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Platelet factors induce chemotactic migration of murine mammary adenocarcinoma cells with different metastatic capabilities

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Summary. The chemotactic response of neoplastic cells (NC) induced by soluble platelet factors was investigated. NC suspensions isolated from murine mammary gland adenocarcinomas having different metastatic capabilities were incubated in Boyden's chambers and challenged with (1) 'Early Platelet Factors' (EP), obtained from the soluble fraction of recently collagen-activated human platelets, and (2) 'Late Platelet Factors' (LP), isolated after 24 hours incubation of the platelet aggregates. Chemotaxis was expressed as the distance travelled by NC through nitrocellulose filters. NC isolated from M3, the tumour line having the stronger metastatic potential, showed a significant chemotactic response towards LP factors, whereas NC from the M2 line exhibiting the lower metastatic behaviour, showed a chemotactic response towards EP factors. Both tumour cell lines lacked motion capability towards the well known chemoattractant peptide N-f-Met-Leu-Phe-Phe as well as to serum, plasma, collagen type I or culture medium. The different chemotactic response of both tumour lines when they were challenged by concentration gradients of factors released by early or late collagen-activated human platelets, confirm a relationship between platelet activity and metastatic capabilities and suggests that platelet chemoattractants might play a role in the metastatic dissemination of these mammary gland adenocarcinomas.

Keywords: chemotaxis, tumour cell, platelets, metastasis, cell migration, mammary gland adenocarcinoma

The metastatic cascade involves the segregation of neoplastic cells from the primary tumour, their entry into blood or lymphatic vessels and ulterior leakage from the circulatory stream, lodgement, survival, and cell growth at distant extravascular tissues (Weiss 1977; Nicholson 1978).

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One of the key steps in the metastatic process is the transmural transport of neoplastic cells and its regulation through factors at the molecular level. *In vivo*, it has been observed that tumour cells are capable of active migration across the vessel wall (Wood 1958), and it has also been suggested that chemotactic mechanisms may play a pivotal role in directing the migration of cancer cells from tumour emboli through the vascular walls (Orr *et al.* 1981). It appears that some tumour cell lines exhibit

a chemotactic response to factors derived from tumour tissue (Hayashi *et al.* 1970), the fifth component of serum complement (Romualdez *et al.* 1975), and conditioned medium from resorbing bone culture (Orr *et al.* 1980).

Some platelets proteins, such as platelet factor 4 (PF4), a cationic platelet secretory protein, are implicated in neutrophil chemotaxis (Mezzano *et al.* 1992). Moreover, platelet growth factors (Allam *et al.* 1992; Liapi *et al.* 1990), lipidic metabolites such as PAF-acether (Konig *et al.* 1983) and eicosanoids derivatives from the lipoxygenase pathway (Turner *et al.* 1975) have been identified as platelet chemotactic factors. Besides leucocytes, it has been shown that an uncharacterized platelet chemotactic factor may recruit macrophages and smooth muscle cells at the early stages of formation of atherosclerotic plaques (Ross & Harker 1976).

Recently, it has been reported that certain platelet metabolites released during platelet activation exhibit chemotactic activity toward some tumour lines. Thus, platelet glycoprotein thrombospondin induces tumour cell chemotaxis and haptotaxis mediated by different molecular domains and may participate in the directed movement of cells in metastasis (Taraboletti *et al.* 1987; Yabkowitz & Dixit 1991). Even though the multistep mechanism of metastasis development can not be attributed to a single cause, the hypothesis that chemotactic response contributes to the ability of tumour cells to start the vascular dispersion and/or the tissue homing deserves further investigation.

The purposes of this study were (1) to evaluate the motion capability of murine mammary gland adenocarcinoma cells with moderate and low metastatic ability, (2) to examine the chemotactic migration of these tumour cells induced by soluble factors released from activated platelets, and (3) to correlate these directional responses with the metastatic potential of tumour lines.

Materials and methods

Mammary gland adenocarcinoma cells

The M3 adenocarcinoma appeared spontaneously in the mammary gland of an inbred female BALB/c mouse strain. It requires a latency of 6–8 days, killing the animals around 30 days post-inoculum, and 30–50% of animals develop lung and liver metastases (Bal de Kier Joffe *et al.* 1983; Eynard *et al.* 1991). The M2 mammary gland adenocarcinoma appeared also spontaneously in an inbred BALB/c female and develops around 10% of lung metastases (Klein *et al.* 1981). Both tumours were characterized as type B by Dunn's classification (Squartini 1979).

