Adhesion molecule expression and response to chemotactic agents of human monocyte-derived macrophages

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SUMMARY

Human monocyte-derived macrophages have been proposed as agents of anti-tumour immunotherapy. The aim of the present study was to investigate *in vitro* the properties of these cells likely to control their recruitment to the sites of inflammation and tumours. The expression of adhesion molecules involved in the binding of monocytes to endothelial cells was modified during monocyte-macrophage differentiation, with a significant increase in CD11c, CD14 and intercellular adhesion molecule-1 (ICAM-1). Monocyte-derived macrophages were sensitive to chemoattractants, in particular to the monocyte-specific chemokine monocyte chemotactic protein-1 (MCP-1). They responded by an increased expression of adhesion molecules and were attracted by the cytokine in an under-agarose migration assay. The migration response, however, decreased after days 4–5 of monocyte differentiation into macrophage. In conclusion, human monocyte-derived macrophages show alterations of surface structures involved in the recognition of inflammatory endothelium. This may explain why the cells are poorly recruited to the sites of inflammation and tumours when introduced into the circulation.

Keywords   macrophages   adhesion molecules   MCP-1   chemotaxis   human

INTRODUCTION

Monocyte-derived macrophages have been recently proposed as agents of immunotherapy in cancer patients [1–3] and administered either in contact with tumours, e.g. in serous cavities, or intravenously. The presence of macrophages in the vascular compartment does not represent a physiological situation, and it may be questioned whether these cells can be recruited to the sites of inflammation and penetrate tumours. The aim of the present work was to investigate, by *in vitro* assays, some of the properties of monocyte-derived macrophages supposedly implicated in their recruitment to the site of inflammation and tumours.

The mechanism of recruitment of blood monocytes to inflammatory foci comprises a two-step process. The cells first adhere to the vascular endothelium before migrating to sites of inflammation, in response to specific chemoattractants. Binding and subsequent stretching of monocytes over the endothelium surface can be inhibited by MoAbs directed against adhesion molecules, either expressed on monocytes or on endothelial cells. Monocyte molecules implicated in the adhesion reaction include CD62L (L-selectin), CD14, β-2 integrins (CD11a, CD11b and CD11c associated as heterodimers with CD18), and the β-1 integrin CD49d (VLA-4) [4]. CD54 (intercellular adhesion molecule-1 (ICAM-1)) is expressed both on monocytes and endothelium, but anti-CD54 MoAb inhibits monocyte binding, presumably by acting on the ICAM-1 molecules of endothelial cells.

Natural chemoattractants specific for monocytes have recently been identified. Monocyte chemotactic proteins (MCP) belong to the chemotactic cytokine family designated chemokines, which selectively affect monocytes and not neutrophils. MCP-1, also called monocyte chemotactic and activating factor (MCAF) [5], MCP-2 and MCP-3 [6], constitutes a group of three low molecular weight proteins with marked peptide sequence similarities. MCP are secreted by normal and tumour cells, but so far only MCP-1 has been studied in detail (reviewed in [7]). Several lines of evidence suggest that MCP-1 plays a major role in the recruitment of tumour-associated macrophages from circulating monocytes (reviewed in [8]).

Adhesion molecule expression and *in vitro* migration were investigated in populations of human macrophages obtained from

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7-day cultures of circulating monocytes under conditions of weak adhesion to culture substrate.

MATERIALS AND METHODS

Cells
Mononuclear cells were isolated from buffy coats of healthy donors by centrifugation on Ficoll gradients (MSL, Eurobio, Paris, France). The cells were washed and processed immediately or cultured in gas-permeable and hydophobic plastic bags (Life-cell; J-BIO, Les Ulis, France). The culture methods were similar to those used for the preparation of autologous macrophages in protocols of cancer immunotherapy [1–3]. Briefly, mononuclear cells were suspended at 5×10^6/ml in Iscove's modified Dulbec-co's medium (IMDM; Biochrom, Berlin, Germany), supplemented with 2% autologous serum and in some experiments with 500 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; Schering-Plough, Levallois-Perret, France). Freshly isolated or 1–7-day cultured mononuclear cells were submitted to an elutria-tion procedure, using a J-6 MC centrifuge (Beckman, Gagny, France) fitted with a Sanderson's chamber. The elutriation pro-ducts were analysed repeatedly during the separation process with a Coulter Counter ZM analyser. The purity of the monocyte-macrophage fractions reached 95%. Cell viability measured by trypan blue exclusion was above 95%. More than 95% pure granulocytes were prepared routinely by depositing 10 ml hepar-inized blood over 10 ml Dextran-Radioselectan mixture. The latter contained 100 v of 9% dextran (T500; Pharmacia, Orsay, France) in saline and 40 v of 38% Radioselectan (Schering) in distilled water. After incubation for 1 h at 37°C and centrifugation at 900 g for 25 min at room temperature, the supernatant was collected and centrifuged. Contaminating erythrocytes were eliminated by brief exposure to a 2% NaCl solution. The cell pellet was resuspended in PBS and adjusted to the desired concentrations.

