufficiently dense to allow the establishment of partially confluent patches of fibroblast monolayer showed a strong tendency to underlap the fibroblasts. Such PMNs appeared to move about as rapidly, while underlapping fibroblast monolayers, as on a cell-free substratum. Both overlapping (Fig. 13 *a*) and underlapping (Fig. 13 *b*) have been observed in cultures fixed in glutaraldehyde and examined in the scanning electron microscope.

When observed with the interference-reflection microscope, the portion of the fibroblast in the immediate vicinity of an underlapping PMN was lifted free of the substratum (Fig. 14). As the PMN moved about beneath the fibroblast, new regions of the fibroblast were lifted off the substratum, and regions formerly underlapped recontacted the substratum. PMNs were able to underlap areas of the fibroblast showing gray interference colors (interpreted as area of close approach of the ventral surface to the substratum; Lochner and Izzard, 1973),[8] but apparently were unable to underlap areas of the fibroblast associated with the dark gray patches or feet (interpreted as areas of very close approach and strong adhesion to the substratum). In the cells depicted in Fig. 14, the underlapping PMN moved freely beneath the fibroblast, detaching its ventral surface from the substratum as it went, save for regions close to patches of feet. After prolonged underlapping, the fibroblast broke off two small portions of cytoplasm associated with the two patches of feet

FIGURE 7 Tracings of selected frames of a time-lapse film showing contact paralysis after collision between leading lamellae of two heart fibroblasts. Contact is made at 7 *a*. The maximal extent of overlapping after contact is shown in 7 *b* (arrow). In 7 *b*, a second contact is made slightly separated from the first contact which results in a small amount of overlapping in 7 *c* (arrow). In 7 *c*, retraction has occurred at the site of the first contact, and, in 7 *d*, has occurred at the site of the second contact. In 7 *d*, both cells are now moving toward the right. The direction of movement of the lower cell has been shifted through 90° as a result of its collision with the upper cell and the subsequent paralysis of its lamellipod along the margins of contact. Intervals between successive frames: *a,b*, 6 min 44 s; *b,c*, 2 min 56 s; *c,d*, 8 min 10 s.

FIGURE 8 Tracings of selected frames of a time-lapse film showing the extensive retraction of the leading lamellae of two heart fibroblasts after collision. Collision occurs at (*a*) with the maximal extent of overlapping shown in (*b*). Paralysis and retraction follows (*c–e*), with long thin retraction fibers being drawn out. Intervals between successive frames: *a,b*, 2 min 50 s; *b,c*, 15 min 20 s; *c,d*, 9 min 20 s; *d,e*, 5 min 20 s.

FIGURE 9 Tracings of selected frames of a time-lapse film showing complete overlapping of a heart fibroblast by a PMN. The PMN moves completely across the dorsal surface of the fibroblast (*b–d*) to the opposite side from which it moves away from the fibroblast (*e*). Intervals between successive frames: *a,b*, 6 min 6 s; *b,c*, 0 min 48 s; *c,d*, 0 min 56 s; *d,e*, 6 min 50 s.

FIGURE 10 Tracings from selected frames of a time-lapse film showing strong overlapping of a heart fibroblast by a PMN. The main body of the PMN moves onto the dorsal surface of the fibroblast (*a–d*), retaining contact with the glass only at the tip of a retraction fiber (*d*). At (*e*) the retraction fiber has contracted, pulling the PMN off the fibroblast and back onto the glass. Initial contact was made 2 min 50 s before (*a*). Interval between successive frames: *a,b*, 0 min 24 s; *b,c*, 0 min 24 s; *c,d*, 0 min 54 s; *d,e*, 0 min 22 s.

TABLE I

Analysis of the Behavior of Rabbit Peritoneal PMNs after Collision with Chick Embryo Fibroblasts in Monolayer Culture

Type of PMN	Type of fibroblast	Number of collisions that resulted in*					
		Complete overlapping	Strong overlapping	Moderate overlapping	Weak overlapping	Underlapping‡	Total number of collisions
Sterile 4 h§	Gizzard	7	41	77	24	8	157
Sterile 14 h	Heart	27	38	52	6	26	149

Data are derived from the analysis of collisons occurring between PMNs and fibroblasts in time-lapse films.

* The degrees of overlapping have the following meanings: *complete*, the PMN relinquishes all contact with the glass to move completely onto the fibroblast; *strong*, at least half of the PMN overlaps the fibroblast; *moderate*, less than half of the PMN overlaps the fibroblast but there is no apparent diminution of pseudopodial activity on the part of the PMN; *weak*, little or no overlapping occurs. Usually pseudopodial activity continues, however.

‡ By underlapping it is meant that part or all of the PMN passes between the undersurface of the fibroblast and the glass substratum.

§ Sterile means that the exudate from which the PMNs were collected was produced by injection of sterile normal saline containing 0.1% glycogen. The time is the time of recovery of the exudate.

indicated by the black arrows. After detachment from the parent cell, the feet faded out.

The absence of contact inhibition of pseudopodial activity in PMN-fibroblast contacts appears to be reciprocal in the sense that the fibroblasts also showed little evidence of contact inhibition of pseudopodial activity (Table II). In most contacts, the leading lamella showed continued ruffling at the sites of contact during and immediately after collision. The retraction of the leading lamella that was occasionally observed (15% of collisions) was restricted in extent and was the result of an underlapping of the leading lamella of the fibroblast by processes of the PMN. Such underlapping probably breaks pre-existent contacts present between the undersurface of the fibroblast and the substratum.

