The Function of c-fms in Hairy-Cell Leukemia: Macrophage Colony-Stimulating Factor Stimulates Hairy-Cell Movement

By J. Burthem, P.K. Baker, J.A. Hunt, and J.C. Cawley

Hairy cells (HCs) and some activated B cells express high levels of macrophage colony-stimulating factor (M-CSF) (CSF-1) receptor, but the functional effects of the cytokine on B cells have not been previously identified. Using video microscopy, image analysis, and migration assays, M-CSF was shown to induce chemokinetic and chemotactic movement of HCs. This movement response involved transition to a highly mobile, rounded cell form and was accompanied by distinctive changes in F-actin polymerization and phosphatase antialkaline phosphatase staining of cytocentrifuge preparations.

Culture conditions. Short-term cell cultures were performed in Hanks' balanced salt solution (HBSS) at 37°C in 5% CO2 in air unless otherwise stipulated.

Adhesive Proteins

The purity of proteins and fragments used in this study was determined to be greater than 90% using sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and Coomassie blue staining of 8% polyacrylamide gels.

Fibronectin (FN) was purified by gelatin-sepharose affinity chromatography according to the method of Engvall and Ruoslahti (fresh frozen human plasma unsuitable for therapeutic use was provided by the Blood Transfusion Service, Liverpool, UK).

Vitronectin (VN) was a kind gift from G. Burns (Department of Cancer Research, Newcastle, NSW, Australia), and human laminin (LN) and type I collagen (Coll) were purchased from the Sigma Chemical Company Ltd (St Louis, MO).

Cytokines and MoAbs

Antihuman M-CSF (catalogue code BDA 15) was supplied by British Biotechnology (Oxon, UK). Other MoAbs used in these studies were anti-β1, -clone 4B4 (Coulier, Hialeh, FL), two anti-β3 antibodies—clone PM6/13 (Soretex, Oxford, UK) and clone 1076 (a kind gift from G. Burns). Two anti-α1 antibodies—clones HP1-3 and HP2-4 (a kind gift from F. Sanchez-Madrid, Hospital de la Princesa, Madrid, Spain) and anti-α2, -clone 13C1, also provided by G. Burns. Human M-CSF was obtained from Eurocetus (Harefield, UK). All other MoAbs used in depletion or immunocytochemistry were purchased from Becton Dickinson (Mountain View, CA).

From the Departments of Haematology and Clinical Engineering, University of Liverpool, UK.

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Address reprint requests to J. Burthem, MRCP, Department of Haematology, Third Floor Duncan Bldg, Royal Liverpool University Hospital, Liverpool, UK L69 3BX.

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**Hairy-Cell Adhesion Assay**

A sensitive hairy-cell adhesion assay was developed using 96-well tissue-culture plates coated by overnight incubation at 4°C with adhesive proteins. Plates were washed with phosphate-buffered saline (PBS) and $10^5$ HC s suspended in HBSS were allowed to adhere for 15 minutes at 37°C. Plates were washed three times with PBS by inversion then fixed for 2 minutes in 2% paraformaldehyde.

The number of adherent HCs was specifically determined by an immunoassay. An anti-CD11c MoAb (obtained from “The Binding Site,” Birmingham, UK) was allowed to bind to the adherent HCs, this antibody was then detected using peroxidase-conjugated F(ab')2; fragments of antimouse polyclonal antibody with colorimetric analysis of o-phenylenediamine (OPD) substrate development.

**Cell-Migration Assay**

Cells were washed and suspended in HBSS at a concentration of $2 \times 10^6$ mL. 30 μL of HBSS or M-CSF in HBSS at various concentrations was pipetted into the bottom wells of a 48-well chemotaxis chamber (Neuro Probe, Bethesda, MD). The upper chamber was then placed on top, with an 8 μm pore presoaked nitro-cellulose filter between the upper and lower wells. M-CSF at various concentrations was placed in the upper wells and then incubated for 4 hours at 37°C in 5% CO₂. After this period, the filters were removed, washed once in PBS, and then fixed in paraformaldehyde solution (1:1 37% paraformaldehyde:H₂O). Filters were stained with hematoxylin, and cell migration was assessed by two observers using the second-cell leading-front technique on a calibrated microscope.²
Form factor

Calculation of the relative contribution of chemokinesis and chemotaxis to the movement response used the methods of Zigmond and Hirsch. In these calculations, only concentrations of M-CSF up to 10³ U/mL were considered because concentrations above this level became less stimulatory to migration, rendering calculations more difficult to interpret.