Adhesion molecule expression
Monocyte and macrophage populations suspended in PBS supple-mented with 0·5% bovine serum albumin (BSA) and 0·1% sodium azide were incubated under the conditions indicated by the manufacturer with one of the following mouse MoAbs: anti-CD11a (clone 25.3), anti-CD11b (clone Bear 1), anti-CD11c (clone BU15), anti-CD18 (clone 7E4), anti-CD49d (VLA-4, clone HP2.1), anti-CD14 (clone RMO 52), anti-CD62L (LAM-1, clone DREG 56) (Immunotech, Marseille, France) and anti-CD54 (ICAM-1, clone 15-2, kindly given by Nancy Hogg, London, UK). After washing, the cells were exposed for 45 min to fluorescein-labelled anti-mouse Fab$'_2$ fragment (Silenus, Eurobio) and washed again. In each experiment, a series of quantification beads coated with known amounts of mouse IgG antibody (Qifikit, Marseille, France) was included and incubated with fluorescein-labelled anti-mouse Fab$'_2$, like the cell samples. It was verified that the various MoAbs were used at saturation, an important prerequisite for the Qifikit test. Cells and beads were analysed in a flow cytometer (FACScan; Becton Dickinson, Pont-de-Claix, France). A calibra-tion curve was drawn by plotting bead fluorescence against number of molecules of mouse immunoglobulin per bead. This allowed expression of mean fluorescence intensity as mean number of antigen sites per cell [9]. In some experiments, before incubation with anti-adhesion molecule antibodies, the cells were suspended in IMDM supplemented with 10% fetal calf serum (FCS) and exposed for 30 min to 10 nM N-formyl-met-leu-phe (fMLP; Sigma,

St-Quentin-Fallavier, France) or to various concentrations of pure human MCP-1 produced and purified as described [10].

Chemotaxis
Chemotactic migration was studied with purified cells, using the 'under agarose' migration technique [11]. Briefly, cell migration was measured at various time intervals after exposure to 10 nM fMLP, human serum or MCP-1 used at 1–100 ng/ml. Purified cell populations were placed in the central wells of the agarose plates while the attractants were deposited in the peripheral wells. PBS was added to a control peripheral well to measure spontaneous cell migration. The maximal distances covered by the cell migration front towards PBS (spontaneous migration: SM) and attractants (orientated migration: OM) were measured. All determinations were done in triplicate. The results were expressed as OM distance and chemotactic index (OM/SM).

RESULTS

Expression of adhesion molecules
The expression of adhesion molecules known to play a role in monocyte adhesion to endothelium was analysed comparatively on freshly isolated monocytes and on monocytes cultured for 7 days. The results are reported in Fig. 1, and show that the expression of certain molecules increased significantly—as judged by Student's *t*-test—during monocyte differentiation to macrophage, namely CD11c ($P \leqslant 0·01$), CD54 ($P \leqslant 0·001$) and CD14 ($P \leqslant 0·05$). The other antigens investigated, CD11a, CD11b, CD18, CD49d (VLA-4) and CD62L (LAM-1), exhibited a roughly equivalent expression on both cell types. Marked individual variations were noted in the expression of the molecules.

Incubation with fMLP or MCP-1 has been reported to up-regulate the expression of adhesion molecules by freshly sampled monocytes [12]. We compared this property in fresh and 7-day cultured monocytes. Thirty-minute exposure to the activators resulted in enhanced antigen expression, as exemplified in the experiment of Fig. 2. MCP-1 and fMLP increased the number of adhesion structures on the cell surface, both on monocytes and macrophages. Synthetic fMLP was a slightly better stimulant than natural MCP–1. The increase in the number of molecules reached 75% for CD11c and was often 25–50% for CD11a, CD11b, CD18, CD14 and CD54.