Pseudopodial Activity after Collision between Nonsterile PMNs and Fibroblasts in Monolayer Culture

PMNs collected from exudates produced by injection of nonsterile saline showed a marked propensity to underlap isolated fibroblasts in monolayer culture. Sometimes as many as four or five PMNs could be found beneath a single fibroblast. Although similar levels of underlapping of fibroblasts in sparse monolayers were not usually observed with sterile PMNs collected from 4-h exudates, high frequencies of underlapping were shown by sterile PMNs collected from 14-h exudates or from 4-h exudates maintained at room temperature overnight. Such PMNs were motile, milling about beneath the fibroblast and occasionally moving partly or fully from beneath the overlying cell. Collision of fibroblasts with the ruffling leading lamellae of flattened PMNs derived from nonsterile exudates was not accompanied by diminution of ruffling activity of either fibroblast (in head-to-head collisions) or PMN. Contact inhibition or contact retraction of pseudopods did not occur. An absence of contact paralysis is thus not the property solely of rounded PMNs but is seen also in flattened cells which possess lamellipods that exhibit ruffling activity.

Contact Inhibition of Overlapping after PMN-Fibroblast Collisions in Monolayer Culture

As noted above, although PMNs show no discernible inhibition of pseudopodial activity upon collision with the margins of fibroblasts and often show marked but temporary overlapping of the exposed surface, they usually do not relinquish all adhesion with the glass to pass completely onto and across the exposed surface of the fibroblast (Table I, compare frequency of "complete overlapping" to frequency of incomplete overlapping). Instead, after a period of contact and overlapping, lasting from a few seconds to many minutes, most PMNs change their direction of movement and move away from the fibroblast (Fig. 15). In other words, collision with fibroblasts may influence the actual path taken by moving PMNs but apparently