Tumours were excised under aseptic conditions and blood clots, necrotic areas and connective tissue were carefully eliminated. Cell suspensions were obtained from small fragments of tissue washed in Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) at pH 7.3, containing 25 μg/ml of gentamicin. They were incubated for 30 minutes at 37°C in a solution of 0.01% pronase (Sigma Chemical Co., St Louis, MO, USA) and 0.24% deoxyribonuclease Type I (Sigma) in Dulbecco's Minimum Essential Medium (DMEM) (Sigma). After centrifugation, the cells were resuspended in DMEM with 10% fetal bovine serum. Cells were washed twice, resuspended in serum-free DMEM and viability was assessed by the trypan blue exclusion test. Only cell preparations with a viability higher than 70% were utilized. As previously reported by others (Bal de Kier Joffe *et al.* 1983), light microscopy examination of smears of these tumours stained with May Grunwald–Giemsa showed only about 1% of non-tumour cells as contaminants.

Platelet preparation

Venous blood was collected into 3.8% sodium citrate (9:1) from four volunteers of each sex (20–40 years old) who had not taken any drug affecting platelet metabolism for at least 7 days before the study. Pooled platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained as previously described (Eynard *et al.* 1990), and the suspensions were utilized within 90 minutes after isolation. Samples of PPP were filtered through 0.2-μm porosity nitrocellulose filter (Sartorius, RFA) to obtain platelet-free plasma (PFP). Samples of 100 ml of PRP were treated with 50 μg/ml of calf skin collagen type I (Sigma) at 37°C under slow stirring. The appropriate collagen concentration to induce maximal platelet aggregation was established as in previous aggregometric assays (Eynard *et al.* 1990). Collagen-stimulated PRP was centrifuged at 100 ***g*** at 4°C and the supernatant was collected. This fraction had soluble factors released during platelet aggregation or 'early platelet factors' (EP). Sedimented platelets were incubated in culture plastic dishes (Falcon) in DMEM without serum for 24 hours in a humidified atmosphere with 5% CO_2 and centrifugated afterwards at 100 ***g***. The supernatant thus obtained had 'late platelet factors' (LP) released by the aggregates during 24 hours following stimulation.

Aliquots of EP and LP factors were kept at −20°C until use. It has been reported that the chemotaxis generating capacity of human platelet extracts is stable for several weeks when stored-cold (Wecksler & Coupal 1973).

