INVASIVE LOCOMOTORY BEHAVIOUR BETWEEN MALIGNANT HUMAN MELANOMA CELLS AND NORMAL FIBROBLASTS FILMED IN VITRO

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SUMMARY

Explants of human malignant melanoma (MM96), normal adult human skin fibroblasts (HSF) and embryonic chick ventricle were confronted in pairs. The 2 outwandering populations in each confrontation eventually met in a situation where each could potentially invade the other. The human explants were artificially prepared from dissociated cells. The primary objective of the study was to compare the relative invasive capacities of the malignant and non-malignant human cell populations against a standard population of chick heart fibroblasts (CHF). Relative invasiveness was also compared for (a) malignant human melanoma cells against human and avian fibroblasts; (b) the 2 different fibroblast populations against MM96; and (c) the 2 fibroblast populations against each other.

Time-lapse films were prepared for each confrontation. Each cell population was also filmed in a free or unconfronted state. The films were analysed in terms of (a) cell speeds in relation to numbers of contacts; (b) the net radial outward velocity; (c) directional frequencies of movements; and (d) cell behaviour resulting from heterologous collisions. Replicate cultures were fixed and stained 24-40 h after junction. Measurements from these indicated relative distances travelled by each population towards the opposing population and towards free space. Nuclear overlap and population density measurements were also recorded.

MM96 cells invaded the standard chick fibroblast population but eventually encountered moderate obstruction. Invasion was mainly by oriented movement between successive pairs of chick cells. Contact paralysis of ruffling of MM96 was not seen. Human fibroblasts did not invade the CHF population. They were contact inhibited by chick cells and typically reversed their direction of movement. MM96 cells invaded the human fibroblast population without obstruction. They were not usually contact inhibited by HSF and several different pathways for locomotory invasion, including overlapping, were involved. Cells of both fibroblast populations were contact inhibited by leading-edge collisions with MM96 cells. Fibroblast invasion of MM96 was by underlapping of orthogonally oriented, bipolar melanocytes or dendritic processes. Human fibroblasts were less obstructed than chick fibroblasts during invasion of MM96. Initiation of invasion of HSF by chick cells was apparently orientation dependent. Invasion by CHF was assisted by the alignment, morphology and contact-mediated withdrawal of the human cells. Heterologous contact inhibition was not apparently defective for either cell type. In all confrontations involving both malignant and non-malignant cells, the extent and pattern of invasion could be related to apparent deficiencies in heterologous contact inhibition of locomotion.

INTRODUCTION

The use of confronted explants as concentrated foci of cell migration for the study of potentially invasive locomotory behaviour of normal and malignant cell populations was pioneered by Abercrombie, Heaysman & Karthauser (1957). In this early investi-
gation and in a more recent study (Abercrombie & Heaysman, 1976), malignant and non-malignant mouse cells were confronted with a standard population of embryonic chick heart fibroblasts. The combined results of these experiments indicated that invasion of the standard fibroblast population by mouse fibroblasts was negligible within a standardized period of time but that cells of different transplantable mouse sarcomas invaded populations of chick heart fibroblasts to varying and apparently specific degrees. Conversely, the standard avian fibroblasts were almost totally obstructed by mouse fibroblasts but encountered varying degrees of obstruction by the different sarcoma populations. In early experiments (Abercrombie et al. 1957) in which 2 mouse sarcomas were each tested against both mouse and chick fibroblasts, the relative degrees of invasiveness of the malignant cells against the two types of normal populations appeared to be similar.

The implications from these results were that, while contact inhibition of locomotion (Abercrombie & Heaysman, 1954) was operative between cells of heterologous (= heterotypic) fibroblast populations, a deficiency in heterologous contact inhibition and possibly also in heterologous adhesion was operative between sarcoma cells and fibroblasts. This postulated deficiency (Abercrombie & Heaysman, 1976) could theoretically operate to produce either overlapping or underlapping by malignant cells, normal cells or both, thus producing varying patterns of invasion.

Although the relative degree of invasion by each type of cell population could be measured from fixed and stained cultures alone (Abercrombie & Heaysman, 1976), the causal patterns of locomotory behaviour of individual cells could not be positively identified without observation. The present investigation was designed to combine evidence from fixed cultures with that from time-lapse films. From films, the detailed behaviour of a limited number of cells could be analysed over a specified period of time. From fixed cultures, as pointed out by Abercrombie & Heaysman (1976), the results of actions of very many individual cells, as seen at a given point in time, could be assessed.

Human populations had not been previously tested in a confrontation system similar to that described above for mouse cells. In the present experiments, explants of malignant and non-malignant human cells were confronted with explants of chick ventricle and, later, with each other. The locomotory behaviour of chick heart fibroblasts had already been quantitatively and qualitatively documented through the many investigations of Abercrombie, Heaysman and associated workers. The retention in confrontation experiments of a readily obtained, easily cultured, uniform population of this kind against which the invasive behaviour of other cell populations could be tested and compared was of obvious importance. The main emphasis of the study was on the behaviour of each pair of confronted populations throughout a standardized 6-h period of time-lapse filming, beginning at approximately the time of the initial junction of heterologous outwanderings between the explants. For comparison with post-junctional effects, the behaviour of each cell type in unconfronted situations was also filmed and analysed. It was hoped that analyses of filmed locomotory behaviour with reference to frequency of direction, speed in relation to numbers and types of contacts, net radial outward velocity and heterologous collision reactions would help
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to explain measurements obtained from fixed-culture analyses of similar confrontations cultured for a post-junctional period of at least 24 h.

Successive comparisons were made of the invasive behaviour of: (i) malignant and non-malignant human cells confronted with a standard avian fibroblast population; (ii) malignant human cells confronted with human and avian fibroblast populations; and (iii) human and avian fibroblast populations (a) confronted with malignant human cells, and (b) confronted with each other.

Abercrombie (1975) defined cellular invasion as the movement of a cell population into an area already occupied by another population. Although this definition in no way eliminates the possible effects of multiplication of one or both cell types and although cell proliferation is typically seen to occur in invasive situations in vitro, the present investigation, as far as film analyses were concerned, was specifically limited to ‘locomotory’ invasion, i.e. invasion associated with the movements of individual cells. Cells which divided during the filming period were not included among those selected for quantitative study of locomotory behaviour.

MATERIALS AND METHODS

Cells

Chick heart fibroblasts (CHF) were cultured as populations emigrating from primary explants of 8-day embryonic chick ventricle.

Adult human skin fibroblasts (HSF) were obtained initially as outwanderings produced from mass cultures of minced dermal tissue. After eventual dissociation and routine subculturing for brief periods, cell suspensions at early passage numbers were stored under liquid nitrogen. Before each experiment, cells were thawed, seeded into flasks and cultured for a few days until adequate numbers were available. They were then used to prepare artificial explants (see below).

Cells of the permanent in vitro line of malignant human melanoma MM96 (Whitehead & Little, 1973) were cultured as required from stocks maintained under liquid nitrogen. A test implantation into ‘nude’ mice (Giovanella, Stehlin & Williams, 1974) kindly carried out by Dr R. Baker, produced small, pigmented tumours after several weeks. In all confrontation experiments artificial explants, prepared directly from cultured cells, were used.

Artificial explants

Type 1. Approximately $10^5$ dissociated cells were seeded into circular wells 5 mm in diameter cut through blocks of surgical stainless steel. The flat bottom surface of the metal rested on a glass coverslip coated with fused collagen (Stephenson, 1975) to which the cells attached. Before sterilization, the back of each coverslip was marked with Gurr’s Glass-Marking ink to indicate the approximate centre of each well. If 2 confronted artificial explants were required, double wells cut in a single block of metal and separated by a gap of slightly more than 1.0 mm were used. The thick cell suspension was left for approximately 6 h before the metal blocks were removed. If phase-contrast examination showed that any leakage of cells had occurred, the cultures were discarded. In general, human fibroblasts attached very well and removal of the chambers left discrete, circular, multilayered areas of cells from which radial migration began rapidly. Adequate adhesion of MM96 cells to the substratum was achieved only after the addition of 10 mM MgCl₂ (Natt, unpublished) to the medium during the initial incubation period.

Type 2. A second type of artificial explant, suitable for human fibroblasts but not for human melanoma cells, was prepared by seeding cells very thickly into glass rings 100 mm in diameter and incubating them for several days on coverslips. The resulting thick mats of cells were then removed and cut into small fragments. When incubated, the outlines of these fragments became rounded as in natural explants and migration of fibroblasts began.
In practice, natural explants of chick heart fibroblasts were confronted with Type 1 artificial explants of HSF or MM96. Type 2 explants of HSF were confronted with Type 1 explants of MM96. After the explants were placed in position and again when initial measurements of the interexplant distance were made, the area between the explants was carefully monitored for the presence of free cells. If these were found and could not be removed by pipetting, the cultures were discarded.