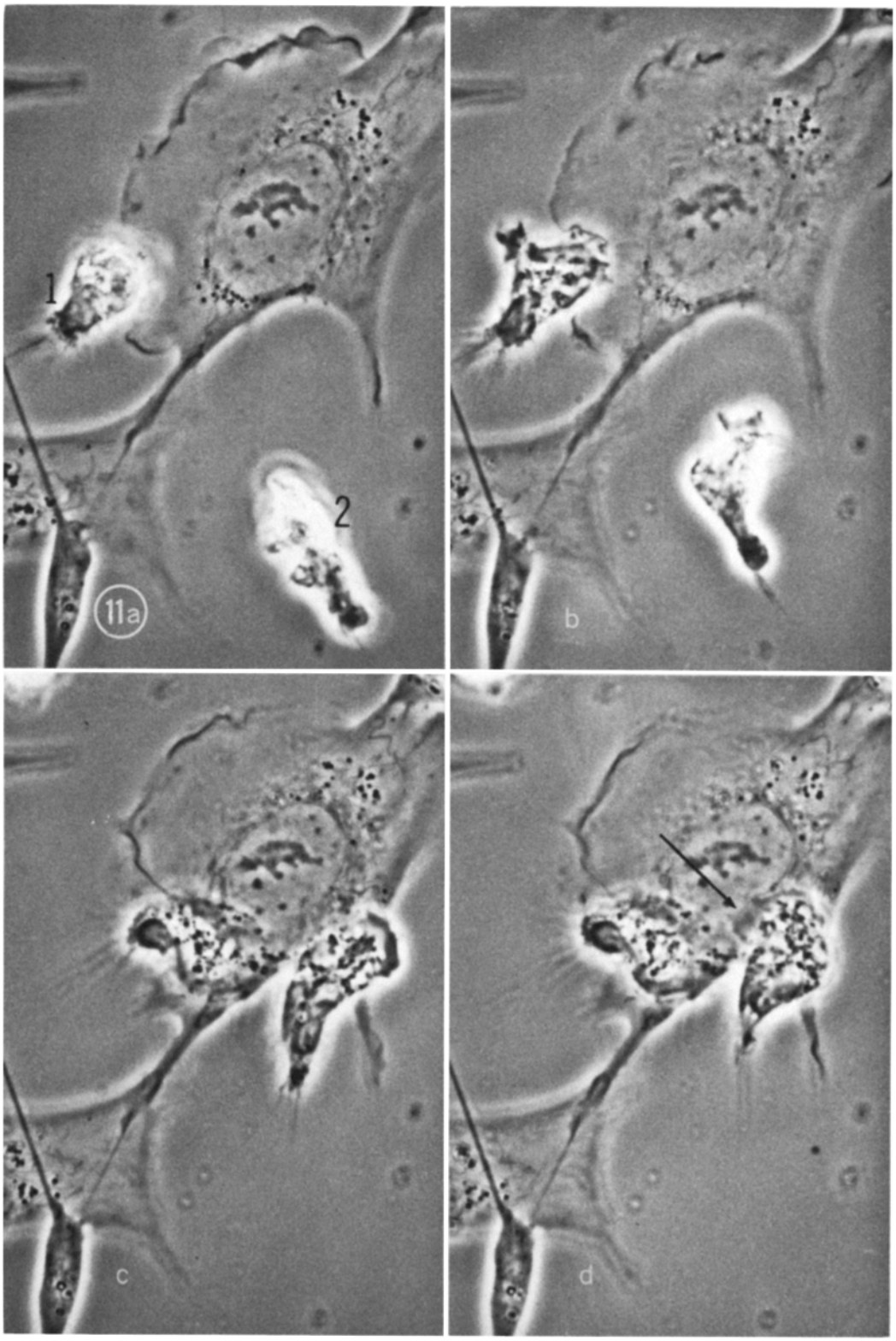

FIGURE 11 Successive phase-contrast photomicrographs showing partial overlapping of the dorsal surface of a heart fibroblast by sterile PMNs. In (*a*) and (*b*), PMN no. 1 overlaps a leading lamella of the fibroblast. In (*c*) and (*d*) the same PMN underlaps the fibroblast. PMN no. 2 contracts and overlaps the fibroblast in (*c*) and (*d*). The plane of focus is very slightly above the glass-medium interface (at about the level of the dorsal surface of the fibroblast). Note the lamellar pseudopod of PMN no. 2 that is being protruded over the dorsal surface of the fibroblast in 11 *d* (arrow).

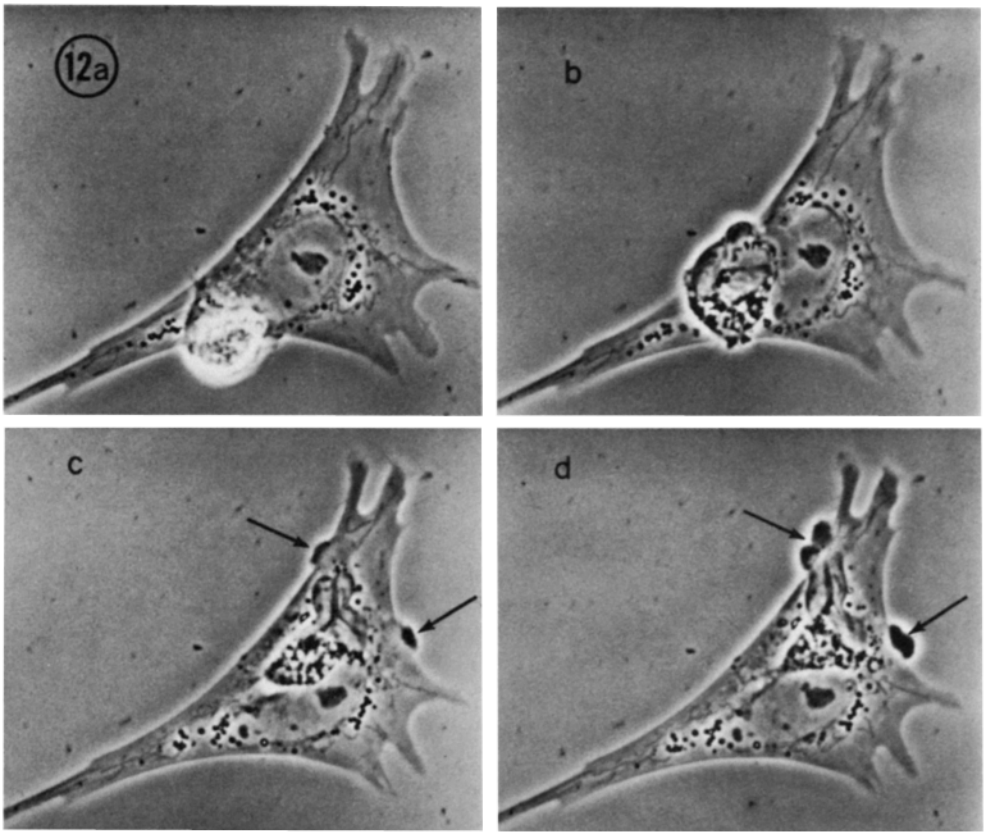

FIGURE 12 Successive phase-contrast photomicrographs showing underlapping of a heart fibroblast by a sterile PMN. Both overlapping and underlapping are present in (*a*), although the area of the PMN beneath the fibroblast is small. In (*b*) the PMN moves entirely beneath the fibroblast, resulting in dramatic changes in the appearance of the PMN. The refractility of the cytoplasm seen in (*a*) is absent and the nucleus is now visible. Underlapping continues in (*c*) and (*d*) with the PMN moving freely beneath the fibroblast. The dark processes in (*c*) and (*d*) (arrows) are tips of pseudopods of the PMN that are peeking out from beneath the fibroblast.

does not affect the locomotory apparatus itself. It appears that the change in direction of movement after collision is an expression of the spontaneous change in direction as a lateral pseudopod gains precedence over the leading pseudopod, as is observed also in free-moving PMNs, rather than being the result of paralysis of the leading pseudopod after contact.