Chemotactic evaluation

Boyden chambers fitted with nitrocellulose filters of 8-μm

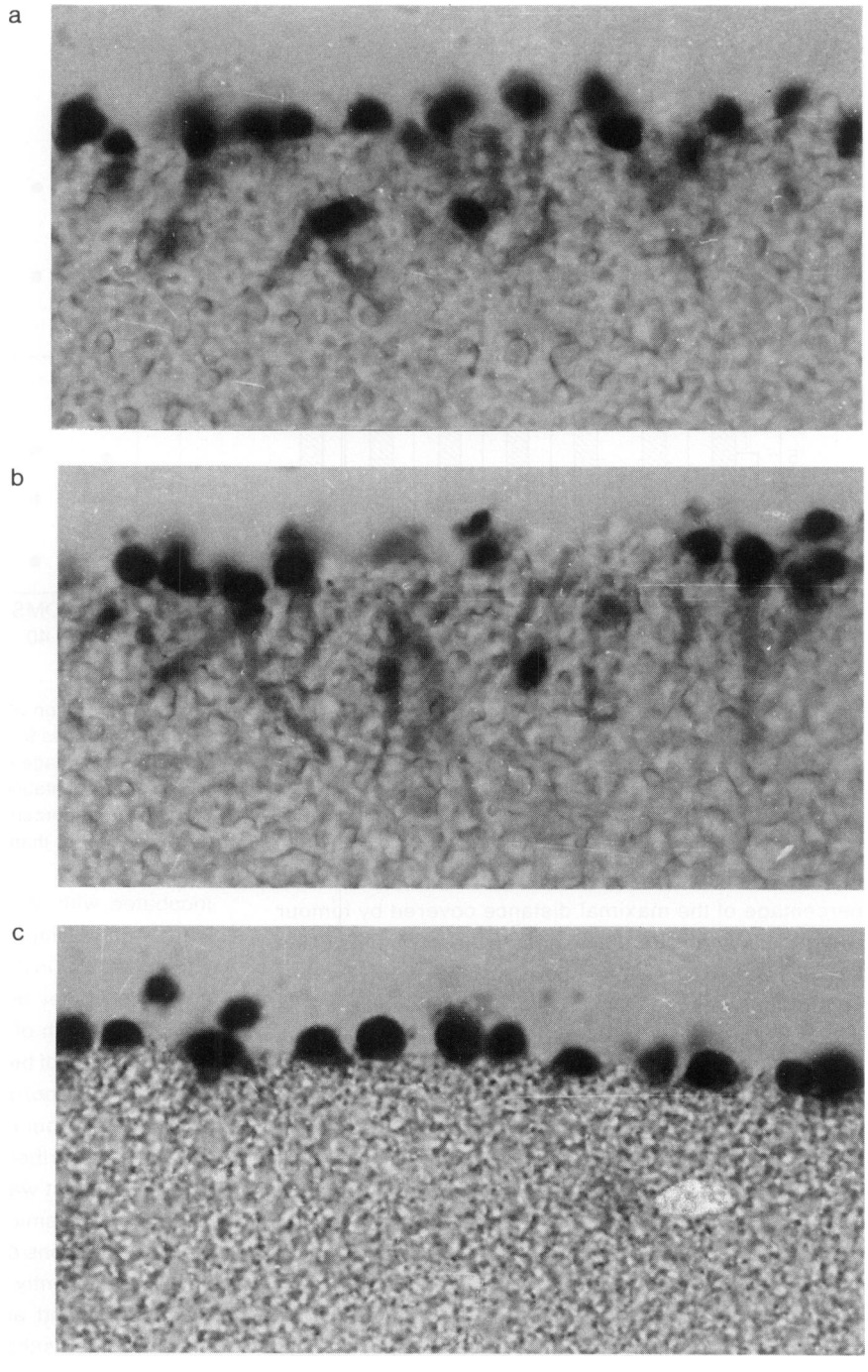

Figure 1. Transverse sections of filters of 8-μm porosity. a, M3 tumour cells confronted with 'late platelet factors'; b, M2 tumour cells confronted with 'early platelet factors' c, M2 cells confronted with DMEM plus serum. H & E. ×670.

porosity (Sartorius, RFA) were used. The lower compartment of the chamber was filled with 200 μl of EP or LP factors while PFP, DMEM, DMEM plus 0.1% serum, DMEM plus 2 mg/ml collagen or DMEM plus 10^{-9} M N-f-Met-Leu-Phen-Phen (Sigma), previously incubated in the absence of platelets, were used as controls. The upper compartment of each chamber was filled with a suspension of neoplastic cells from M3 or M2 lines (2×10^6 cells/ml). After incubation for 24 hours at 37°C, the filters were fixed *in situ* with 4% formaldehyde in PBS, stained with haematoxylin and eosin, dehydrated in ethanol and embedded in paraffin. Cell or cytoplasmic translocation was determined on 24 fields taken at random from three semi-serial transversal sections of filters in each experi-

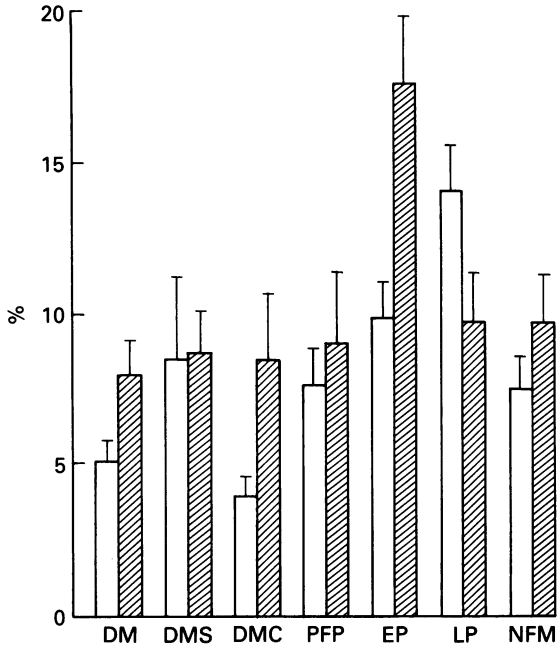

Figure 2. Migration of cells from □, M3 and ■, M2 tumour cell lines expressed as percentage of the thickness of the filter. DM, DMEM; DMS, DMEM plus serum; DMC, DMEM plus collagen; PFP, platelet-free plasma; EP, early platelet factors; LP, late platelet factors; NFM, DMEM plus N-F-Met-Leu-Phen-Phen synthetic peptide. Vertical bars represent the SEM.