**Medium**

The fluid medium consisted of RPM1 1640 (Moore, Gerner & Franklin, 1967) supplemented with 15% foetal calf serum and buffered with HEPES (6.67 g l⁻¹) and NaHCO₃ (2.0 g l⁻¹). The substrate throughout was fused collagen (Stephenson, 1975).

**Fixed cultures**

For each type of confrontation, 10 coverslip cultures were prepared by the same techniques as those used for filmed cultures (see above). The period of incubation up to the time of junction varied with the outward velocities of the confronted populations. Fixation in formol-saline was carried out 24-40 h after junction of the heterologous outwanderings. The cultures were stained with Mallory's aqueous haematoxylin.

**Measurements from fixed cultures**

These were adapted from those of Abercrombie & Heaysman (1976). Some additional measurements were necessitated by the difficulty of identifying the initial edges of Type 1 artificial explants once cell migration had begun. For this reason, measurements of the inter-explant space and of the distance from the central marker to the edge of the artificial explant (Fig. 1) were made immediately the metal culture chambers had been removed and attachment of the opposing explants had occurred. A second set of measurements was made on the living cultures before junction of the opposed outwanderings. After fixation, additional distance measurements were recorded (Fig. 1) and the extent of the invasion zone for each confrontation and an invasive index for each cell type (Abercrombie & Heaysman, 1976) were calculated. Homologous and heterologous nuclear overlap indices (Abercrombie & Heaysman, 1954; Abercrombie, Lamont & Stephenson, 1968) were obtained for cells in each invasion zone. Homologous indices were calculated for areas of comparable density in the free 'side' outwanderings and for the region immediately behind the invasion zone. Estimates of population densities were obtained solely from cells in the limited areas in which overlap counts were made.

**Films (Table 1)**

The interval between frames was 15 s and objective magnifications were 25 or 16 times. Each film sequence ran for at least 6 h, which was the standard period chosen for analysis. Photographic enlargements of standard size were later prepared from every 40th frame. The gap between each of the 36 successive photographs from frames 0–1440 represented a 10-min interval.

**Measurements from films**

Most methods used were adaptations of those of Abercrombie & Heaysman (1953, 1966). For filming purposes, confronted explants were aligned so that the common radius joining their centres was parallel to either the long or short axis of the photographic field (Fig. 2). From the enlargement of the first frame of each film the outline of the rectangular camera field was traced. The centre of the nucleus of each cell selected for analysis (Table 1) was then marked and traced from successive photographs spanning the entire film. Each selected cell was numbered and colour-coded for identification. The distance covered as a consequence of nuclear displacement during each 10-min interval was measured and recorded to the nearest 0.5 mm. The direction of movement in relation to one or other of 4 fixed quadrants (Fig. 2) was also recorded.

The net radial outward velocity of each cell is the effective distance travelled away from the explant within a specified time. In order to try to relate this to fixed culture measurements of
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Fig. 1. Diagram, not drawn to scale, of measurements from cultures of confronted explants before and after fixation. \(a, c\) are centres of opposing explants, \(b_1\) and \(s_1\) are measurements of the distance from the centre \(a\) to the 'between' and 'side' edges of an artificial explant before migration has begun. \(b_2\) and \(s_2\) are between and side measurements of the migration area or outwandering before junction with the confronting population occurs. \(b_3\) and \(s_3\) are estimated positions at junction, obtained from the previous data and the interexplant distance. \(b_4\) and \(s_4\) are measurements of the post-junction between and side distances. In simplest terms the invasive index can be calculated as \(\frac{(b_4 - b_3)}{(s_4 - s_3)}\) (measurements are based on those of Abercrombie & Heaysman, 1976).

Table 1. Experimental design for confrontations, films and fixed cultures

<table>
<thead>
<tr>
<th>Cell types and situations</th>
<th>No. of 6-h films</th>
<th>No. of cells selected for analysis from each film (total)</th>
<th>No. of fixed and stained cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chick heart fibroblasts (CHF)</td>
<td>3</td>
<td>10 (30)</td>
<td></td>
</tr>
<tr>
<td>not confronted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human skin fibroblasts (HSF)</td>
<td>3</td>
<td>10 (30)</td>
<td></td>
</tr>
<tr>
<td>not confronted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human malignant melanoma (MM96)</td>
<td>3</td>
<td>10 (30)</td>
<td></td>
</tr>
<tr>
<td>not confronted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MM96 confronted with CHF</td>
<td>6</td>
<td>5 of each type (60)</td>
<td>10</td>
</tr>
<tr>
<td>HSF confronted with CHF</td>
<td>6</td>
<td>5 of each type (60)</td>
<td>10</td>
</tr>
<tr>
<td>MM96 confronted with HSF</td>
<td>6</td>
<td>5 of each type (60)</td>
<td>10</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td>*<em>27</em></td>
<td>*<em>270</em></td>
<td><strong>30</strong></td>
</tr>
</tbody>
</table>

*In several films, particularly those including the relatively large, elongated human fibroblasts, the specified number of cells with all their contacts could not be traced throughout the entire sequence. Three additional films were prepared to provide the full number of confronted cells for analysis. Five additional unconfronted HSF cells were also analysed.
distances travelled between the explants (Abercrombie et al. 1957; Abercrombie & Heaysman, 1976), the net outward velocity was considered only in relation to the common radius between the explants (Fig. 3). In the case of unconfronted explants, an arbitrary radius, aligned parallel to one axis of the photographic field, was chosen. The net velocity, converted to \( \mu m \ h^{-1} \), was expressed as a single measurement for each unconfronted cell. Two successive net velocity measurements were recorded for each cell for which pre- and post-junction data were available.

The numbers of homologous and heterologous contacts were recorded at 10-min intervals for each selected cell. Where relevant, the time of the first heterologous contact was identified.

Fig. 2. Diagram, not drawn to scale, of the orientation of direction-quadrants in relation to a pair of confronted explants and to the rectangular photographic frame. \( a \) = centre of an explant from which cells are migrating. Between the explants \( a \) and \( b \) any cell theoretically may move in any one of the 4 directions indicated. \( ro \) = radially outward, away from the explant; \( ri \) = radially inward, towards the explant; \( lo \) = laterally or tangentially outward; \( li \) = laterally or tangentially inward. For cells migrating from explant \( b \) the reverse terminology applies. For unconfronted explants an arbitrary radius is selected and aligned with one axis of the photographic field.

Fig. 3. Diagram, not drawn to scale, illustrating the measurement of net radial outward velocity of a single cell before and after junction. \( a \) and \( b \) represent centres of confronted explants. \( p_1 \) is the initial, prejunction position of the nucleus of an individual cell. \( p_2 \) is the position of the same cell at the time of its first contact with a heterologous cell. \( p_3 \) is the post-junction position of the same cell at the end of the film. The actual measurements of net velocity are made in relation to the radial axis between \( a \) and \( b \). \( v_1 \) represents the net prejunction velocity and \( v_2 \) the net post-junction velocity. The mean values for all cells, measured initially in \( \mu m \), are converted to \( \mu m \ h^{-1} \). For unconfronted cultures, a fixed, arbitrary radius is chosen.
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so that pre- and post-junction measurements could be separated. All contacts, whether stable or changing (Abercrombie & Heaysman, 1953), were included together in subsequent calculations.

Standardized criteria for the selection of cells for locomotory analysis were adopted. Cells were observed in succession, beginning with those nearest the leading edge of the outwandering. Those which divided, left the field, or had contacts outside the field were eliminated for purposes of analysis. Table 1 lists the numbers of cells selected from each film.

Heterologous collisions were assessed as far as possible in terms of contact inhibition reactions. Available examples of collision reactions unobstructed by neighbouring cells were too few for quantitative assessment.

For statistical calculations, data from all films illustrating a specific confrontation or situation were combined. Because the timing of each film confrontation was arranged to begin at or soon after junction of the outwanderings, the total numbers of pre-junction data were usually too low to be of valid comparative use. Comparisons were therefore made between post-junction measurements and those from unconfronted populations of appropriate culture age.

RESULTS

Unconfronted populations

General description of cells

The classical description of chick heart fibroblasts emigrating from explants (Abercrombie & Heaysman, 1953) was mirrored in films prepared for the present study, even though substrata and media differed widely between the two investigations. The continuous, two-dimensional meshwork of migrating cells (Fig. 4A) consisted of individuals moving predominantly away from the parent explant while frequently breaking contacts and forming new ones. Mitotic divisions caused the temporary rounding of proliferating cells, the daughter cells subsequently forming their own contacts with neighbours and joining in the formation of the expanding monolayer.