Protein coating of filters. Nitro-cellulose filters were soaked overnight at 4°C with specific adhesive protein (VN 20 µg/mL, LN 20 µg/mL, FN 50 µg/mL, Coll 50 µg/mL). Filters were washed in HBSS immediately before use. The number of cells adherent to the surface of coated filters appeared to be relatively independent of the coating protein. This allowed direct comparison of results as the leading-front migration analysis is relatively insensitive to small fluctuations in cell number.

Table 1. Video-Tracking Analysis of HC Movement in Response to M-CSF

<table>
<thead>
<tr>
<th>M-CSF (U/mL)</th>
<th>Below</th>
<th>Medium</th>
<th>10²</th>
<th>10³</th>
<th>2.5 x 10³</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-CSF†</td>
<td>32 ± 9</td>
<td>96 ± 7</td>
<td>114 ± 7</td>
<td>184 ± 7*</td>
<td></td>
</tr>
<tr>
<td>10² U/mL</td>
<td>50 ± 9</td>
<td>102 ± 10</td>
<td>114 ± 7</td>
<td>130 ± 10</td>
<td></td>
</tr>
<tr>
<td>10³ U/mL</td>
<td>78 ± 7</td>
<td>117 ± 11</td>
<td>110 ± 4</td>
<td>132 ± 7</td>
<td></td>
</tr>
<tr>
<td>2.5 x 10³ U/mL</td>
<td>96 ± 2*</td>
<td>122 ± 7</td>
<td>132 ± 13</td>
<td>106 ± 13†</td>
<td></td>
</tr>
</tbody>
</table>

The results display representative data from a single patient; similar findings were obtained in three other patients. Measurements are given as the migration of the two leading-front cells (µm). The standard errors of the means are derived from the means of 10 measurements by two observers on duplicate samples (ie, 40 observations at each concentration).

Use of MoAbs in cell-migration assay. One hundred microliters of washed patient cells (2 x 10³/mL) was incubated for 30 minutes at 4°C with specific anti-integrin chain MoAbs at saturating concentrations. The cells were then diluted to a concentration of 10⁹/mL with HBSS and 50 µL of this suspension used per test (performed exactly as described above).

Time-Lapse Video Microscopy

Cover slips were incubated overnight with substrata proteins and washed in PBS. 100 µL hairy cells or activated tonsillar B lymphocytes were added at 2 x 10⁵/mL and incubated for 10 minutes at 37°C in 5% CO₂ and then gently rinsed in warmed RPMI 1640 medium. The coverslips were placed into 24-well plates and 1 mL of RPMI 1640 medium was placed over them. The preparations were observed using an Olympus CK-2 inverted microscope (Olympus Optical Co, London, UK) with attached JVC TK-1085E color video camera (JVC, Tokyo, Japan). Images were either captured directly by photographing the monitor screen or quantitatively by measurement of cell movement tracks traced from the screen.

Table 2. Checkerboard Analysis of HC Movement in Response to M-CSF

<table>
<thead>
<tr>
<th>M-CSF (U/mL)</th>
<th>Above</th>
<th>10²</th>
<th>10³</th>
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</table>
Fig 3. Time-lapse video observation of a motile tonsillar B cell exposed to M-CSF (1,000 U/mL). Tonsill B lymphocytes (greater than 80% M-CSF receptor expression) were adherent to adhesive protein coated slides as described. A single responsive cell is illustrated (T0). After addition of M-CSF, further modest elongation occurs with anterior ruffling and shape change (T5 to T10). Subsequently (T15 to 35), the cell loses its tail and becomes rounded, more phase bright, and more motile. No such changes were seen in the absence of M-CSF.