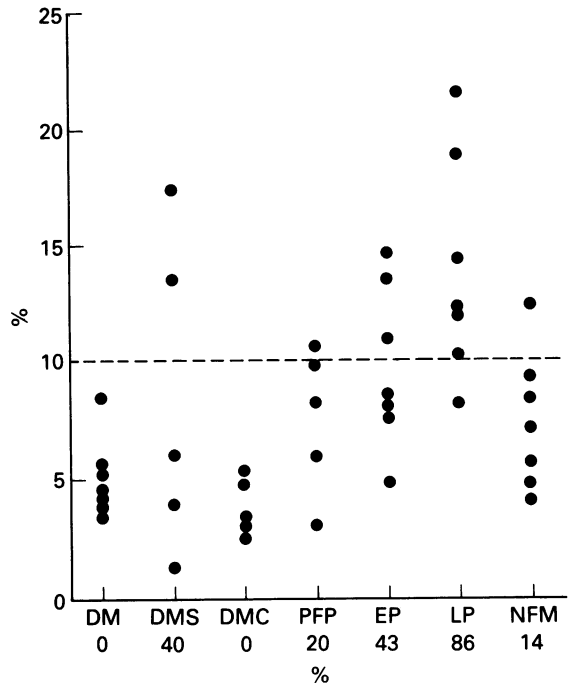

Figure 3. Migration of cells from M3 tumour cell line. Each point corresponds to one assay. Broken line represents the selected percentage over which displacement took place towards the substance tested. Numbers at the bottom correspond to percentage of experiments with values of cell migration higher than 10%. See Figure 2 for details.

mental condition. Chemotaxis was expressed as the percentage of the maximal distance covered by tumour cell translocations from the upper to the lower side of the filter in relation to the total thickness of the filter. Ten per cent of the whole filter thickness, corresponding to the average diameter of the tumour cells, was considered as a threshold criterion to establish cell migration. Each assay of tumour cell chemotaxis was performed at least ten times. The variability of samples is expressed as mean ± standard error. The significance of differences between data was determined by ANOVA test (Steel & Torrie 1987). A probability of $P < 0.01$ was selected as the criterion of significance for M3 and M2 cells. The Tukey test, indicated for simultaneous means comparison of several sets of data, was also employed (Steel & Torrie 1987), and the significance value for both cell lines was selected at the level of alpha = 0.05.

Results

Movement of whole tumour cells or their cytoplasmic translocations through the filter toward the lower compartment of the Boyden's chamber showed evidence of active cell motility on both tumour lines assayed. As may be seen in Figure 1, microscopic observations of filters incubated with M3 and M2 cell lines, upon stimulation with LP and EP factors, showed small tumour cells wholly embedded within the filter's network, whereas the majority of the bigger ones exhibited cytoplasmic prolongations of a length of 40–50 μm directed toward the lower attractant side of the filter. In some experiments in which filters of 12-μm porosity were used, small as well as large M3 and M2 tumour cells penetrated into the filter as far as the above described cytoplasmic prolongations (data not shown). Thus, it was evident that penetration of tumour cells or cytoplasmic ('lamellipodial') translocations were clear expressions of cell motility. This active cell migration was differently expressed by the M3 and M2 lines of mammary gland adenocarcinoma cells depending on the confronted substances.

M3 tumour cells showed a positive chemotactic response when they were challenged with LP factors (Figure 2, Table 1). In different experiments it was shown that cells from 6 out of 7 (86%) M3 tumours exceeded the criteria established for cell migration towards LP factors (Figure 3). Although M3 tumour cells also showed a chemotactic tendency towards EP factors (Figure 2), the rate of oriented migration was below the limit of significance (Table 1).

Table 1. Statistical comparison of the chemotactic behaviour of M3 and M2 tumour cell lines.

	DM	DMS	DMC	PFP	LP	EP
DMS						
M3	3.34					
M2	0.69					
DMC						
M3	1.21	4.55				
M2	0.48	0.20				
PFP						
M3	2.45	0.89	3.66			
M2	1.07	0.39	0.59			
LP						
M3	8.82*	5.49	10.04*	6.38*		
M2	1.79	1.11	1.31	0.72		
EP						
M3	4.64	1.30	5.85	2.19	4.19	
M2	9.57*	8.89**	9.09*	8.49*	7.77*	
NFM						
M3	2.34	0.99	3.56	0.11	6.48*	2.29
M2	1.74	1.06	1.26	0.66	0.06	7.83*

Numbers represent comparison (Tukey's test) of chemotaxis of tumour cells confronted to different factors. Minimal significant differences for M3 = 6.21 and for M2 = 7.63.