Emigrating human skin fibroblasts were arranged typically as a monolayer of radially oriented, more or less parallel, elongated cells (Fig. 4B) except at the free margins of outwanderings (Fig. 4C). Here the parallel arrangement was less clearly defined. Irregularly oriented, sometimes free cells, with moderately expanded but often deeply divided leading lamellae, were seen. Elsewhere, elongated, tapering forms predominated, sometimes with very long, thin, terminal processes. Viewed at low magnification in stained preparations, the nuclei of stained cells often gave the impression of being in rows. This appearance was misleading in terms of the cells themselves as the ends of successive cells tended to dovetail between others. Irregular terminal and lateral contacts occurred but parallel cells were typically separated by narrow zones of free space along at least part of their length. The general speed of movement seen in films appeared to be much slower than that of chick heart fibroblasts. In every film, most cells were moving radially outwards from the explant but usually one or two could be seen travelling in the opposite direction between adjacent, outwardly moving, parallel cells. Recently formed mitotic daughter cells could be seen (a) both to travel inwards, (b) both to travel outwards, or (c) to move individually in opposite directions.

Populations of the human melanoma line MM96 (Fig. 4D–F) were highly pleomorphic. Changes of form could be seen in individual cells during the course of a film. Cell shape varied from rounded to polygonal, stellate, multidendritic or bipolar.
Fig. 4. Unconfronted cell populations emigrating from explants. Peripheral regions of outwanderings. All photographs are enlargements from parts of 16-mm frames, all to the same scale. The arrow indicates the direction of radial outward movement. A, Embryonic chick heart fibroblasts. B, adult human skin fibroblasts emigrating from artificial explant. Area slightly behind free edge, to show parallel alignment. C, adult human skin fibroblasts. Part of free edge of outwandering from artificial explant. D, E, human malignant melanoma cells from permanent in vitro line, MM96. Outwanderings from two different artificial explants. F, MM96 outwandering from an explant of a tumour resulting from a test implantation of MM96 cells in a nude mouse. The outwandering is shown for comparison with those of the artificial explants used in confrontations.
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Although the cells were known to be capable of producing pigment, this was not an obvious feature of the rapidly growing cultures filmed. Each population as a whole made slow but predominantly outward progress although individual cells displayed little recognizable polarity. Some cells appeared merely to oscillate within a confined area and the impression of poor adhesion to the substratum was very strong. MM96 cells had a thick, dense, compact appearance compared with that of fibroblasts. Expanded lamellae, if they occurred, were transient. Ruffling associated with small lamellae at the ends of bipolar cells or the multiple processes of stellate cells was often intense, as was intermittent blebbing. Mitotic divisions were frequent and newly formed cells often remained on top of older cells. Association to form irregular, 3-dimensional clusters was common. Most of the morphological characteristics listed above have previously been reported for other, widely different populations of cultured malignant cells (Barski & Belehradek, 1965; Veselý & Weiss, 1973; Abercrombie & Heaysman, 1976).

Measurements recorded from films of unconfronted explants

Movements of migrating avian and human fibroblasts were overwhelmingly outward from the explant and in a radial direction (Table 2). When lateral-outward frequencies were added to radial-outward values, total outward movements accounted for 76% (CHF) and 73% (HSF) of all measured movements over a 6-h period. MM96 cells, although demonstrating a significant outward direction of movement, nevertheless had a much lower percentage of movements in either radial-outward (Table 2) or total outward directions (57%) than the fibroblast populations. They also displayed a relatively greater number of 'zero' measurements, i.e. intervals during which movement was too slight to be recognized on the scale of measurements employed. These zero values could not be assigned to any specific direction and were therefore omitted from Tables 2 and 3.

Regressions (Table 3) of speed ($Y$) against numbers of contacts ($X$) in unconfronted cells moving in radial-outward and radial-inward directions were compared by analysis of covariance (Snedecor & Cochran, 1967, p. 435). No distinction was made between stable and changing contacts. For chick fibroblasts moving outwards the regression, as indicated by $r^2$ (Table 3), accounted for only a very small amount of the variability of the speed. In the opposite direction, i.e. inwards, towards the explant, the relationship of numbers of contacts to cell speed became much more important, the regression accounting for 12% of the variance of cell speed. The steepness of the slope was significantly increased and the adjusted mean speed (Table 3) was significantly reduced compared with values recorded for outward-moving cells.

When similar regressions for human skin fibroblasts travelling in opposite directions were compared (Table 3), the slopes were almost identical. As in the case of chick fibroblasts, human cells travelling inwards moved significantly more slowly than those travelling away from the explant. The correlation coefficients were both significant and the value of $r^2$ was only slightly greater in an inward direction than for outwardly moving cells (Table 3).

Comparisons of similar regressions relating to the speed of MM96 cells (Table 3)
Table 2. Percentage frequencies of cells in unconfronted populations emigrating from explants

<table>
<thead>
<tr>
<th>Population</th>
<th>Direction</th>
<th></th>
<th>Chi-squared value (d.f. 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Radial</td>
<td>Lateral</td>
<td></td>
</tr>
<tr>
<td></td>
<td>outward</td>
<td>inward</td>
<td></td>
</tr>
<tr>
<td>Chick heart fibroblasts (CHF)</td>
<td>54</td>
<td>22</td>
<td>1048</td>
</tr>
<tr>
<td>Human skin fibroblasts (HSF)</td>
<td>52</td>
<td>21</td>
<td>1078</td>
</tr>
<tr>
<td>Human malignant melanocytes (MM96)</td>
<td>29</td>
<td>28</td>
<td>914</td>
</tr>
</tbody>
</table>

Before pooling the frequencies from all cells of each type, tests for heterogeneity of the individual cell values were carried out and found to be non-significant. The Chi-squared test for goodness-of-fit for each set of pooled data was based on a hypothetical expected value of N/4 for each quadrant. All results shown were highly significant (P < 0.001). Refer to Fig. 1 for orientation of quadrants.

Table 3. Comparisons of regressions of speed (Y) on number of contacts (X) for unconfronted cells travelling in a radial direction away from or towards the explant

<table>
<thead>
<tr>
<th>Population ...</th>
<th>Chick heart fibroblasts</th>
<th>Human skin fibroblasts</th>
<th>Human melanoma cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direction of movement</td>
<td>Outward</td>
<td>Inward</td>
<td>Outward</td>
</tr>
<tr>
<td>N</td>
<td>568</td>
<td>117</td>
<td>566</td>
</tr>
<tr>
<td>Mean contact no. (X)</td>
<td>3.63</td>
<td>3.21</td>
<td>4.84</td>
</tr>
<tr>
<td>Y adj (adjusted mean speed)</td>
<td>33.5</td>
<td>26.5</td>
<td>17.8</td>
</tr>
<tr>
<td>b (= reg. coefficient = slope)</td>
<td>-0.0170</td>
<td>-0.0543</td>
<td>-0.0259</td>
</tr>
<tr>
<td>Comparison of slopes</td>
<td>F = 6.97</td>
<td>F = 0.0054</td>
<td>F = 0.1209</td>
</tr>
<tr>
<td>d.f. 1, 681</td>
<td>d.f. 1, 745</td>
<td>d.f. 1, 460</td>
<td></td>
</tr>
<tr>
<td>Comparisons of intercepts (a)</td>
<td>F = 10.3</td>
<td>F = 6.8</td>
<td>F = 3.4</td>
</tr>
<tr>
<td>d.f. 1, 682</td>
<td>d.f. 1, 746</td>
<td>d.f. 1, 461</td>
<td></td>
</tr>
<tr>
<td>r (correlation coefficient)</td>
<td>-0.1216</td>
<td>-0.3473</td>
<td>-0.2636</td>
</tr>
</tbody>
</table>

Y = speed = log (Y + 1), measured in mm/10 min. The adjusted mean speed (Y adj.) = mean speed adjusted for the removal of any dependence on the value of X. Values for adjusted means are shown after conversion to mm h⁻¹. Intercept (a) = adjusted mean speed when X = 0. Small letters above b values indicate significance of t for test of (b = 0). Probability P < 0.05 is indicated by p, P < 0.01 by s, and P < 0.001 by c.
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indicated no difference in slopes or adjusted means. Only the outward slope displayed even a probably significant difference from zero and the correlation coefficients were non-significant. Therefore, for unconfronted MM96 cells, little evidence was found of a linear relationship between speed and homologous contact number.

An estimate of effective outward progress was provided by the net radial outward velocity (Fig. 3). The mean values of this measurement (Table 4) confirmed visual impressions, that chick heart fibroblasts advanced outwards much more rapidly than cells of either HSF or MM96 populations. Of the 3 types, the malignant cells made by far the slowest effective progress.