Image Analysis

Cells were prepared as for time-lapse video microscopy and stained with Rose-Bengal. An image analysis system (Improvision, Coventry, UK) was used to quantify cell morphology automatically, without subjective human interaction. Preparations were examined with a Carl Zeiss Jenaval microscope (magnification x 320) (Zeiss, New York, NY) and an attached Hitachi KP140 solid-state camera (Hitachi Co, Tokyo, Japan). The final image was analyzed using an Apple Macintosh IIfx/20/80 computer (Apple Computers, Cupertino, CA). Size and resolution was 540 x 476 x 8 bits deep, resulting in a grey image of the smear with 256 levels of grey. The preparations were scanned and images of greater than 600 cells were taken for each sample. To analyze these images, the system used PRISMVIEW ANALYSIS SOFTWARE (Analytical Vision Inc, Raleigh, NC). The image from the slide was converted into a binary image. A selective deletion was performed removing noncellular objects from the binary image on the basis of cross-sectional area. Because some of the cells were not stained completely evenly throughout the cell, a fill procedure was used to fill the weaker stained areas. The morphology of each individual cell was then measured. The parameters that best described cell form in this study were selected from the multiple parameters available. These were:

\[
\text{Area} = \frac{1}{2}(X_k Y_{k+1} - X_{k+1} Y_k)
\]

\[
\text{Form Factor} = 4\pi \text{Area}/\text{Perimeter}^2
\]

Scanning Electron Microscopy

Cells were cultured exactly as described for time-lapse video microscopy. The cells were washed in PBS and fixed in warm 2.5% gluteraldehyde in 0.2 mol/L sodium cacodylate pH 7.4 for 30 minutes. Preparations were then washed and desiccated before sputter coating according to standard protocols. Microscopy was performed using a Joel JSM-35C (Tokyo, Japan). All EMs shown in this study were visualized at 35 KV and magnification of x 1,500 using 30° table tilt.

F-Actin Studies

Immunofluorescence microscopy. Cell cultures on protein-coated coverslips were fixed for 2 minutes in 2% paraformaldehyde,
M-CSF STIMULATES HC MOVEMENT

Mean fluorescence

![Graph showing mean fluorescence over time with and without M-CSF](image)

**Fig 4.** FACS analysis of the F-actin content of HCs after stimulation with M-CSF (100 U/mL).

then permeabilized with methanol before staining with rhodamine phalloidin (Molecular Probes, Inc, Eugene, Oregon). Preparations were mounted in 1:1 glycerol:PBS and visualized using fluorescence microscopy at an excitation of (550 to 580).

**Immunofluorescent fluorescence-activated cell sorter (FACS) analysis.** NBD phallacidin (Molecular Probes) was used with a simultaneous fixing, permeabilization, and fluorescence technique (5 to 10 U/mL of fluorescent phallotoxin, 50 to 100 µg/mL lysophosphatidyl choline and 3.7% formaldehyde). Hairy cells were incubated with or without M-CSF (1,000 U/mL) at 37°C and preparations were sampled at the various time points. Phallacidin solution was added to the samples at 4°C for 20 minutes before analysis on a FACScan (Becton Dickinson) using Lysis version II software counting 10,000 events. Integrin receptor analysis used MoAbs to α protestors, β protestors, and relevant class-specific control MoAbs, together with fluorescein isothiocyanate-labeled second-layer detection antibodies. HCs were analyzed before exposure to M-CSF, and at 30 and 180 minutes after incubation with the cytokine.

**Statistical Methods**

Filter-migration assays were statistically evaluated using the Wilcoxon signed rank sum test using matched-pair observations; for comparison of calculated (predicted) cell movement in filters versus actual (observed) cell movement, a paired t-test of difference was used. Image analysis data were collated and analyzed statistically using SAS version 6.04 (SAS Institute, Marlow, Bucks, UK). The data were analyzed using an unbalanced analysis of variance (ANOVA) F test, applying the Tukey’s studentized range test and the Waller-Duncan k-ratio t test. The combination of these tests not only tested the differences between groups of data, but also enabled the data to be grouped according to significant effects.

**RESULTS**

**M-CSF Induces a Biphasic Change in HC Morphology, Which Is Associated With Cell Motility**

*Hairy cells.* Approximately 40% of a given population of HCs are mobile on VN, and therefore, this surface was chosen for initial studies. No immediate change in morphology was observed after the addition of M-CSF (1,000 U/mL). However, when analyzed by time-lapse video microscopy, elongated HCs (an appearance that correlates well with HC motility) were seen to undergo further elongation (over 10 minutes) and then to become phase bright and contract their processes (complete by 30 minutes) (Fig 1). Rounded or spread cells did not elongate, but simply became phase bright (not shown).