The degree of contact inhibition of nuclear overlapping was determined from fixed and stained mixed monolayer cultures, maintained under culture conditions nearly identical to those employed for filming. The overlap analysis was performed according to the procedures set forward by Abercrombie et al. (1957). Since cell margins are difficult to delineate where cells abut one another, the criterion for overlapping was that the center of the nucleus of one cell lie over the nucleus of a second cell. The data are presented in Table III. The overlap index expresses the degree of contact inhibition of nuclear overlapping by comparing the observed number of nuclear overlaps to the number of nuclear overlaps that would be expected if the cells were distributed over the substratum at random. An overlap index of 100% indicates that cells are randomly distributed; a value less than this indicates that cells show inhibition of overlapping of their nuclei. As can be seen from rows 1 and 2 of Table III, both fibroblasts and 4-h sterile PMNs are contact inhibited from overlapping fibroblasts in mixed cultures maintained on glass cover slips. The

degree of inhibition is strong to moderate and is statistically significant. Comparing the data from the nuclear overlap analysis to the observations made on living cultures, one can assert that although sterile PMNs lack detectable contact paralysis in collision with fibroblasts and readily overlap the margins of fibroblasts, they rarely overlap fibroblasts to a degree that produces superposition of cell nuclei.

There was no inhibition of PMN-fibroblast overlapping for nonsterile PMNs collected from the rabbit at 12 h (Table III, row 3). The overlap index of 140% suggests preferential overlapping, although the significance of the difference from 100% was at only the 10% level of confidence ($\chi^2(1) = 3.52$, $P = 0.10$–0.05). Careful examination of these cultures indicated that the increased overlapping was due to underlapping of fibroblasts by the PMNs. The same morphological criteria for distinguishing underlapping from overlapping as was discussed previously for living cultures apply for these fixed cultures.

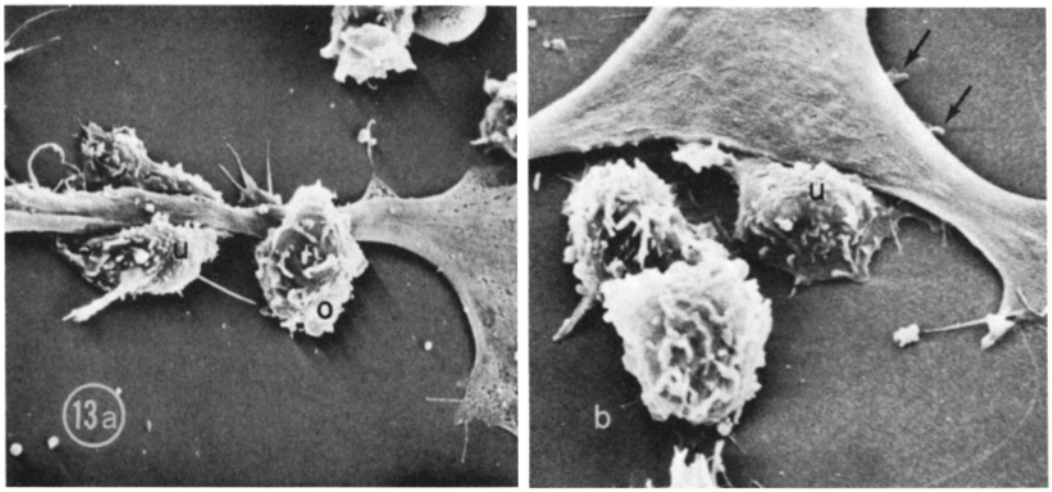

FIGURE 13 Scanning electron micrographs of sterile PMNs and chick heart fibroblasts in monolayer culture showing overlapping (*o* in *a*) and underlapping (*u* in *a* and *b*) of fibroblasts by PMNs. In (*b*) the processes indicated by the arrows belong apparently to the PMN that has underlapped the fibroblast.

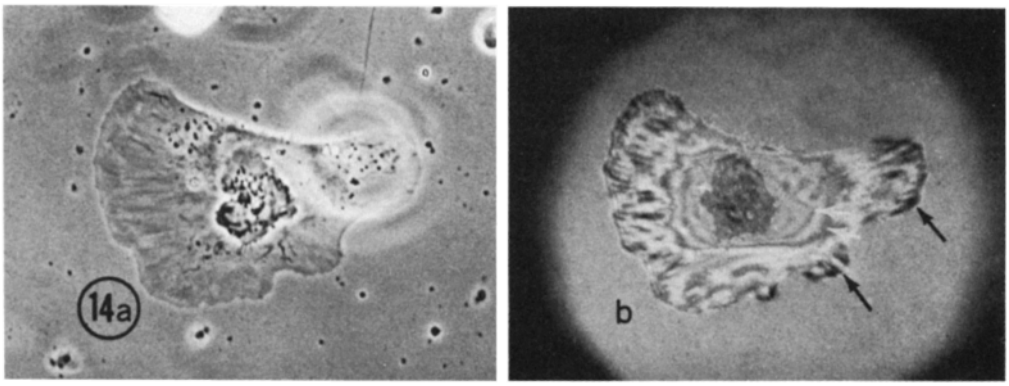

FIGURE 14 Phase-contrast (*a*) and interference-reflection micrographs, taken a few seconds apart, of a PMN underlapping a fibroblast. In this case (also recorded on film) the PMN moved freely beneath the fibroblast, separating the undersurface of the fibroblast from the substratum (indicated by the higher order interference fringes in *b* indicated by the white arrows) save in regions where the fibroblast showed "feet". Eventually, the fibroblast separated itself from two small portions of its own cytoplasm associated with the feet indicated by the black arrows.