Under-agarose migration of monocytes and macrophages
The migratory properties of monocytes cultured for various times were investigated. Neutrophils from healthy donors were included in all experiments as standard controls. The responses to two chemoattractants, human serum as complement source and fMLP, were compared (Fig. 3). The maximal migration distance of monocytes was shorter than that of neutrophils ($P \leqslant 0·001$, Student's *t*-test). For example, after 1·5-h migration to fMLP, neutrophils had moved $2·14 \pm 0·10$ mm ($n = 9$), while monocytes moved only $1·17 \pm 0·14$ mm ($n = 7$). A migration period of 3 h *versus* 1·5 h improved the migration distance to fMLP for mono-cytes, but not for neutrophils. Monocyte migration distance decreased during differentiation to macrophages, particularly after day 4. The migration distance of monocytes cultured for 7 days became unmeasurable. In contrast, the chemotactic index of freshly isolated monocytes was higher than that of neutrophils: $2·59 \pm 0·31$ and $2·10 \pm 0·16$, respectively, after 1·5-h migration ($P \leqslant 0·1$), and $3·26 \pm 0·48$ *versus* $1·85 \pm 0·1$ after 3-h
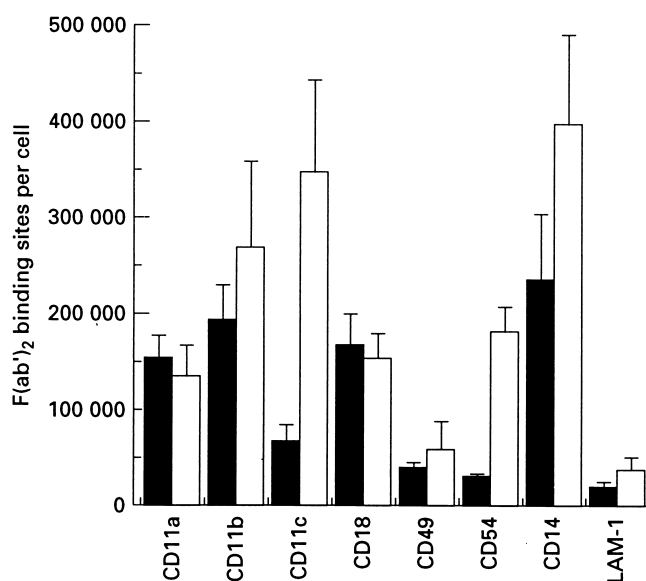
Fig. 1. Differential expression of adhesion molecules on monocytes separated from freshly sampled or from 7-day cultured mononuclear cells. Mononuclear cells were cultured in hydrophobic plastic bags and monocytes were separated by elutriation. The number of antigen sites per cell was determined using a kit of calibration beads. The results represent the means of five experiments and are expressed as numbers of antigen sites ± s.e.m. ■, Monocytes; □, macrophages.

migration ($P \leqslant 0{\cdot}01$). The migration index remained elevated during the culture of monocytes, as long as migration distance could be measured.

As MCP-1 can be produced in normal and tumour tissues and can activate macrophages [7,8], the effect of pure natural MCP-1 on macrophage migration was evaluated. The cytokine was active on fresh monocytes and had little effect on neutrophils (not shown). In the experiment presented in Table 1, use was made of monocytes cultured for 4 days, a period enabling migration distances to be quantified. At 1 ng/ml, MCP-1 produced a maximal chemotactic effect. However, the migration distance and chemotactic index were higher when macrophages were stimulated with fMLP or serum. Increasing the migration time to 18 h did not improve the results.

DISCUSSION

The culture of blood monocytes in plastic bags or on Teflon foils has been shown to preserve cell viability for several weeks and to enable monocyte differentiation into macrophage-like cells. After 7 days of culture, the cells show a 10-fold surface enlargement and display numerous membrane protrusions [13]. They exhibit phagocytosis and are cytotoxic for U937 tumour cells, in contrast to fresh monocytes [14,15]. They also express new antigen markers, such as transferrin receptors, Fc receptor-III (CD16), vitronectin receptor (CD51), and antigens recognized by MoAbs MAX.1, MAX.3 and 25F9 [16–18]. Properties like cytotoxic activity and the expression of maturation antigens increase during culture and reach a plateau after 5–10 days, suggesting that this interval corresponds to the time required for monocytes to complete their maturation into macrophages. For this reason, 7-day cultured monocytes were considered here as mature macrophages.