Table 4. Net radial outward velocities. Comparisons of means of unconfronted and post-junction populations

<table>
<thead>
<tr>
<th>Cell populations and confrontations</th>
<th>Unconfronted Mean ± s.e.</th>
<th>N</th>
<th>Post-junction Mean ± s.e.</th>
<th>N</th>
<th>t</th>
<th>d.f.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human malignant melanocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Against CHF</td>
<td>1.60 ± 0.55</td>
<td>30</td>
<td>1.71 ± 0.36</td>
<td>27</td>
<td>0.2</td>
<td>55 n.s.</td>
</tr>
<tr>
<td>(b) Against HSF</td>
<td>3.24 ± 1.08</td>
<td>24</td>
<td>1.4 ± 0.52</td>
<td>24</td>
<td>52 n.s.</td>
<td></td>
</tr>
<tr>
<td>Human skin fibroblasts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Against CHF</td>
<td>6.43 ± 1.36</td>
<td>35</td>
<td>-7.23 ± 2.8</td>
<td>27</td>
<td>4.7</td>
<td>60 P &lt; 0.001*</td>
</tr>
<tr>
<td>(b) Against MM96</td>
<td>5.20 ± 2.57</td>
<td>28</td>
<td>0.5 ± 0.5</td>
<td>28</td>
<td>61 n.s.</td>
<td></td>
</tr>
<tr>
<td>Chick heart fibroblasts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Against HSF</td>
<td>17.47 ± 1.52</td>
<td>30</td>
<td>13.8 ± 1.79</td>
<td>27</td>
<td>1.6</td>
<td>55 n.s.</td>
</tr>
<tr>
<td>(b) Against MM96</td>
<td>5.02 ± 1.98</td>
<td>28</td>
<td>5.0 ± 1.98</td>
<td>28</td>
<td>56 P &lt; 0.001</td>
<td></td>
</tr>
</tbody>
</table>

Values are recorded in μm h⁻¹.

* For this comparison only, a variance ratio test was significant. P < 0.001 was therefore accepted as a minimal level of significance for the t-test.

Confronted populations

Human malignant melanoma cells (MM96) versus chick heart fibroblasts (CHF)

(a) Qualitative observations from films. At junction, a slowly moving, irregularly distributed outwandering of MM96 cells met a chick fibroblast population of coherent, intensely monolayered cells of roughly comparable density. The chick cells were strongly oriented outwards. Outriding MM96 cells apparently entered the fibroblast sheet only when they could insert a leading edge, typically the actively ruffling narrow tip of a bipolar cell, between 2 adjacent chick cells. Once entry had been effected, invading MM96 cells then continued to move slowly outward between successive chick cells, taking on the orientation of the latter (Fig. 5). Sometimes several melanocytes followed each other in a row.

When the leading edge of a melanoma cell met either the leading edge or the side of a chick cell, the malignant cell exhibited no lessening of its typically intense membrane activity and no obvious contraction or withdrawal. In the crowded conditions often seen in films and at the magnifications employed, it was usually impossible to determine the exact extent of any overlapping or underlapping by melanoma cells.
Fig. 5. Interactions between MM96 cells and chick fibroblasts. Enlargements of parts of 16-mm frames from a 6-h film. Arrows show radial outward direction of movement of each population. The sequence shows the outward progression of the MM96 cell $m_1$ between successive pairs of chick fibroblasts. Refer to Results for general descriptions. Cells are numbered for identification of their relative positions in successive sequences.
However, in head/side collisions it is likely that in at least some cases the leading edge of a melanocyte was extended beneath the chick cell to meet the fibroblast next in line. In collisions involving the leading edges of both heterologous cells, underlapping or overlapping appeared to be minimal and the chick cell itself was contact-inhibited.

In early, less-crowded film sequences, it could be seen that after a heterologous collision the obstructing chick cell typically pulled apart from one or more adjacent fibroblasts and moved into free space (Fig. 5). The melanoma cell moved forward to occupy the vacated area and made contact with the chick cell or cells next in line. In later sequences of some films in which crowding of the chick population was intense and free space for contact-inhibited chick cells was greatly reduced, outriding MM96 cells appeared to be temporarily blocked. In no case was there evidence of a melanoma cell actively moving outwards on top of a fibroblast sheet.

Table 5. Comparisons of frequencies of movements outward from the explant in (a) unconfronted and (b) post-junction situations

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Movement comparisons</th>
<th>Movements outwards ((ro+lo)) as percentage of total</th>
<th>(N) (total)</th>
<th>Chi-squared value (d.f. 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human malignant melanoma (MM96)</td>
<td>Not confronted</td>
<td>56.9</td>
<td>914</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Post-junction v. CHF</td>
<td>56.8</td>
<td>860</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>Post-junction v. HSF</td>
<td>58.7</td>
<td>661</td>
<td>0.6</td>
</tr>
<tr>
<td>Human skin fibroblasts (HSF)</td>
<td>Not confronted</td>
<td>73.2</td>
<td>1078</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Post-junction v. CHF</td>
<td>56.7</td>
<td>665</td>
<td>229  (P &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>Post-junction v. MM96</td>
<td>60.1</td>
<td>546</td>
<td>28.4 (P &lt; 0.001)</td>
</tr>
<tr>
<td>Chick heart fibroblasts (CHF)</td>
<td>Not confronted</td>
<td>76.1</td>
<td>1048</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Post-junction v. HSF</td>
<td>73.5</td>
<td>880</td>
<td>16.9 (P &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>Post-junction v. MM96</td>
<td>56.0</td>
<td>836</td>
<td>84.9 (P &lt; 0.001)</td>
</tr>
</tbody>
</table>

Chi-squared values were derived in each case from 2 x 2 contingency tables comparing frequencies in a post-junction situation with those recorded for an unconfronted population.

\((ro+lo) = (radially outwards + laterally outwards) = total movements away from the explant)\.

In all films, soon after junction of the MM96-CHF outwanderings, a distinct slowing down of the chick fibroblasts was seen as they became progressively crowded and compacted. Most heterologous collisions involving leading edges resulted in contact inhibition of CHF followed either by change of direction, or by blockage and temporary immobilization. However, chick fibroblasts were able to pass beneath dendritic processes of MM96 cells or beneath elongated, orthogonally oriented bipolar melanocytes which were presumably attached to the substratum at each end. Chick cells were also seen to squeeze through small gaps between adjacent melanocytes.

(b) Measurements obtained from films. After junction of MM96 cells with CHF, the net radial outward velocity (Table 4) and the frequency of outward movements (Table 5) of the melanoma cells were unchanged compared with their values in unconfronted MM96 cultures. Multiple regressions of speed of MM96 cells against...
numbers of homologous and heterologous contacts could not be compared directly with regressions for unconfronted cells in which only homologous contacts were present. For confronted situations, multiple regressions of speed against homologous and heterologous contacts of cells travelling radially outward and inward were compared by analyses of multiple covariance (Table 6). As in unconfronted situations, the adjusted mean speeds of MM96 cells were similar in both directions. However, the relationship between speed and contacts, which was non-significant for unconfronted MM96 cells, increased in importance in the presence of chick cells.

After junction with MM96, the net outward velocity and the frequency of outward movements of the chick cells were significantly decreased compared with their values in unconfronted populations (Tables 4, 5). These indications of severe obstruction by the melanoma cells were probably supported also by the fact that the post-junctional adjusted mean speed for outwardly moving chick cells became only marginally higher than that of chick cells travelling back towards the explant (Table 6). The positive value (0.0350) of the partial regression coefficient $b_z$ and the high significance of its difference from zero (Table 6) have not yet been adequately explained. A subjective impression that chick fibroblasts temporarily increased their speed while underlapping MM96 cells or their processes may perhaps be relevant.

At the beginning of the filming period, the mean densities of MM96 and CHF in the camera field were 477 and 837 cells mm$^{-2}$. After 6 h, the MM96 population had increased by 54% and that of CHF by only 6%. Although the relative contributions of mitosis and immigration were not assessed, the implication of severe obstruction of the fibroblasts seemed clear.

Measurements from fixed cultures and comparisons with measurements from films. At junction, MM96 cells had travelled only 30% of the mean distance covered by chick fibroblasts in the same period (Fig. 6). These measurements, which were based on estimates (Abercrombie & Heaysman, 1976) and for which precise times were not recorded, could not be compared directly with net outward velocity measurements from films but nevertheless reflected the large difference in MM96 and CHF velocities. Between junction and fixation, MM96 cells travelled approximately 87% of the distance covered by CHF cells in the same time and contributed almost 47% to the invasion zone (Fig. 6). When the between-explant distances traversed by MM96 and CHF after junction were compared with the free 'side' distance for each population (Figs. 1, 6), the invasive index of the chick fibroblasts was found to be severely depressed (Table 7). The profound post-junctional obstruction of chick cells as recorded from films was therefore confirmed by the fixed-culture measurements.

The invasive index of MM96 cells indicated a probably significant reduction from unity (Table 7). As no significant blockage of melanoma cells was demonstrated from film analyses, its effect presumably became recognizable only in the more crowded conditions of the post-filming period.