Adhesion of PMNs to Confluent Fibroblast Monolayers

The observations reported in the preceding section permit the conclusion that in vitro the rabbit peritoneal PMN shows contact inhibition of total overlapping of fibroblasts without showing contact inhibition of pseudopodial activity during collision with fibroblasts. This mode of behavior conforms to that of type II contact inhibition as defined in the introductory paragraphs. It has been suggested (Abercrombie, 1970; Martz and Steinberg, 1973, 1974; Martz et al., 1974) that the monolayering observed in cases of type II contact inhibition may be an expression of a stronger adhesiveness of cells for the artificial tissue culture substratum (serum-coated glass in our experiments) than for the exposed surfaces of other cells. In order to examine this proposal for the case of contact inhibition of overlapping of fibroblasts by PMNs, we compared the extent of adhesion of PMNs to serum-coated glass to their extent of adhesion to confluent monolayers of fibroblasts. As can be seen by inspection of the data presented in Table IV, the adhesive behavior of the PMNs conforms to the expectations of the hypothesis. After half an hour in culture, PMNs adhere in larger numbers to serum-coated glass than they do to fibroblasts.

Caution must be exercised, however, in interpreting these data since the parameters of real interest to the differential adhesion hypothesis, the relative strengths of adhesion, are defined in terms of interfacial free energies of adhesion (Phillips,

TABLE II

Analysis of the Behavior of the Lamellipods of Chick Embryo Fibroblasts after Collision with Rabbit Peritoneal PMNs

	Result of collision			
Type of fibroblast	No inhibition of lamellipodial activity	Local retraction of lamellipod	Uncertain	Total number of collisions observed
Gizzard	15	5	2	22
Heart	28	3	0	31

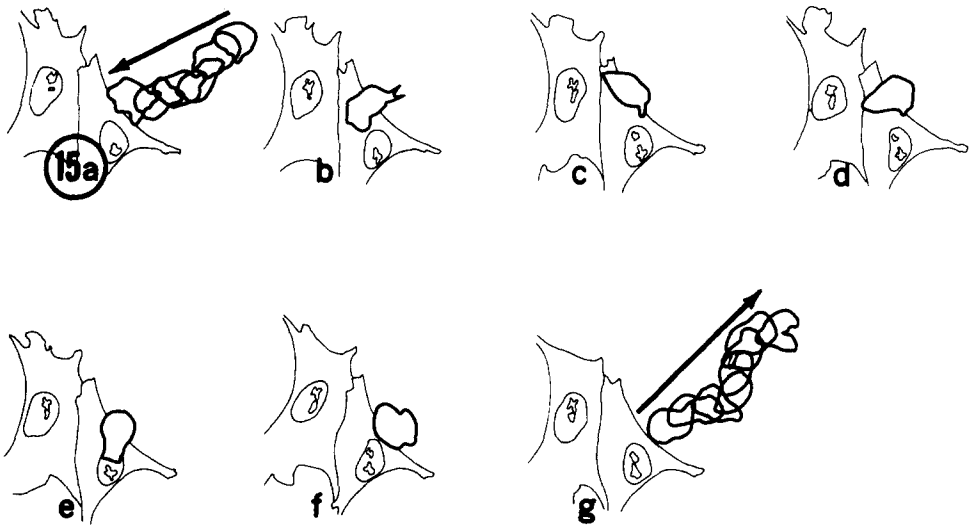

FIGURE 15 Tracings of selected frames of a time-lapse film showing partial overlapping and eventual change in direction of a PMN after collision with a gizzard fibroblast. The PMN moves towards and contacts the fibroblast in (*a*) (successive tracings show the outline of the PMN at 0.5-min intervals as it approaches the fibroblast). In *b–e* the PMN overlaps the fibroblast. In *f* the PMN ceases overlapping and then moves away from the fibroblast in (*g*) (in [*g*], successive tracings of the PMN represent its outline at 0.5-min intervals as it moves away from the fibroblast). In (*a*) and (*g*) the arrows represent the directions of movement of the PMN. In (*g*) the direction of movement of the PMN is the reverse of that shown in (*a*), a change in direction of movement which was a result apparently of its collision with the fibroblast. Intervals between successive frames: *a,b* (the final position of the PMN in (*a*) is taken as zero time), 0 min 40 s; *b,c*, 1 min 0 s; *c,d*, 0 min 6 s; *d,e*, 1 min 48 s; *e,f*, 1 min 32 s; *f,g* (the initial position of the PMN is taken as the position for determination of the interval), 0 min 38 s.

TABLE III

Analysis of Frequency of Overlapping in Cultures Containing Rabbit Peritoneal PMNs and Chick Embryo Fibroblasts

Type of PMN	Type of fibroblast	Time*	Observed values: Nuclear points: Hits on F nuclei: By F nuclei	By P nuclei	Misses of F nuclei: By F nuclei	By P nuclei	Random points: Hits on F nuclei	Misses of F nuclei	Expected‡ values for overlapping F nuclei: By F nuclei	By P nuclei	Overlap§ index: F on F	P on F	χ^2 (1): F on F	P on F	P: F on F	P on F
		h									%	%				
Sterile, 3.5 h	Gizzard	3	2	10	312	725	73	2,327	9.6	22.4	21	45	6.3	6.2	0.025–0.010	0.025–0.010
Sterile 3.5 h	Heart	3	3	14	411	630	135	1,865	29.8	43.5	10	32	23.2	19.3	<0.005	<0.005
Nonsterile 12 h	Heart	4.5	3	38	493	529	139	2,761	23.8	27.2	13	140	18.7	3.5	<0.005	0.10–0.05

Data are derived from counts of number of nuclear overlaps in cultures fixed and stained 2–5 h after addition of PMNs to sparse cultures of fibroblasts.