* Significantly different.

DM, DMEM; DMS, DMEM plus serum; DMC, DMEM plus collagen; PFP, platelet-free plasma; EP, early platelet factors; LP, late platelet factors: NFM, DMEM plus N-F-Met-Leu-Phen-Phen synthetic peptide.

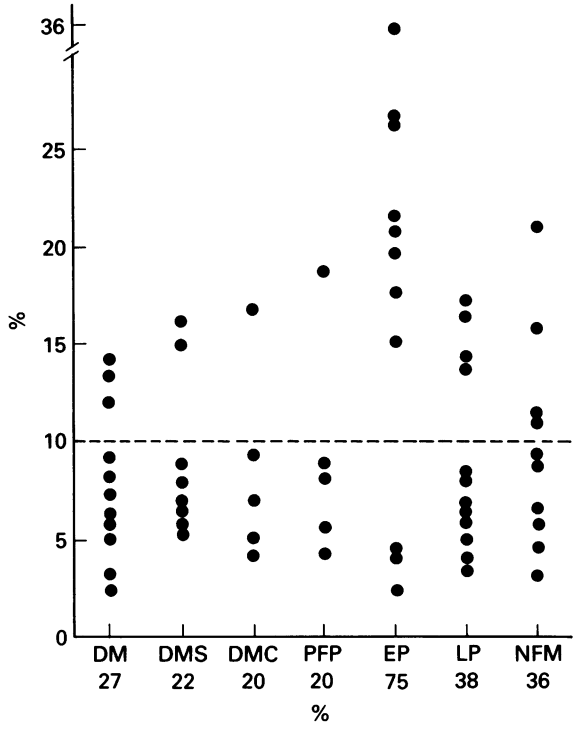

Figure 4. Migration of cells from M2 tumour cell line. Each point corresponds to one assay. Broken line represents the selected percentage over which displacement took place towards the substance tested. Numbers at the bottom correspond to percent of experiments with values of cell migration higher than 10%. See Figure 2 for details.

On the other hand, as may be observed in Figure 2 and Table 1, the chemotactic response of cells isolated from M2 tumours was significantly increased towards EP factors in comparison to the controls ($P < 0.01$). In fact, in separate assays, 9 out of 12 (75%) preparations of M2 tumour cells migrating towards EP factors exceeded the threshold considered as an indicator of cell migration, whereas only 20% of controls reached that value (Figure 4). In contrast, LP factors failed to modify significantly the oriented movement of M2 cells.

In both M3 and M2 tumour cell lines, the N-f-methionyl peptide failed to induce a significant chemotactic response (Figures 2–4; Table 1).

Discussion

Evidence indicating that tumour cell movement contributes importantly to invasion and hence to metastasis formation is scarce (Crissman *et al.* 1988), as is that concerning the formation of platelet thrombi in this process (Wood 1958; Orr *et al.* 1981).

Most studies of platelet biology involve events occurring during the first minutes following their activation. However, in-vivo observations on experimental intravascular clots indicate that platelets remain closely attached to tumour cells for 24 hours (Hayashi *et al.* 1970). Furthermore, a time sequence of the steps following a tail vein injection of single cell suspensions of Lewis lung carcinoma and of a mammary adenocarcinoma showed that the thrombus formed by platelets and arrested tumour cells remains closely attached for at least 24 hours (Crissman *et al.* 1988). On the other hand, a single subcutaneous injection of platelet pellet induced a strong chemotactic response of polymorphonuclear leucocytes after a delay of 8 days (Braunstein *et al.* 1980).

The results from the present study show that cells from M3 and M2 tumour lines exhibiting a different metastatic potential revealed an active migration ability. This motion behaviour was evident upon their challenge with soluble factors released in the early or late stages of the platelet metabolic activity that follows their activation.