We examined these changes quantitatively using image analysis. The average spread area of the HCs increased over the period of cell elongation (0 to 15 minutes, Fig 2A), and then became reduced to below control levels by 30 minutes (Fig 2A). This biphasic response was also observed when form factor (FF) was analyzed. After 20 minutes incubation with cytokine, the FF increased markedly (Fig 2B), confirming a shift in the shape of the whole population towards a circular form (FF is a measure of the deviation of cell shape from the circular form, and has a maximum value of 1 = perfect circle; it provides a useful measure of cell shape in motility studies). However, during the first 10 minutes, despite the elongation of individual cells noted above (and reflected in increased area), there was no marked change in the FF of the population as whole. This probably reflects our observation that only a proportion of cells undergo the elongation response and that image analysis parameters are relatively insensitive to lymphocyte elongation (as has been noted previously).

Time-lapse video microscopy showed that both the elongation and rounded phases of M-CSF-induced shape change reflected increased HC motility. During the elongation phase, the HCs moved by typical lymphocyte crawling, with the anterior pole of the cells advancing short distances, leaving the elongating tail to its rear. During the rounded, phase-bright period, all cells showed intense cell-membrane movements and a proportion became substantially more mobile. These observations were quantitated by cell tracking of the time-lapse video observations performed at low power (Table 1). This analysis indicated that M-CSF recruited previously immobile cells to move, and approximately doubled the distance traveled by individual cells in the absence of a chemotactic gradient (=chemokinosis).

To examine these observations by an alternative technique and also to determine the relative importance of chemotaxis versus chemokinosis, the effect of M-CSF was measured in a filter-migration assay. As is shown in Table 2, M-CSF induced both chemokinesis and chemotaxis. When the relative importance of the two effects was analyzed using the methods of Zigmond and Hirsch, chemokinesis was shown to be the major effect, whereas a more minor but consistent chemotactic effect was also demonstrable (P < .1). To confirm that M-CSF was the active agent responsible for the chemotaxis and chemokinesis observed in the filter-migration assay, this was performed after preincubation of the M-CSF with an excess of neutralizing antibody. In these experiments, the chemotaxis and chemokinesis were reduced to the levels observed in control experiments (M-CSF effect significance, P < .001).

*Tonsillar B cells.* HCs share many features with normal activated B cells. In particular, we have shown that such B
cells, like HCs, express M-CSF receptor. Therefore we examined in a preliminary way the effect of M-CSF on activated tonsillar B lymphocytes. Using time-lapse video microscopy, M-CSF was shown to induce a proportion of cells to undergo similar morphologic changes to those described above in association with a motility change in HCs (Fig 3).

The Changes in HC Movement Induced by M-CSF Are Accompanied by Biphasic Changes in F-Actin Polymerization and Distribution

When F-actin was semiquantitatively examined by FACS measurement of NBD-phallacidin binding, a biphasic response to M-CSF was again observed. F-actin polymerization increased markedly during the first 10 minutes after addition of M-CSF (Fig 4); thereafter, levels decreased gradually, but remained elevated above controls at 30 minutes (Fig 4).

The intracellular distribution of F-actin was examined by fluorescence microscopy and the changes correlated with the changes in total F-actin and with the morphologic responses to M-CSF noted earlier. During the elongation phase of the HC response to M-CSF, intense staining for F-actin appeared at the blunt leading edges (lamellipodia) of the stimulated cells (Fig 5E); F-actin became progressively less obvious in the trailing cell pole (uropod). Subsequently (10 to 30 minutes after M-CSF addition), a further marked change in F-actin occurred such that the overall reactivity became somewhat reduced and the cells became more diffusely stained (Fig 5F).

Because changes in peripheral cytoplasmic actin are associated with cell-surface ruffling in motile cells, such ruffling was examined by scanning electron microscopy. During the elongation phase (Fig 5B), the HC surface became strikingly smooth (corresponding to the phase-dark appearances (Fig 1, 0 to 10 minutes). As the cells became rounder, surface ruffles returned (Fig 5C), and presumably

Fig 5. Scanning EM (top row, left to right, A through C) and F-actin (bottom row, left to right, D through F) appearances of HCs stimulated with M-CSF.
Fig 6. M-CSF has no effect on cell adherence to purified adhesive proteins. The adherence of HCs to multwell plates coated with purified adhesive proteins was determined in the presence (white) or in the absence (black) of M-CSF, the gray bar indicates the effect of 15 minutes preincubation with the cytokine. Error bars are calculated from the data of four separate patients.

account for the phase-bright appearance of Fig 1. Despite the rounding of cells between 10 to 30 minutes, polarity of cells was still apparent (Fig 5, C and F), although the trailing uropod was frequently less obvious.