* Time between addition of PMNs to cover slips bearing 1-day old sparse cultures of fibroblasts and fixation.

‡ The expected number of overlaps, assuming that cells are distributed randomly, are calculated from formulae cited in Materials and Methods.

§ Overlap index is the observed number of overlaps divided by the expected number of overlaps, expressed as percent. 100% indicates that nuclei are distributed randomly (i.e. there is no contact inhibition of overlapping).

TABLE IV

Comparison of Adhesion of PMNs to Serum-Coated Glass with Their Adhesion to Confluent Monolayers of Chick Embryo Heart Fibroblasts, Assayed after 0.5 h in Culture

Experiment no.	Cell suspension: number of PMNs in 1 ml of medium	Number of PMNs/100 μm^2 adherent to	
		Fibroblast monolayers	Glass
1	2.43×10^6	18.8 (2)*	80.6 (2)
	2.08×10^6	12.6 (2)	79.4 (2)
	1.70×10^6	7.0 (3)	58.4 (2)
	1.57×10^6	7.5 (3)	38.4 (2)
	1.18×10^6	4.1 (2)	23.8 (2)
	0.55×10^6	3.0 (2)	10.2 (2)
2	2.64×10^6	8.0 (3)	51.4 (1)
	2.22×10^6	7.5 (3)	36.8 (1)
	1.70×10^6	6.3 (3)	29.6 (1)
	1.37×10^6	3.6 (3)	19.5 (1)
	0.95×10^6	3.2 (3)	10.6 (1)
	0.44×10^6	1.3 (2)	5.1 (1)
	0.25×10^6	0.8 (2)	3.0 (1)

* Figure in parentheses is number of replicates.

1969; Martz et al., 1974). These later parameters are not obtained by our measurements which are, in essence, kinetic measurements of relative degrees of adhesion. What are required, instead, are techniques which determine equilibrium states of systems (Phillips and Steinberg, 1969). Thus, the correspondence of our adhesion studies with the expectations of the differential adhesion hypothesis may merely be fortuitous.

The data in Table V showing the time course of adhesion indicate that the number of PMNs adherent to serum-coated glass is relatively constant from 0.5 h (the earliest time studied) until the termination of the experiment (6 h). As noted above, the number of PMNs adherent to fibroblast monolayers is markedly less than this at 0.5 h. By 2 h, the number of PMNs associated with the fibroblast monolayer has increased to become nearly as high as the number adherent to the glass. The majority of these PMNs are flattened and are identical in appearance to the PMNs in living cultures engaged in underlapping. At high magnification, careful focusing reveals that the flattened PMNs of 2-h cultures are lying ventral to the fibroblasts (i.e. between the fibroblasts and the glass). The number of PMNs associated with fibroblast monolayers is thus an adequate measure of adhesiveness only at time intervals short enough that underlapping (and a resultant passive trapping of PMNs) is not significant. Association of added cells with collecting monolayers by underlapping represents a potential artifact when the technique is used to assay cellular adhesiveness (Walther et al., 1973, Roseman et al., 1974).

DISCUSSION

The hypothesis under examination in the present study is that the fundamental factor which allows a cell to be invasive is that it lacks contact inhibition of activity of its pseudopodial apparatus when it collides with host tissue cells. One prediction which this hypothesis makes is that all invasive cells should lack contact inhibition of pseudopodial activity upon collision with host cells. Such a relationship has been described for cells of certain sarcomas (Abercrombie, 1958, 1961 *a*, *b*, 1967 *a*, *b*, 1970; Abercrombie and Ambrose, 1958; Vesely and Weiss, 1973). The present study examines the

TABLE V

Time-Course of Adhesion of PMNs to Serum-Coated Glass and to Confluent Monolayers of Chick Heart Fibroblasts

Experiment no.	Time	Number of PMNs/100 μm^2 adherent to	
		Fibroblast monolayers	Serum-coated glass
	h		
1	0.5	17.3 (2)*	43.5 (1)
	1	19.1 (2)	40.8 (1)
	2	31.6 (2)	39.9 (1)
	3	37.9 (2)	38.5 (1)
	4	32.8 (1)	42.4 (1)
	5	40.3 (2)	43.0 (1)
	6	39.9 (2)	39.6 (1)
2	0.5	3.7 (3)	18.8 (2)
	1	4.3 (2)	—
	2.5	10.7 (2)	—
	4	10.4 (3)	20.2 (3)
3	0.5	4.5 (3)	21.98 (3)
	1.0	15.4 (3)	—
	1.5	21.1 (3)	—
	2.0	22.6 (3)	—
	3.0	22.3 (3)	—
	4.0	20.1 (3)	24.9 (3)

* Figure in parentheses is number of replicates.

hypothesis with rabbit peritoneal neutrophils (PMNs).