When such macrophages are radiolabelled with [111]indium and injected intravenously into patients, they sojourn for a short time in the lungs and accumulate in the liver and spleen from hours 24 to 72 post-injection, but do not appear to be recruited in tumours. Measurements performed more than 72 h after injection would not be reliable, due to the short half-life of the isotope and its insufficient binding to the cells ([2], and personal unpublished observations). The first step of monocyte recruitment in inflammation relies on the adhesion of these cells to inflamed endothelium. The question therefore arises whether mature macrophages retain the monocyte adhesion molecule equipment required for adhesion to endothelium.

Participation of molecules of the β-2 integrin family in the attachment of monocytes to endothelium has long been known. CD11a, CD11b and CD18 play a particularly important role, whereas contradictory results were reported with CD11c [4]. In the present study, the expression of CD11c on mature macrophages was greatly increased compared with fresh monocytes, a result consistent with *in vivo* observations showing that CD11c was highly expressed on tissue macrophages [19]. The α (CD11a, CD11b and CD11c) and β (CD18) chains of β-2 integrins are known to constitute $\alpha1$-$\beta1$-heterodimers, and the number of CD18 surface molecules per cell was expected therefore to be equivalent to the sum of all α chains. Surprisingly, the number of CD18 molecules was relatively low, suggesting that some α chains were not paired with β chains. This was more pronounced with macrophages than with uncultured monocytes.
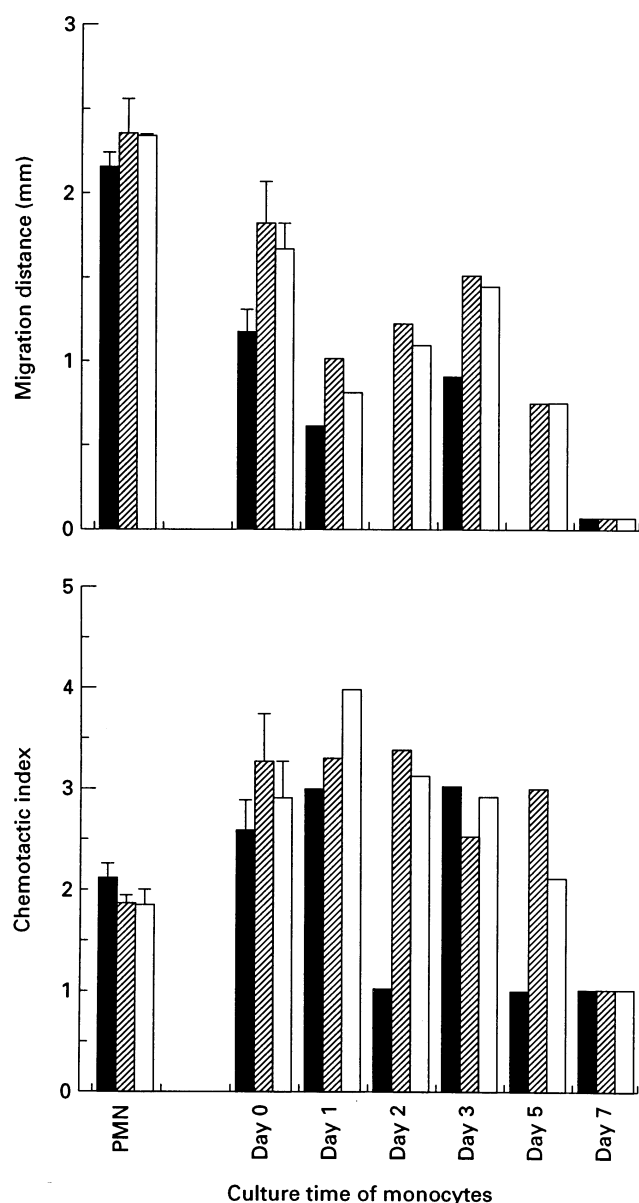
CD14 has recently been classified with a new category of leucine-rich adhesion molecules and found to be implicated at an early stage of monocyte attachment to endothelium [20]. CD14 was greatly enhanced during differentiation of monocytes to macrophages. In contrast, macrophages cultured on glass or collagen substrate expressed less CD14 antigen [21], suggesting that antigen expression depends greatly on culture conditions. No marked variations in CD62L or CD49d were noted during monocyte maturation to macrophage. CD54 (ICAM-1) is a common adhesion molecule and was therefore included in this investigation, although its expression on monocytes does not seem to interfere with monocyte binding to endothelium. The expression of CD54 was greatly enhanced during monocyte differentiation, confirming results reported by others [22].