Involvement of Different Adhesive Proteins in the M-CSF-Induced Movements of HCs

In a simple adherence assay, HCs displayed strong adherence to VN and FN, but weaker adhesion to laminin and collagen. M-CSF had no measurable effect in this assay (Fig 6). However, when HCs were examined by video microscopy on different protein surfaces, the M-CSF-stimulated cells were seen to become motile on LN and collagen (surfaces on which they were previously sessile).

Therefore, these phenomena were examined in a more formal way by measuring migration in the type of filter-migration assay used earlier, but in which the nitro-cellulose filters were coated with different adhesive proteins. In medium alone, HCs showed a capacity to migrate on all surfaces tested (Table 3). Migration was least into uncoated filters and slightly greater into LN- and Coll-coated filters. After M-CSF stimulation, migration over LN and Coll was markedly enhanced (Table 3). As expected from our observation on 2-D surfaces, unstimulated HCs migrated significantly into the VN-coated filters, the cells also migrated well into FN-coated filters but this migration was less than into a VN surface. After M-CSF stimulation, the two surfaces evoked different responses, movement increasing in relation to VN, but remaining the same for FN (Table 3).

In a parallel study of HC behavior on different substrata, we showed that the cells engaged different integrins when interacting with FN- versus VN-coated surfaces. On FN, β1 but not β3 integrins are involved, whereas on VN, α3β3 is the sole integrin engaged. The present demonstration that M-CSF enhances HC migration on VN, but not on FN suggested that the cytokine has a differential effect on the different motilities mediated by individual integrins. Therefore, in particular, we postulated that M-CSF specifically enhanced α4β1-mediated motility.

Therefore, we tested the effect of various specific anti-integrin MoAbs on HC movement in the presence or absence of M-CSF (Fig 7). On an FN surface, MoAbs to α4 or β1 mimicked the effect of natural ligand (VN) and enhanced HC motility—this was unaffected by M-CSF.

Table 3. HC Migration Through Adhesive Protein-Coated Nitro-Cellulose Filters

<table>
<thead>
<tr>
<th>Adhesive Protein-Coated Nitro-Cellulose Filters</th>
<th>Pre-M-CSF</th>
<th>Post-M-CSF</th>
<th>Change in Response to M-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coll</td>
<td>187 ± 18</td>
<td>310 ± 34</td>
<td>65%</td>
</tr>
<tr>
<td>LN</td>
<td>142 ± 8</td>
<td>274 ± 29</td>
<td>92%</td>
</tr>
<tr>
<td>FN</td>
<td>227 ± 21</td>
<td>223 ± 17</td>
<td>-2%</td>
</tr>
<tr>
<td>VN</td>
<td>269 ± 26</td>
<td>348 ± 43</td>
<td>29%</td>
</tr>
<tr>
<td>Uncoated</td>
<td>120 ± 8</td>
<td>167 ± 8</td>
<td>39%</td>
</tr>
</tbody>
</table>

All measurements represent the mean of twenty observations on each of three patients and are given in μm.

* M-CSF was below the filter only, at a concentration of 10^5 U/mL.
stimulatory effect and may have weakly inhibited movement on FN. When these experiments were performed in the presence of M-CSF, the cytokine enhanced the movement induced by \( \alpha_{\beta_3} \) MoAb. In contrast, the cytokine had no effect on either the baseline movement on FN (no MoAb) or on that observed in the presence of \( \alpha_x \), \( \beta_1 \) or isotypic control antibodies.

Quantitative FACS analysis of \( \beta_1 \), \( \alpha_x \), and \( \beta_3 \) chain expression showed that after up to 180 minutes exposure to M-CSF, there was no change in the levels of expression of any of these integrin chains. These data, taken together with the specific effects of M-CSF on \( \alpha_{\beta_1} \)-mediated movement suggest to us that the cytokine has a specific qualitative effect on the response mediated by \( \alpha_{\beta_3} \) engagement.