PMNs are highly invasive in vivo. Their principal function is to phagocytose bacteria present in wounds and infections. In this capacity, they represent a rapidly mobilized first line of defence against bacterial infection (Fruhman, 1970; Speirs, 1970). Their invasive abilities are employed to allow them to pass from the circulation across the vascular endothelium (Florey and Grant, 1961; Marchesi and Florey, 1960; Hurley, 1964) into the tissue spaces. Once within the tissue spaces, the cells are able to migrate freely between tissue cells (Clark et al., 1936; Allison et al., 1955; Grant et al., 1962; Graham et al., 1965; Cliff, 1966; Wood et al., 1968) to the actual site of infection. PMNs have been shown to be able to respond chemotactically to a number of factors (Wilkinson, 1974). Presumably, the chemotactic response is important in directing cells to the precise sites of infection (but see Allison et al., 1955; Cliff, 1966; Florey and Jennings, 1970, p. 128). The results of our studies of the invasive abilities displayed to aggregates of chick embryo fibroblasts demonstrate that this invasive ability is retained in vitro and validate the use of embryonic fibroblasts as host tissue cells in the studies of cell interaction in monolayer culture.

The studies of cell interaction in monolayer culture demonstrate that rabbit peritoneal PMNs *do not* exhibit contact inhibition of pseudopodial activity during collisions with embryonic fibroblasts and thus conform in their behavior with the sarcoma cells studied previously (Abercrombie, 1970; Vesely and Weiss, 1973). A loss of contact inhibition of pseudopodial activity seems in large part to be reciprocal in the sense that most collisions do not result in diminution of activity of the leading lamella of the fibroblast. The occasional cases of retraction of leading lamellae which were observed were associated with local underlapping of the leading lamella by processes of the PMN.

In addition to the prediction tested in the present study, the contact paralysis hypothesis suggests a number of predictions regarding the behavior of noninvasive cells. If the hypothesis is correct, noninvasive cells should show contact paralysis during a high percentage of collisions with other noninvasive cells. Data regarding this prediction are not complete enough to evaluate its validity. Vesely and Weiss (1973) report a high incidence of paralysis of ruffled membranes after head-to-head collisions of nontransformed fibroblasts. The high frequency of underlapping observed in head-to-side collisions of fibroblasts (Abercrombie, 1970; Armstrong and Armstrong, 1973; Boyde et al., 1969; DiPasquale and Bell, 1974; Harris, 1973 *b*) perhaps does not represent an important exception to this requirement since large portions of the undersurfaces of cells posterior to their leading lamellae are not adhering to the substratum (Ambrose, 1961; Boyde et al., 1972; Chambers and Fell, 1931; Goodrich, 1924; Harris, 1973 *b*) and actual contact between colliding cells in these circumstances may be limited. Several laboratories, however, have reported that even in apparently confluent monolayers of fibroblasts (Abercrombie and Heaysman, 1954; Bell, 1972; Martz, 1973; Martz and Steinberg, 1974) and epithelial cells (Weiss, 1958, 1959; Dye, 1971; Steinberg, 1973; Garrod and Steinberg, 1973), cells exhibit continued milling about, which suggests that paralysis of locomotion of cells is not adequate to halt completely the movement of cells apparently in contact with other cells around their entire perimeters.

A second prediction that the hypothesis makes for noninvasive cell behavior is that noninvasive cells should be stationary in solid tissue masses. Recent studies from this (Armstrong and Armstrong, 1973) and other (Elsdale and Bard, 1972; Wiseman and Steinberg, 1973) laboratories do not support this prediction. Instead, cells of noninvasive cell types do mill about in at least some solid tissues (although not in all, see Weston and Abercrombie, 1967). On the basis of these data, it is suggested that an absence of contact paralysis may invariably accompany the ability for invasion and may perhaps even be necessary for display of invasive behavior, but the exact role of contact paralysis in maintaining the architecture of normal tissues is still uncertain.

The inhibition of overlapping demonstrated by rabbit peritoneal neutrophils to sparse cultures of chick embryo fibroblasts differs from the results of studies reported by Oldfield (1963) on the behavior of chicken circulating PMNs cultured with chick heart fibroblasts grown as confluent monolayers emigrating from explants of heart tissue. The overlap index of PMNs vis-à-vis fibroblasts obtained by Oldfield indicates a lack of inhibition of overlapping. There are several possible explanations for the difference in results. A species

difference in behavior of PMNs may be important. Oldfield used circulating PMNs whereas we used peritoneal PMNs (which have performed one episode of invasion to reach the peritoneum before they are collected). A third and probably crucial difference, however, is in the design of the experiment. Most of our observations were performed within 4 h after seeding PMNs onto *sparse* cultures of fibroblasts. Under these conditions, underlapping is only moderately common (Table I, row 1).

Underlapping is far more common if PMNs are cultured in apposition to *dense* cultures of fibroblasts. When studied by time-lapse filming, PMNs in contact with dense monolayers underlap the fibroblasts in large numbers and show rapid locomotion beneath the fibroblast monolayers. Presumably, this readiness to underlap dense or confluent monolayers results from a reduction in the extent of contact of fibroblasts to the substratum under these conditions as compared to fibroblasts in sparse cultures (Harris, 1973 *b*). Underlapping of confluent monolayers represents a serious artifact of the collecting-monolayer studies of adhesiveness (Table V) if time periods greater than 0.5 h are used. Since the fibroblasts used by Oldfield were present as monolayers, the overlapping which she recorded may have been underlapping of the fibroblasts by the PMNs. Unfortunately, the fixed and stained cultures used in her study have been lost, so it is not certain that this explanation is the correct one.