The homologous nuclear overlap indices of MM96 and CHF in the invasion zone were much increased compared with indices for the same cells at roughly comparable total cell densities elsewhere (Table 8). Both indices were nevertheless significantly lower than the values expected had the distribution of their nuclei been random.
Table 6. Relationships of speed (Y) of cells travelling in 2 possible directions to numbers of homologous (X₁) and heterologous (X₂) contacts. Summarized results of multiple regressions and multiple covariance analyses based on post-junction data from paired confrontations.

<table>
<thead>
<tr>
<th>Populations, confrontations and directions</th>
<th>N</th>
<th>Y adj</th>
<th>X₁</th>
<th>X₂</th>
<th>b₁</th>
<th>b₂</th>
<th>R² (%)</th>
<th>F for heterogeneity of regressions (d.f.)</th>
<th>F for difference between intercepts (a) (d.f.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM96 v. CHF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outward</td>
<td>271</td>
<td>14.7</td>
<td>1.5</td>
<td>3.2</td>
<td>-0.0155*</td>
<td>0.0054</td>
<td>2.4</td>
<td>(2, 455)</td>
<td>0.65</td>
</tr>
<tr>
<td>Inward</td>
<td>190</td>
<td>14.6</td>
<td>1.6</td>
<td>3.1</td>
<td>-0.0138*</td>
<td>-0.0207*</td>
<td>6.3</td>
<td>(1, 457)</td>
<td></td>
</tr>
<tr>
<td>CHF v. MM96</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outward</td>
<td>289</td>
<td>24.8</td>
<td>3.1</td>
<td>2.2</td>
<td>0.0099</td>
<td>0.0350*</td>
<td>6.2</td>
<td>4.89*</td>
<td>4.23*</td>
</tr>
<tr>
<td>Inward</td>
<td>192</td>
<td>20.9</td>
<td>3.4</td>
<td>1.8</td>
<td>-0.0095</td>
<td>-0.0078</td>
<td>0.5</td>
<td>(2, 475)</td>
<td>(1, 477)</td>
</tr>
<tr>
<td>MM96 v. HSF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outward</td>
<td>233</td>
<td>18.9</td>
<td>1.3</td>
<td>1.7</td>
<td>-0.0042</td>
<td>0.0131</td>
<td>1.1</td>
<td>1.71*</td>
<td>4.24*</td>
</tr>
<tr>
<td>Inward</td>
<td>166</td>
<td>16.7</td>
<td>1.3</td>
<td>2.0</td>
<td>-0.0022</td>
<td>-0.0058</td>
<td>0.6</td>
<td>(2, 393)</td>
<td>(1, 395)</td>
</tr>
<tr>
<td>HSF v. MM96</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outward</td>
<td>222</td>
<td>22.6</td>
<td>3.6</td>
<td>1.5</td>
<td>-0.0159</td>
<td>-0.0325*</td>
<td>3.5</td>
<td>0.34</td>
<td>1.92</td>
</tr>
<tr>
<td>Inward</td>
<td>141</td>
<td>20.6</td>
<td>3.5</td>
<td>1.2</td>
<td>-0.0221*</td>
<td>-0.0353*</td>
<td>11.3</td>
<td>(2, 257)</td>
<td>(1, 359)</td>
</tr>
<tr>
<td>CHF v. HSF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outward</td>
<td>155</td>
<td>17.9</td>
<td>3.2</td>
<td>3.9</td>
<td>0.0195*</td>
<td>-0.0086</td>
<td>13.1</td>
<td>8.45*</td>
<td>14.44*</td>
</tr>
<tr>
<td>Inward</td>
<td>290</td>
<td>20.6</td>
<td>2.6</td>
<td>4.0</td>
<td>-0.0318*</td>
<td>-0.0371*</td>
<td>16.3</td>
<td>(1, 439)</td>
<td>(1, 441)</td>
</tr>
</tbody>
</table>

Y = speed = log (Y + 1), measured in mm/10 min. Y adj = mean speed adjusted for the removal of any dependence on the values of X₁ and X₂. Values for adjusted means are shown after conversion to μm h⁻¹. Intercept (a) = adjusted mean speed when X₁ = 0 and X₂ = 0. b₁ and b₂ are the partial regression coefficients. The squared multiple correlation coefficient, R², indicates the percentage of the variance of Y attributable to the regression of Y on X₁ and X₂. Small letters above b values indicate the significance of t for (b = 0). Probability P < 0.05 is shown by p, P < 0.01 by s, and P < 0.001 by c.
Fig. 6. Diagrams illustrating relative degrees of invasion of confronted cell populations (modified after Abercrombie & Heaysman, 1976). $a$ is the radius of a Type 1 artificial explant (stippled); $b$ is an arbitrary standardized interexplant distance. For each confrontation, the 2 partly superimposed central columns represent the mean distances travelled between the explants by each cell population in relation to the common radius (see Fig. 1 and Table 7). The invasion zone common to the 2 populations is shaded black. The thick bar across the invasion zone represents the point of junction. On either side of this, the post-junction distance travelled by each cell type is shown. The illustrated penetration of the MM96 explant by CHF and HSF populations was possible because of the irregular arrangement of the tumour cells at the edge of the explant. The position of the junction line reflects the relative velocities of each population. The incubation period before and after junction differed for each confrontation and was related to the relative velocities of the cells involved. The unshaded lateral columns represent the 'side' or unconfronted outwanderings for the same periods.

(Abercrombie & Heaysman, 1954). The relatively low heterologous overlap index was consistent with the pattern of collision behaviour observed in films. In heterologous overlaps for which focus checks were carried out, the chick cell was found between the melanocyte and the substratum. It is likely that most of these examples were due to underlapping by chick cells.

**Human malignant melanoma cells (MM96) versus human skin fibroblasts (HSF)**

(a) Qualitative observations from films. MM96 cells were not usually contact inhibited by collisions with human skin fibroblasts, although isolated examples were seen of melanocyte withdrawal after heterologous contact. Paralysis of membrane activity did not occur and after most head-on collisions the melanoma cell either moved outward
after the contact-inhibited fibroblast withdrew or remained in more or less its original position.

Several invasive mechanisms or pathways appeared to be available to MM96 cells, depending on circumstance. (i) Progression was sometimes by diffusion-like movement into free space between elongated, aligned fibroblasts. In this type of situation the melanoma cells were themselves elongated and bipolar in form. (ii) In some observed heterologous collisions involving leading edges, a melanoma cell was seen to move forward on to the upper surface of the contacting fibroblast (Fig. 7). Both cells reacted violently, repeatedly changing their relative positions but with the characteristically rounded, blebbing, malignant cell remaining on the upper surface of the fibroblast.

Table 7. Between: side ratios and invasive indices

<table>
<thead>
<tr>
<th>Confrontation</th>
<th>Between: side ratios</th>
<th>Invasive index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before junction</td>
<td>After junction</td>
</tr>
<tr>
<td>MM96</td>
<td>0.89 ± 0.28</td>
<td>0.78 ± 0.14</td>
</tr>
<tr>
<td>CHF</td>
<td>0.96 ± 0.03</td>
<td>0.56 ± 0.07</td>
</tr>
<tr>
<td>MM96</td>
<td>1.12 ± 0.14</td>
<td>0.87 ± 0.14</td>
</tr>
<tr>
<td>HSF</td>
<td>1.07 ± 0.04</td>
<td>0.71 ± 0.04</td>
</tr>
<tr>
<td>HSF</td>
<td>0.99 ± 0.2</td>
<td>0.52 ± 0.09</td>
</tr>
<tr>
<td>CHF</td>
<td>1.06 ± 0.04</td>
<td>0.87 ± 0.09</td>
</tr>
</tbody>
</table>

Refer to Fig. 1 for explanation of measurements. Pre-junction measurements were recorded from living cultures. Post-junction measurements were obtained from the same cultures after fixation and staining. N = 10 in each case.