Macrophage migration in *in vitro* assays was first assessed by means of a conventional under-agarose migration assay. The chemotactic index of monocytes remained as elevated as that of monocytes at the beginning of the culture, but dropped suddenly on day 5 or 7, when macrophage displacement could no longer be estimated. Seven-day cultured monocytes exhibited, however, some sensitivity to chemoattractants since they responded by increased expression of adhesion molecules. This suggested that the effect of chemoattractants on migration could not be evidenced because macrophages adhered too strongly to the culture vessel. The observation that monocyte adhesion to plastic is mediated by CD11c favours this hypothesis. Anti-CD11c MoAb modifies monocyte binding to, and migration on, plastic surfaces, while anti-CD11a and anti-CD11b MoAbs are inefficient [23]. Consequently, the diminution of migration distance during monocyte-macrophage maturation could be related to the strongly enhanced expression of CD11c that occurred during the process. This hypothesis was supported by our previous observation [24] that 7-day cultured monocytes migrated across tumour cell aggregates.
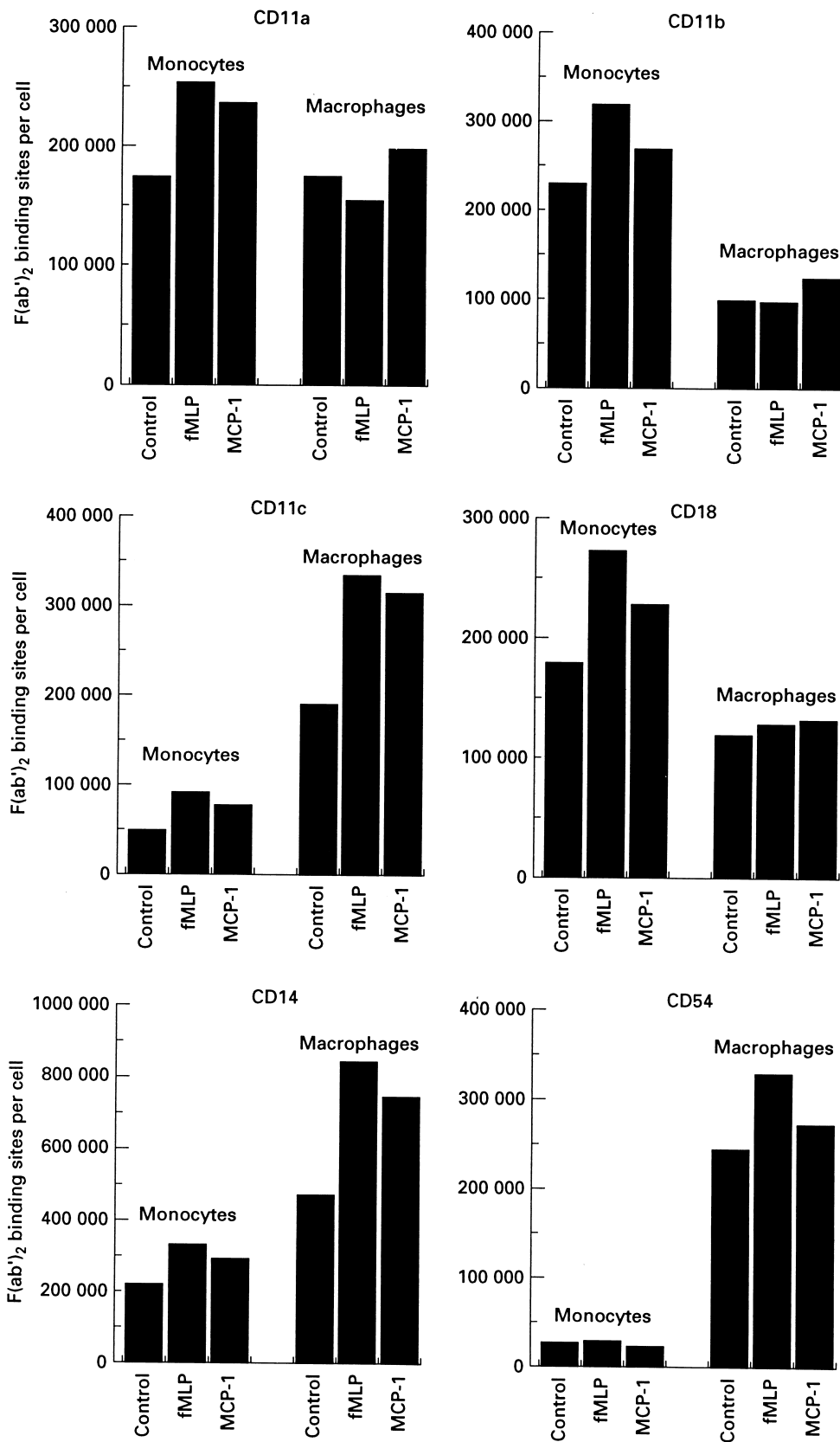
Fig. 2. Enhancement of adhesion molecule expression by N-formyl-met-leu-phe (fMLP) and monocyte chemotactic protein-1 (MCP-1) on uncultured monocytes (monocytes) and on 7-day cultured monocytes (macrophages). The two cell types were sampled from the same donor, and were activated by incubation at 37°C for 30 min in the presence of 10 nM fMLP or 10 ng/ml MCP-1. The results are expressed as numbers of antigen sites. One representative experiment out of three is illustrated.