A problem of interest is the mechanism underlying the absence of detectable contact inhibition of pseudopodial activity in PMN-fibroblast collisions. Of the several factors that may contribute to this phenomenon, a few deserve special mention: (*a*) fibroblasts apparently require some time to manifest contact inhibition of pseudopodial activity and contact retraction after initial collision.[5] Possibly the lack of similar phenomena with PMNs stems from their more vigorous pseudopodial activity—a given PMN pseudopod may never remain long enough in contact with the surface of the fibroblast for contact inhibition and retraction to occur; (*b*) although the mechanism of contact inhibition of pseudopodial activity has not been established, an attractive hypothesis is suggested by observations of Heaysman and Pegrum (1973 *a*). On the basis of fine-structural studies that document the rapid (0.5–1-min) formation of *adhaerens*-type intercellular junctions after collision between normal fibroblasts (but not between noncontact-inhibited sarcoma cells, Heaysman and Pegrum, 1973 *b*), one can propose that inhibition and retraction of pseudopods after contact is the result of the contractile activities of microfilaments associated with these junctions. If this proposal is correct, a defect in intercellular adhesion and junction formation may represent the basis of the absence of contact inhibition of pseudopodial activity observed in the case of PMN-fibroblast contacts.[6] It must be noted that PMNs do adhere to fibroblasts after collision: they are able to pull themselves onto the exposed surfaces of contacted fibroblasts, sometimes losing contact entirely with the glass, and PMNs often show long stretched retraction fibers connecting with fibroblasts as they move off; (*c*) The mode of locomotion of fibroblasts may differ from that of PMNs. At a morphological level, the vigorous cytoplasmic streaming associated with the locomotion of PMNs is absent in fibroblasts. During forward movement, the PMN sometimes shows periods when the region of contact with the substratum is confined to the posterior uropod, especially during bouts of "looping" movement. These aspects of PMN movement suggest the rear-contraction model developed to account for the streaming-associated locomotion of large amoebae (Mast, 1926). Although presently of doubtful applicability to the actual mechanism of movement of amoebae of the *Chaos-Amoeba* group (Allen, 1972), the model may be valid for the movement of some amoebae (Griffin, 1964), and may as well be an important feature of PMN locomotion. If this is true, the location of the contractile apparatus responsible for movement in the PMN would differ from that in the fibroblast where the machinery for motility is almost cer-

[5] Abercrombie (1970, p. 128) states that "... the effect of contact is often delayed for 10–20 min after the cells have apparently met, which allows time for some degree of superimposition of one cell on the other." See also Figs. 7, 8.

[6] In some cases of heterotypic collision, contact inhibition of pseudopodial activity is nonreciprocal, occurring with one partner but not the other (Heaysman, 1970; Vesely and Weiss, 1973). If the hypothesis is correct, nonreciprocal contact inhibition might be the result of asymmetry in the degree of microfilament polymerization subjacent to cell junctions. Such asymmetry has been observed in *adhaerens*-type junctions formed between chick embryo pigmented retinal cells and cardiac myoblasts (Armstrong, 1970, Fig. 10).

tainly contained within the anterior-placed lamellipodium (Abercrombie et al., 1972; Spooner et al., 1972). It may be that an anterior placement of the contractile apparatus involved in locomotion is necessary to render it sensitive to contact. If movement is dependent on contractility at the posterior end of the cell, contacts made at the anterior end may have little effect on the continued protrusion of pseudopodia.

The suggestions made above are not yet established by experimental analysis. Of the three, hypothesis (*b*) is most readily susceptible to test. It requires that PMNs should not make *adhaerens*-type junctions with fibroblasts. Fine-structural studies (currently in progress) should be able to evaluate the validity of the prediction.

Finally, brief note might be made of the nature of contact between the PMN and the substratum as it relates to the mechanism of cell movement. Studies with the interference-reflection microscope showed that even rapidly moving PMNs lack the focal sites of very close approach to the substratum (feet) that are evident in moving fibroblasts, but show instead only the regions of gray interference that in the fibroblast have been interpreted as regions of less close approach (Lochner and Izzard, 1973).[3] It seems likely that fibroblast feet observed in the interference-reflection microscope are identical to the "plaques" described from the fine structural studies of Abercrombie et al. (1971). The plaques are located primarily beneath the leading lamella and are focal sties of close approach to the substratum and have associated bundles of microfilaments. The microfilament bundles pass obliquely backward in the cytoplasm, inserting in the plaque at one end and in the cortical microfilament network along the dorsal margin of the cell at the other end. It has been suggested that the microfilament bundles are instrumental in drawing posterior regions of the fibroblast toward the leading lamella during forward locomotion and that the plaques serve as the sites of very strong adhesions to the substratum necessary for this process (Abercrombie et al., 1972).

The absence of feet in PMNs suggests that they may lack this entire apparatus for moving posterior regions of the cell forward. It is suggested that this function is served, instead, by the vigorous streaming of cytoplasm that accompanies forward movement. Whether cytoplasmic streaming is driven by uropod contraction or by some other mechanism is not known.

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