**DISCUSSION**

The present report clearly shows that M-CSF stimulates HC movement, and therefore, the findings point to a functional role for the cytokine in B-lymphocyte biology. The rapidity with which the M-CSF induced HC movement and the purity of the cell preparations used excluded accessory cell or secondary cytokine effects; blocking with an anti-M-CSF antibody confirmed that the observed responses were attributable to the M-CSF in the preparations.

The movement response was biphasic. The initial crawling phase was typical of the lymphocyte locomotion well described by others. In contrast, the later highly mobile, rounded form of cell has not been previously associated with lymphocyte motility. The rounded cells possessed a generally increased motile potential (chemokinesis) and also to a lesser extent an ability to migrate along a gradient of M-CSF (chemotaxis). These findings are, we believe, novel for a cell type, although M-CSF has been shown to be chemotactic (but not chemokinetic) for monocytes. Furthermore, we have been able to relate the M-CSF response in HCs to cytoskeletal changes and to integrin/substratum interactions—phenomena likely to be of general importance in lymphocyte and monocyte movement.

The biphasic motility response was accompanied by biphasic cytoskeletal changes. Unlike normal circulating B cells, HCs are known to have plentiful F-actin, but the significance of this has not been determined. Here we show that after M-CSF treatment, the polymerization state of actin was enhanced and its intracellular distribution specifically rearranged.

The effect of M-CSF on HC migration varied with the adhesive protein substratum. Unstimulated HCs are motile on VN and this movement was enhanced by M-CSF. The cells are poorly motile on Coll and LN, but also become highly motile on these proteins after exposure to M-CSF. In contrast, the limited HC movement observed on FN was unaffected by the cytokine. These different response patterns suggested the involvement of distinct adhesive protein receptors.

Here and in other studies, we have shown that the \( \alpha_{\beta_3} \) integrin of HCs is concerned with their motility—a concept fully in accord with the involvement of this receptor in the movement of other cell types. The data we present indicate that M-CSF enhances the motility mediated by engagement of \( \alpha_{\beta_3} \) (either by VN itself or by \( \alpha_x \) or \( \beta_3 \) MoAb stimulation), but not that mediated by FN receptors. Therefore, the present data show that M-CSF affects a specific integrin function (\( \alpha_{\beta_3} \)). Furthermore, we show that this differential effect is not the result of a quantitative change in the integrins concerned. Therefore, the precise mechanism remains to be established, but presumably involves either a direct qualitative change in \( \alpha_{\beta_3} \) or an effect on signaling pathways downstream of the receptor. Similarly, the mechanism of M-CSF-enhanced movement on Coll and LN has not been established; it may be mediated by a qualitative change in \( \alpha_{\beta_3} \) or by some other mechanism.

We suggest that the observations presented here are important in the characteristic tissue distribution of HCs in HCL. HCL is characterized by a close association between HCs and endothelial cells/macrophages, and by extensive migration of HCs into the red pulp of the spleen. Moreover, the marrow is heavily involved by HCs and contains an extensive FN matrix. M-CSF can be produced locally at high concentrations (eg, by stimulated endothelial cells or by macrophages in the immediate substratum). This we propose induces HCs to become highly motile and allows them to penetrate the Coll- and LN-rich basement membrane. Subsequent migration might then be directed by certain substratum adhesive proteins. For example, VN is known to be rich in spleen and, in the presence of M-CSF, HCs are likely to invade extensively on this substratum. In contrast, M-CSF is likely to be less important in FN-rich areas such as the bone marrow.

Finally, many observations initially made in relation to HCs have proved to be of general importance for normal B cells. A very recent study has detected low levels of M-CSF receptor on circulating B cells, and we have shown that activated tonsill B cells express the receptor in substantial amounts. Also, two animal studies have reported an unexplained lymphopenia after M-CSF administration. These observations, together with our preliminary finding that activated tonsill B cells, after exposure to M-CSF, undergo morphologic changes associated with motility, lead us to suggest that M-CSF will prove to have a general role in promoting the migration of B cells within tissues.

**ACKNOWLEDGMENT**

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The function of c-fms in hairy-cell leukemia: macrophage colony-stimulating factor stimulates hairy-cell movement

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