Table 8. Nuclear overlap indices and nuclear densities recorded from fixed cultures

<table>
<thead>
<tr>
<th>Cells</th>
<th>Density (cells mm⁻¹)</th>
<th>Homologous overlap index</th>
<th>Heterologous overlap index</th>
<th>Density (cells mm⁻¹)</th>
<th>Homologous overlap index</th>
<th>Density (cells mm⁻¹)</th>
<th>Homologous overlap index</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM96 v.</td>
<td>1310</td>
<td>77.2</td>
<td></td>
<td>2815</td>
<td>33.3</td>
<td>1722</td>
<td>54.5</td>
</tr>
<tr>
<td>CHF</td>
<td>933</td>
<td>48.3</td>
<td></td>
<td>2096</td>
<td>15.6</td>
<td>1742</td>
<td>21.0</td>
</tr>
<tr>
<td>MM96 v.</td>
<td>485</td>
<td>118.3</td>
<td></td>
<td>1761</td>
<td>43.3</td>
<td>1093</td>
<td>52.1</td>
</tr>
<tr>
<td>HSF</td>
<td>195</td>
<td>95.5</td>
<td></td>
<td>513</td>
<td>21.8</td>
<td>569</td>
<td>18.7</td>
</tr>
<tr>
<td>HSF v.</td>
<td>233</td>
<td>21.6</td>
<td></td>
<td>591</td>
<td>29.2</td>
<td>644</td>
<td>32.3</td>
</tr>
<tr>
<td>CHF</td>
<td>460</td>
<td>22.4</td>
<td></td>
<td>1363</td>
<td>13.9</td>
<td>870</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Nuclear overlap indices are shown as percentages of observed overlaps in relation to expected values. Densities are approximations based only on cell numbers recorded during overlap counts. All values are presented here as mean figures without confidence limits. N = 10 for MM96 v. CHF and HSF v. CHF. N = 9 for MM96 v. HSF.
Fig. 7. Interaction between a human skin fibroblast and a human malignant melanocyte from the permanent in vitro line MM96. Enlargements of parts of frames from a 16-mm film sequence. The total time from A to D was approximately 3-5 h. In C and D, the melanocyte was on the dorsal surface of the fibroblast. Arrows indicate direction of outward movement of each population.
(iii) Sometimes an MM96 cell had a long dendritic process in contact with a fibroblast some distance away. If the MM96 cell body became detached, it was carried by recoil into the fibroblast-occupied area. This mechanism appeared to be a form of retraction clumping (Harris, 1974).

In almost all observed collisions involving leading edges of MM96 cells and HSF, the fibroblasts were contact-inhibited. Collision was followed by apparent contraction, withdrawal and change of direction. In head-side collisions, human fibroblasts passed freely beneath elongated, bipolar melanocytes or under dendritic processes.

(b) Measurements obtained from films. Compared with their values in unconfronted situations, the net outward velocity and the frequency of outward movements of MM96 cells remained unaltered after junction of MM96 and HSF populations (Tables 4, 5). When the effects of contacts were standardized, the adjusted mean speed of MM96 travelling outwards against HSF cells was marginally increased compared with that for cells travelling inwards towards the explant (Table 6). These measurements all implied lack of obstruction by the fibroblasts.

After junction with MM96, human fibroblasts displayed a non-significant decrease in net outward velocity (Table 4) and a significant reduction in frequency of outward movements (Table 5). No difference was found in post-junctional adjusted means of HSF populations travelling away from or towards the explant (Table 6).

The mean density of MM96 cells initially in the camera field was 161 cells mm$^{-2}$. The fibroblast density was 171 cells mm$^{-2}$. By the end of the filming period, the MM96 population had increased by 35% and the HSF population by 43%.

(c) Measurements from fixed cultures and comparisons with measurements from films. At junction, MM96 cells had travelled 29% of the mean distance covered by the human fibroblasts. Between junction and fixation, MM96 cells travelled approximately 52% of the HSF distance for the same period and contributed 34% to the invasion zone (Fig. 6). However, when the effects of differing population velocities were eliminated by comparing the post-junctional between-explant distance for each population with the side distance for the same period (Figs. 1, 6), the high degree of invasiveness of the malignant cells became apparent. The MM96 invasive index did not differ significantly from unity (Table 7), indicating negligible obstruction by the human fibroblasts. The HSF invasive index was significantly less than unity (Table 7), indicating obstruction by MM96. During the filming period, this obstruction was shown mainly by a reduction in frequency of outward movements. It seems likely that the blocking effect increased with time, presumably as pathways between the tumour cells or beneath their processes became fewer for the relatively large human fibroblasts.

The MM96 homologous overlap index for cells in the invasion zone (Table 8) was very much higher than in either the area immediately behind this zone or in the side outwandering, even though both these regions had denser populations. The value of the index was no different from that expected if the nuclei were randomly distributed, implying a very low intensity of homologous contact inhibition of movement. The low HSF homologous overlap index (Table 8) implied a high degree of homologous contact inhibition (Abercrombie & Heaysman, 1954). The relatively high HSF-
MM96 heterologous index (Table 8) suggested some degree of failure of heterologous contact inhibition. This was confirmed from observations of filmed collisions. In every heterologous overlap recorded from fixed cultures and in every film sequence in which focus checks were made, the melanocyte lay on the upper or dorsal surface of the fibroblast.

**Adult human skin fibroblasts (HSF) versus embryonic chick heart fibroblasts (CHF)**

(a) **Qualitative observations from films.** Human skin fibroblasts were typically much larger than chick heart fibroblasts (Fig. 8). Early post-mitotic human cells were approximately the same size as the largest chick fibroblasts but could be distinguished quite easily from the latter by morphological and cytological features such as alignment, arrangements of processes and general phase-contrast appearance of nuclei and cytoplasm.

The post-junctional pattern of behaviour of the 2 cell types in any given culture seemed to be largely determined by the orientation of the human cells at the time the outwanderings met. If the peripheral human cells were radially aligned, chick cells advancing outward in a radial direction were eventually able to pass between parallel human cells to achieve what was by definition (Abercrombie, 1975) invasion of the HSF population. Sometimes the peripheral area of the CHF population was in the form of a radially oriented wedge, the cells at the tip being the first to penetrate between the human cells.

When the leading edges of radially oriented human fibroblasts collided with those of chick cells, they appeared to undergo contact inhibition of locomotion, followed by a retreat in the opposite (i.e. inward) direction. The retreat, which seemed to be a direct response to heterologous contact, was apparently begun as a contact retraction (Weiss, 1958). This appeared to give an initial impetus to the inward movement of the human fibroblasts but even when subsequent separation from the contacting chick fibroblasts was seen to occur, the human cells continued to retreat at what seemed to be relatively high speed.

Peripheral chick cells involved in head-on collisions with human fibroblasts were

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**Fig. 8. Interactions between confronted human skin fibroblasts and chick heart fibroblasts.** Enlargements of parts of 16-mm frames. Total time from A to C was approximately 3 h. Cells $h_1$-$h_8$ are human fibroblasts; all other cells are chick fibroblasts. $h_1$-$h_8$ are radially aligned cells at the periphery of an HSF outwandering from an explant outside the camera field, on the right of the frame. Before junction, HSF cells were moving in the direction of the HSF arrow. $h_4$ and $h_5$ are recent mitotic products. The chick fibroblasts are advancing radially from left to right. In A, as a result of heterologous collision, $h_1$ has begun to move back towards the HSF explant. $h_2$ and $c_1$ are still in contact after a collision. $c_2$ is moving into free space between $h_2$ and $h_3$. In B, $h_4$ has retreated as a result of contact retraction from $c_1$ but has produced a new outwardly-directed leading edge in the space between $h_1$ and $h_3$. The leading edge of $c_2$ has collided with this and the locomotion of both cells has become contact inhibited. $c_1$ has moved outward into the space between $h_1$ and $h_3$. $c_1$ cells $h_1$-$h_3$ and $h_4$ have all moved inward from their original positions. $h_4$ has become surrounded by chick cells and is still close to its original position at junction. $h_4$ and $c_2$ are pulling apart from each other and $c_2$ has become contact inhibited by an advancing chick cell.
Filmed human melanoma and fibroblast invasion
themselves usually in close contact from behind with crowded and compacted cells of their own population. Under these circumstances, it was often difficult to assess their heterologous contact reactions. In crowded cultures also, the leading chick cells characteristically remained in contact with the leading human fibroblasts and successively occupied space vacated by them. Frequently, while remaining in contact with a human fibroblast, chick cells gradually moved laterally around its edge until they could enter the space between it and the adjacent human cell.

In less-crowded conditions, chick fibroblasts were seen to undergo contact inhibition of locomotion after their leading edges collided with those of human fibroblasts. In one relatively sparse culture in which the human fibroblasts at the periphery of the HSF outwandering had an open, radial and parallel orientation (Fig. 8), examples of contact inhibition on the part of both chick and human cells were clearly seen after heterologous collision. In these cases, what appeared to be adhesion strands were visible between the heterologous cells as they retracted from each other. Slight differences in behaviour of the chick cells appeared to be dependent on the amount of available free space. The same film (Fig. 8) also showed that chick heart fibroblasts could pass quite freely between adjacent human cells, regardless of lateral contacts, until they were stopped by head-on collision with part of a HSF lamella (Fig. 8).

The combined effect of heterologous collision, seen in almost all films, was the progressive retreat of human skin fibroblasts, in many cases right out of the camera frame (Fig. 9). This meant that the rule adhered to in all other cases, of analysing movements of each cell throughout an entire 6-h period, had to be relaxed for some
human fibroblasts. Occasionally (Fig. 8), a human cell became completely surrounded by chick heart cells and isolated from the main HSF population.