M3 tumour cells, having a higher metastatic potential, exhibited a stronger chemotactic response toward LP soluble factors released from masses of collagen-activated platelets during incubation for 24 hours. In this context, it is worth mentioning that M3 tumour cells showed a clear ability to form stable platelet aggregates in both mice and human platelet-rich plasma *in vitro*

(Eynard *et al.* 1989; Pasqualini & Eynard 1993). According to the available literature, the observed tumour cell chemotaxis could mainly result from the following mechanisms: (1) the activity of a putative platelet protein fraction which in turn induces the formation of a serum factor exhibiting chemoattractant activity (Wecksler & Coupal 1973); (2) the activation of the fifth component of the complement system (Orr *et al.* 1978), and/or (3) the action of chemotactic molecules derived from arachidonate via the lipoxygenase pathway (Goetzl & Pickett 1980). From our results, pathways 1 and 2 may be discarded, insofar as our experiments were performed with serum-free media. Moreover, when in additional controls M3 tumour cells were alternatively challenged in the Boyden chamber with DMEM plus serum as well as with collagen or platelet-free plasma in the lower compartment, no significant effects on tumour cell motility were observed. Hence, any chemotactic activity of a serum contaminant or the collagen used as platelet stimulant can be discarded. The third mechanism also seems unlikely since, although leucocytes generate substantial amounts of 5,12-hydroxyeicosatetraenoic acid (5-12-HETE) through lipoxygenase activity, having strong leucotactic activity (Goetzl & Pickett 1980), this di-HETE is not synthesized by platelets (Lagarde 1988). On the contrary, in that study it was shown that 12-HETE, the most abundant lipoxygenase metabolite produced by platelets, had only a weak chemotactic activity for leucocytes. Nevertheless, the chemotactic activity of 12-HETE with respect to the neoplastic cells used in our study remains to be established. The present findings suggest that the chemotactic activity elicited by platelet aggregates may be due to soluble components released by the platelets themselves during their 24 hours incubation.

M2 cells, the low metastatic line, respond chemotactically to those soluble factors released earlier during platelet aggregation. In-vitro studies carried out at our laboratory indicate that M2 tumour cells are unable to induce either isologous or heterologous stable platelet aggregation (Pasqualini & Eynard 1993). Thus, chemotactic response to EP factor and the lack of tumour cell-induced platelet aggregation appear not to be related phenomena in M2 tumour cells as well as in other types of metastatic cells (Spearman *et al.* 1992). The mechanism by which M2 tumour cells respond to chemoattractants present in EP factors could be much more complex. EP-fraction contains metabolites liberated by collagen-activated platelets suspended in isologous plasma during the first minutes after the irreversible aggregation. During the release reaction, platelets liberate thrombospondin, a glycoprotein present in their alpha granules, which induces tumour cell chemotaxis and may have a role in malignant breast tissue invasiveness (Taraboleti *et al.* 1987; Wong *et al.* 1992). Other platelet proteins having enzymatic activity lack chemoattractant properties but they may induce the formation in the plasma of a poorly characterized protein fraction with a strong leucotactic potential (Wecksler & Coupal 1973). These proteins are active at pH 7.4, thus discounting platelet lysosomal cathepsins which are active at pH 3.4–4.0. Furthermore, aggregating platelets could activate C5 complement fraction, a known chemoattractant for many neoplastic cells (Orr *et al.* 1978).

The peptide N-f-Met-Leu-Phen-Phen, a strong chemoattractant for leucocytes (Showell *et al.* 1976) and some tumour cells (Wass *et al.* 1981), was unable to induce significant translocation of M3 and M2 cells, suggesting that the receptor-mediated mechanism of chemotactic response for these tumour lines is elicited by an alternative pathway.

The microscopic finding that the cytoplasmic lamellopodia of the tumour cells of M3 and M2 lines penetrate into the network filter is consistent with the results of other workers who showed that transforming growth factor-beta 1 stimulates the invasion of pulmonary adenocarcinoma cells accompanied by dramatic morphological changes that included the appearance of numerous long pseudopodia and changes in the motile behaviour of the cells (Mooradian *et al.* 1992). On the other hand, the fact that the bigger tumour cells are preferentially arrested on the top of the filter suggests a certain stiffness of the nuclear or perinuclear cytoskeleton components, insofar as the flexibility of normal cells traversing narrow labyrinths is a well known phenomenon (Newgreen 1989).

In conclusion, we have shown that M3 and M2 tumour lines of murine adenocarcinoma of the mammary gland express an active migratory behaviour. This property is differently expressed by tumour cells with different metastatic capabilities, depending upon the challenging factors that are apparently released by platelets at diverse metabolic stages. The molecular nature of these putative 'early' and 'late' factors is unknown and will be the subject of future investigations.

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