Monocyte-derived macrophages responded to MCP-1, both by an orientated migration and by an increased expression of adhesion molecules. Thus, effects which had been reported initially with freshly separated monocytes, were found here to apply also to more mature forms obtained by culture. MCP-1 is produced by a

Table 1. Chemotactic activity of monocyte chemotactic protein-1 (MCP-1), N-formyl-met-leu-phe (fMLP) and serum on human monocytes purified by elutriation from 4-day cultured blood mononuclear cells

Migration time (h)	Chemoattractants				
	MCP-1 (10 ng/ml)	MCP-1 (1 ng/ml)	MCP-1 (0·1 ng/ml)	FMLP (10 nM)	Human serum
1·5	0·15 (1·43)	0·15 (1·43)	0·10 (1·29)	0·55 (2·37)	0·15 (1·43)
3·0	0·15 (1·27)	0·30 (1·67)	0·10 (1·20)	0·55 (1·92)	0·60 (2·00)
18·0	0·20 (1·36)	0·25 (1·45)	0·10 (1·18)	0·55 (1·58)	0·75 (1·83)

The cells were deposited in agarose wells and allowed to migrate randomly or against three chemoattractants: MCP-1 at various concentrations, fMLP, and human serum. The results represent the mean of three determinations and are expressed in maximal migration distance (mm) and in chemotactic index (in parentheses).

variety of cells, including tumour cells, and seems to play an important physiological role in the recruitment of monocytes by tumours [8].

Some of the problems raised by the utilization of monocyte-derived macrophages in anti-tumour immunotherapy are also encountered in other forms of cellular immunotherapy. Expanded populations of lymphokine-activated killers (LAK) or of tumour-infiltrating leucocytes (TIL) represent, like macrophages, anti-tumour effector cells that have circulating precursors, i.e. natural killer (NK) cells, lymphocytes and monocytes, respectively. These precursors can be physiologically recruited in tumours, but the activated effectors do not seem to have this property. Of [111]indium-labelled LAK introduced into the circulation, 99% are taken up outside the tumours [25], and CTL access to tissue antigen is also known to be restricted *in vivo* [26]. If the margination of the cells in liver or spleen sinusoids depends on the expression of identified adhesion molecules, trials to condition the cells by modulating the expression of these molecules before injection might be proposed. At present, the only way to allow cell–cell contact between tumour and effector cells is by intralesional administration. This technique, however, requires that the injected cells can diffuse throughout tumour nodules, a property displayed by human blood monocyte-derived macrophages, as judged by their *in vitro* penetration of tumour cell aggregates [22].

Fig. 3. Under-agarose migration of monocytes cultured for various periods of time. Blood mononuclear cells were cultured in the presence or absence of granulocyte-macrophage colony-stimulating factor (GM-CSF). Monocytes were separated after the time of culture indicated, deposited in agarose wells and allowed to migrate randomly or against two chemoattractants, 10 nM N-formyl-met-leu-phe (fMLP) and human serum. Polymorphonuclear leucocytes (PMN) from healthy donors were included as controls. Migration times were 1·5 and 3 h. Maximal distance (mm) of migration and chemotactic index were determined for PMN, uncultured monocytes and monocytes cultured for 1–7 days. Each determination represents the mean of five to nine experiments for PMN and uncultured monocytes and one to three determinations for monocytes at each day of culture. Error bars (s.e.m.) are represented when there were more than three determinations. ■, fMLP 1·5 h; ▨, fMLP 3 h; □ , serum.

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