In 2 films, cells of the free edge of the confronting HSF population were aligned more or less at right angles to the prevailing radial arrangement. In these cases, approaching chick cells could not pass beyond the outriding human cells and, after making contact, turned aside either to right or left. In another film of a very crowded culture of chick cells, all the human fibroblasts retreated except one large orthogonally oriented cell. This appeared to become stretched and at least one central area became detached from the substratum. Through this tunnel, chick cells advanced in a continuous stream.

(b) Measurements obtained from films. After junction with CHF, the net outward velocity of human fibroblast populations was so severely reduced compared with the unconfronted HSF figure that it became a negative value (Table 4). Clearly, by the end of the filmed post-junction period, many human cells had retreated to take up a position closer to the HSF explant than they had occupied at the time of junction. The HSF frequency of outward movements was also highly significantly reduced after junction with CHF (Table 5). The corresponding increase in numbers of movements in the reverse direction towards the explant was mainly radial-inward. Multiple covariance analysis (Table 6) showed that the adjusted mean speed of human fibroblasts moving back towards the explant was faster than that of cells moving outwards against the HSF population.

No significant alterations were detected in either the net outward velocity (Table 4) or the frequency of outward movements (Table 5) of chick heart fibroblasts after junction with HSF. The post-junctional adjusted mean speed of the chick cells (Table 6) was significantly greater for cells moving outward against HSF than for cells moving inward towards the CHF explant. These results gave no evidence of CHF obstruction by human fibroblasts.

At the beginning of filming, the mean densities of HSF and CHF in the camera field were 117 and 317 cells mm$^{-2}$. After 6 h, the mean density of chick cells had increased by 153%, while that of the HSF population had decreased by 8%.

(c) Measurements from fixed cultures and comparisons with measurements from films.

In fixed cultures the area of greatest penetration by chick fibroblasts was typically the narrow zone related to the common radius between the explants, where the invading chick cells could sometimes be seen as a wedge or spearhead. This pattern was by no means universal. In some cultures there were subsidiary wedges, while in others no massed formations could be distinguished. At the sides of the narrow invasion strip in most cultures, cells of both HSF and CHF populations were oriented orthogonally to the radial axis, suggesting a mutual turning aside after junction.

The high variability in the distances travelled by human fibroblasts after junction with CHF was reflected in the confidence limits of the HSF invasive index (Table 7). In 4 out of the 10 fixed cultures, HSF populations displayed 'negative' invasion against chick heart cells, i.e. radial measurements made to the furthest human cell between the explants (Fig. 1) did not reach as far as the estimated position of HSF at junction. In all of these cultures, a 'wedge' pattern of chick cells was seen in the
invasion zone. In another 4 cultures, the furthest human cell was almost at or very slightly beyond the estimated junction position. In the remaining 2 cultures, the leading human fibroblasts were some distance beyond the estimated point of junction.

The very low, negative HSF invasive index (Table 7), which represented the mean of the conditions described above, implied that the human fibroblasts which had travelled furthest between the explants in the post-junction period were maintained at approximately the level they had occupied at junction (Fig. 6). From filmed observations, this condition could be explained in terms of human cells 'trapped' and surrounded by chick fibroblasts near the point of junction (Fig. 8). These isolated human cells appeared to make very slow progress in any direction. The negative measurements recorded from individual fixed cultures (see above) were obviously explained by the post-collision reversal of movement of human fibroblasts recorded from films. Unfortunately, the frequent loss of inwardly moving human fibroblasts from the camera field usually prevented any assessment of how long the reverse movement continued. No direct evidence was seen of the limited HSF invasion of chick populations recorded from 2 fixed cultures (see above). However, the net outward velocity measurements recorded for individual human cells were highly variable and included a number of positive values.

The unobstructed invasion of HSF populations by chick fibroblasts, well documented from film analyses, was confirmed by the CHF invasive index which did not differ significantly from unity (Table 7 and Fig. 6). In the invasion zone (Table 8), the total numbers of nuclear overlaps were very small and in some cultures were completely absent. The heterologous overlap index, which was lower than either of the homologous indices in the invasion zone, was consistent with the high intensity of heterologous contact inhibition observed in films. In each of the 4 heterologous overlaps recorded, the chick cell was found between the human fibroblast and the substratum.

**DISCUSSION**

Theoretical considerations relating to the use of confronted explants in the study of invasion have been formulated and discussed in detail by Abercrombie (1975) and Abercrombie & Heaysman (1976). The specific reasons for the present study were (a) the introduction of human material into the same type of confrontation system as that already established (Abercrombie et al. 1957; Abercrombie & Heaysman, 1976), and (b) the combination and assessment of evidence from time-lapse films as well as from fixed cultures. Results of the test comparisons listed in the Introduction are considered in sequence below.

**Invasive behaviour of malignant and non-malignant human cells against a standard avian fibroblast population**

MM96 malignant human melanoma cells invaded the standard chick fibroblast population but eventually encountered moderate obstruction from the chick cells. Adult human skin fibroblasts did not invade the standard CHF population. The special features of this latter confrontation of normal fibroblasts will be discussed on page 415.
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An apparent anomaly between results of film analyses and those from fixed cultures requires to be resolved for MM96-CHF confrontations. Post-junctional net outward velocity and frequency of outward movements of MM96 were not reduced from their unconfronted values; nor did MM96 post-junctional outward and inward mean speeds, adjusted after standardization of contact effects, differ from each other. Yet measurements from fixed cultures showed that the mean outward distance travelled by MM96 cells after meeting the chick fibroblast population was probably reduced compared with the distance MM96 cells travelled towards free space at the side of the explant.

This blockage of MM96 cells was apparently related to the heterologous collision reactions of the tumour cells with the chick fibroblasts. The melanoma cells moved outward in free space between chick fibroblasts, rather than over or under the latter. Underlapping by anterior extensions of MM96 cells beneath orthogonally oriented fibroblasts was not uncommon but locomotion of entire tumour cells was not actually seen in such cases. Essentially, progression of MM96 cells was by fibroblast-oriented diffusion through areas of free space successively vacated by chick cells. As long as space was available for occupation by contact-inhibited fibroblasts, little apparent check to MM96 cells occurred, as was confirmed by quantitative results from films. However, as seen in late sequences of some films in which chick cells were intensely crowded and compacted, outriding MM96 cells were halted by closely packed fibroblasts which were themselves temporarily unable to move. Presumably, with increasing time, blockages of this kind became sufficiently numerous and severe to affect the invasive index.

The heterologous collision behaviour of MM96 against CHF can be interpreted in more than one way from the data at present available. Its exact nature must remain in doubt until films taken at a higher microscope resolution and covering longer post-junctional periods are available. One interpretation is that collisions of leading edges of MM96 and CHF resulted in non-reciprocal contact inhibition (Heaysman, 1970) in the sense that MM96 cells did not exhibit paralysis of ruffling and apparent contraction as seen in contact inhibition between normal fibroblasts (Abercrombie & Ambrose, 1958). Nor did they undergo obvious retraction or change of direction. However, unlike the previously reported examples of neoplastic cells involved in non-reciprocal reactions (Heaysman, 1970; Vesely & Weiss, 1973), MM96 cells were not seen to move on to the upper surfaces of chick fibroblasts when free space either between or beneath the latter was unavailable. It is not, of course, certain that overlapping by MM96 did not occur during the post-filming, pre-fixation period, but the relatively low heterologous overlap index recorded from fixed cultures probably does not support this suggestion. Statistically adequate collision data are undoubtedly necessary to resolve the problem.
Invasive behaviour of malignant human cells confronted with avian and human fibroblast populations

Human melanoma cells were more efficient in their invasion of human skin fibroblasts than of chick heart fibroblasts. Against human fibroblasts, the net outward velocity and the frequency of outward movements of MM96 cells were not reduced from their unconfronted values and even showed trends towards increases. After junction of HSF, the mean outward speed of MM96, adjusted for removal of any dependence on numbers of contacts, was probably greater than the adjusted mean inward speed. The invasive index recorded from fixed cultures was not reduced from unity and the entire picture of MM96 invasion of human fibroblasts was one of total lack of obstruction. This was in contrast to the invasion of chick fibroblasts by MM96 (see above), during which the tumour cells encountered moderate blockage.

Part of the reason for the facilitated invasion of MM96 against HSF was probably the much lower density of the human fibroblast population compared with that of the chick cells. However, the main reason appeared to lie in the non-reciprocal heterologous contact inhibition between MM96 cells and human fibroblasts and the variety of possible avenues for locomotory progression of the tumour cells. In particular contrast to the behaviour of MM96 against CHF was the active movement of melanoma cells on to the upper surfaces of human fibroblasts. Examples of MM96 cells on top of fibroblasts were quite numerous but in only a few cases were the actual collision reactions preceding the overlap clearly seen. In these examples, overlapping by MM96 followed head-on heterologous collisions involving leading edges of both cells. Statistically adequate figures for the incidence of overlapping were not available but it obviously did not occur in all head-on collisions. In perhaps the majority of cases, MM96 cells, which exhibited no obvious contact paralysis (Wolpert & Gingell, 1968), merely moved forward on the substratum as the contact-inhibited fibroblast withdrew.

The relatively high MM96-HSF overlap index from fixed cultures and the fact that almost all the observed heterologous overlaps had a rounded MM96 cell on top of a fibroblast, suggested that active overlapping by MM96 continued to operate during the post-filming period.

Invasive behaviour of human and avian fibroblast populations confronted with malignant human melanoma cells

Human skin fibroblasts were relatively more efficient in their invasion of human melanoma cells than were chick fibroblasts. However, each fibroblast population encountered severe obstruction by MM96 and neither invaded the malignant population as effectively as each fibroblast population was itself invaded by the melanoma cells.

For both CHF and HSF, obstruction by MM96 was indicated during the filming period by significant reductions in frequencies of outward movements. The net outward velocity of the chick cells was also severely reduced but that of the human fibroblasts was unaffected. This difference may have been a function of the much greater densities of both cell populations in MM96-CHF confrontations. After standardization of the effects of contacts, post-junctional adjusted mean speeds of
Filmed human melanoma and fibroblast invasion

HSF travelling outward and inward were similar but the adjusted mean speed for chick cells moving outwards against MM96 was probably greater than for inward-travelling CHF. A possible explanation for this apparent anomaly is that the only chick cells which could continue moving outward were those which had access to free space between or beneath MM96 cells.

Cells of both HSF and CHF were contact-inhibited by collision of their leading edges with those of MM96 cells. In both cases, any reciprocal invasion of the melanoma population by fibroblasts appeared to take place by underlapping of bipolar melanocytes, dendritic processes, or by movement through narrow gaps between adjacent MM96 cells. Details of actual heterologous contact in these predominantly head-side encounters were often obscure. If no heterologous contact occurred, underlapping by fibroblasts could be regarded as diffusion-like movement into free space; if the fibroblast leading edge was involved, underlapping represented a failure of contact inhibition of locomotion of CHF or HSF.

Confrontation of normal human skin fibroblasts and chick heart fibroblasts

In view of the results of Abercrombie et al. (1957) and Abercrombie & Heaysman (1976) in relation to confrontations of normal mouse and chick fibroblasts, it could perhaps have been expected that chick heart fibroblasts and normal adult human skin fibroblasts would also exhibit negligible mutual invasion. The strongly invasive behaviour of chick heart fibroblasts and the negative invasion by HSF as actually observed in CHF-HSF confrontations therefore requires critical evaluation.

For human skin fibroblasts, analyses from films showed that the net outward velocity became negative, the frequency of outward movements was very severely reduced and the mean speed of outward-travelling human cells, after any dependence on numbers of contacts had been removed, became significantly less than that of cells moving back towards the HSF explant. All these values were supported by direct observations that, after contact with chick fibroblasts, most human fibroblasts retreated in the direction of the HSF explant. Still further evidence of this change of direction came from the overall decrease in HSF mean density during the filming period. Occasionally, outriding human fibroblasts near the original junction zone became surrounded by chick cells and were unable to move freely. The observed occurrence of cells in this position helped to explain the relatively low negative invasive index recorded from fixed cultures.

Against human fibroblasts, chick heart cells displayed an unaltered frequency of outward movements and a slightly but non-significantly reduced net outward velocity. The post-junctional adjusted mean speed of CHF moving towards the HSF population was increased compared with that of inward-moving chick cells. These results, together with a CHF invasion index which was non-significantly reduced from unity, gave no indication of obstruction by the human cells.

As far as could be judged from direct observation, which was often difficult because of the crowding outward of the chick fibroblasts, heterologous contact inhibition of locomotion was not deficient for either type of fibroblast. Adhesion, paralysis of ruffling, apparent contraction and, where possible, retraction and separation of the
heterologous cells appeared to occur essentially as seen during homologous contact inhibition of chick fibroblasts (Abercrombie & Ambrose, 1958.) The relatively low heterologous overlap index and the fact that overlapping or underlapping of either type of fibroblast by the other was very rarely seen, provided additional evidence for a high intensity of CHF-HSF heterologous contact inhibition. The retreat of a human fibroblast after a heterologous collision appeared to be initiated by a contact retraction (Weiss, 1958).

In most cultures, the leading chick cells were themselves so crowded by homologous neighbours that retraction and change of direction were not possible and the easiest route lay in following closely behind retreating human fibroblasts. The ability of the latter to change their direction of movement from outward to inward was undoubtedly assisted by the generally open, parallel alignment at the periphery of the HSF outwanderings, together with the associated system of spaces between the elongated adjacent cells. The question of how long the inward movement could continue cannot be determined until prolonged filming of HSF-CHF confrontations at low magnifications can be carried out.

Because of the sustained inward movement of contact-inhibited human cells, the post-junctional change from anisotropic to isotropic contact inhibition (Abercrombie, 1975) which presumably occurred after junction of the normal mouse and chick fibroblasts studied by Abercrombie & Heaysman (1976) and which effectively prevented invasion by either cell type, would not have taken place for chick fibroblasts in CHF-HSF confrontations.

A problem of great interest which is yet quite unsolved in any quantitative sense and which appears to be relevant to the facility of human fibroblasts for moving in either outward or inward directions, is the reported importance of orientation in relation to contact inhibition of locomotion of these cells. From a study of human foetal lung fibroblasts, Elsdale (1968) claimed that if the approach path of 2 fibroblasts was greater than 20°, contact inhibition of locomotion would occur, while if the angle of approach was below 20° contact inhibition would be absent and the pathways of the 2 cells would become parallel. This mechanism was invoked by Elsdale to explain the initiation and maintenance of the parallel arrays characteristic of human fibroblasts. Martz & Steinberg (1973), in discussing Elsdale's observations, classified the locomotory pattern of foetal human lung fibroblasts as contact inhibition of orthogonal movement. Harris (1974) referred to it as directional contact inhibition. The implication that head-on collisions of human fibroblasts approaching each other along the same pathway would not result in contact inhibition is particularly intriguing in view of the observed sensitivity of HSF to direct head-on collisions with chick fibroblasts.

The initiation of invasion of HSF by chick fibroblasts appeared to be largely dependent on how the peripheral human cells, i.e those reached first by the chick fibroblasts, were oriented in relation to the common radius between the opposing explants. This was the line along which invasion was most likely to begin and which was also the axis of the narrow strip along which invasion was measured in fixed cultures (Abercrombie et al. 1957; Abercrombie & Heaysman, 1976). If the peripheral human fibroblasts in this zone had a radial polarity such that the leading chick cell
approached them on more or less parallel pathways, not only could head-on, heterolo-
gous collisions involving leading edges occur, resulting in withdrawal of the human
cells, but also the relatively small, highly motile chick cells could eventually enter
spaces between the much larger, elongated and intensely aligned human fibroblasts.
If, on the other hand, the parallel human fibroblast array at the time of junction was
guarded by cells of the free edge which were orthogonally aligned with respect to the
interexplant radius, the radial approach path of the advancing chick cells was at least
temporarily blocked. Head-side collisions were followed by turning and progression
of the chick cells to right or left.

General comparisons with mouse: chick confrontations

The results from the present investigations appear to show that malignant and non-
malignant human cells, like malignant and non-malignant mouse cells (Abercrombie
& Heaysman, 1976), displayed differences in invasive capacity against a standard
population of normal embryonic chick fibroblasts. Results obtained by Abercrombie
& Heaysman (1976) from 3 different murine sarcomas showed that cells from different
types of tumours invaded the CHF population to different degrees and that their
relative invasive capacities appeared to bear some degree of relationship to their known
invasiveness in host mice. In the case of human tumours, comparative information of
this kind is not yet available.

The explanation presented by Abercrombie & Heaysman (1976) for the invasion of
the standard fibroblasts by different sarcomas and of sarcoma populations by fibro-
blasts was that a deficiency in heterologous contact inhibition of locomotion and
perhaps also in heterologous cohesion was present in each case. Our results suggest
that similar deficiencies were probably associated with heterologous contact relation-
ships between malignant human melanoma cells and each fibroblast population. How-
ever, in confrontations of normal human and chick fibroblasts, heterologous contact
inhibition did not appear to be defective for either cell type. Invasion of human fibro-
blasts by chick cells, which was perhaps the first reported example of invasion of one
normal fibroblast population by another of different origin, was essentially by diffusion-
lke movements into free space. It was also undoubtedly assisted by the actual abandon-
ment of territory by retreating human cells.

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