Metastatic competence of BW5147 T-lymphoma cell lines is correlated with in vitro invasiveness, motility and F-actin content

Hendrik Verschueren, Imme Van der Taelen,* Joëlle Dewit, Jos De Braekeleer, and Patrick De Baetselier*

Pasteur Instituut van Brabant, Brussels and *Instituut voor Moleculaire Biologie, Vrije Universiteit Brussel,
Sint-Genesius-Rode, Belgium

Abstract: The aim of our study was to investigate whether the level of actin polymerization plays a role in the motile and tissue infiltrating behavior of malignant lymphoma cells. For a panel of cell lines derived from the murine BW5147 T-cell lymphoma, we had previously shown a correlation between experimental metastasis formation and in vitro monolayer invasion. We have analyzed the motility and the F-actin content of six non-metastatic, noninvasive (meta-inv) and five metastatic, invasive (meta-inv) variants of BW5147. Fourier analysis of cell contours was used to quantify shape changes of cells. All meta-inv lines rapidly protruded and retracted pseudopodia, whereas only one of the six meta-inv lines showed this type of motility. Flow cytometry of cells stained with fluorescein-labeled phalloidin showed that the motile meta-inv cell lines have a higher F-actin content than their nonmotile meta-inv counterparts. The results indicate that in lymphoma cells a high level of actin polymerization is a prerequisite for the formation of pseudopodia, which in turn are necessary for infiltration of the cells into tissues, and eventually for efficient metastasis formation. A corollary of this conclusion is that regulation of actin polymerization is a possible target for intervention aimed at moderating the spread of malignant lymphoma. J. Leukoc. Biol. 55: 552–556; 1994.

Key Words: metastasis • invasion • motility • acting • lymphoma

Lymphocytes are unique as they recirculate continuously from the blood and lymph into extravascular tissues and back. This migratory life style implies that lymphocytes are able to penetrate into nonlymphoid tissues, in order to reach all cells that carry foreign antigens on their membrane. Malignant lymphomas may inherit this tissue-infiltrating capacity from their normal ancestors, and as a consequence, invade and destroy most organ tissues. Despite the obvious importance of lymphocyte and lymphoma migration, there have been limited efforts at understanding the cytoskeletal dynamics that govern the crawling movements of these cells, in comparison with, for example, fibroblasts or polymorphonuclear neutrophil granulocytes. It has been shown that the transition from a resting to a locomotor state in lymphocytes is characterized by an increase in F-actin [1], but we know of no investigations where actin polymerization has been related to invasion of lymphoid cells into biological substrates.

As a model system for the study of lymphoid cell invasion and motility, we use a family of established cell lines, all derived from the murine BW5147 T-lymphoma, and analyze their behavior when they are confronted with a precultured monolayer of fibroblastlike cells [2]. This approach appears to be relevant, as we and others have shown that there is good agreement between invasive potential of T-cell lines in the monolayer invasion assay (MIA) and their capacity to extravasate and metastasize to various visceral organs after iv. injection in syngeneic mice [3, 4].

We have provided arguments in favor of the role of active cell movement in lymphoma invasion by showing that a metastatic, invasive variant, BW-O-Lil, performed active shape changes by protruding and retracting pseudopodia, whereas the noninvasive parental BW-O line did not [5]. For a panel of BW5147-derived lines we have shown that an irregular cell shape, indicative of protrusive activity, is indeed a hallmark of invasive, metastatic variants [6]. On the basis of these observations, and of a detailed analysis of video recordings of BW-O-Lil cells moving through a fibroblastic monolayer, we have suggested that protrusion of pseudopodia and constriction of the cell body are essential for efficient penetration of these lymphoma cells, e.g., through a fibroblast monolayer in vitro or a vessel wall in vivo [7].

In this paper we confirm and extend this link between metastatic competence and invasiveness on the one hand, and the capacity of the lymphoma cells to perform active shape changes on the other. In addition, we use quantitative approaches to show that invasiveness and cell motility can be correlated with F-actin content.

We have used 11 related cell lines. The original BW5147 line, a T-cell lymphoma from AKR mice, was obtained from the Salk Institute (La Jolla, CA). This original population will further be called BW-O. BW-6T6 theoretically is the same cell line, but it came to us via several intermediate laboratories. It differs from BW-O by a number of chromosomal abnormalities. The other cell lines were derived from BW5147 by somatic cell fusion, either incidentally in vivo or deliberately in vitro. The in vivo fusion partner was a normal host T-lymphocyte, for in vitro fusion the CTL-D T-cell line or a macrophage-like cell line had been used. One line is a subclone from a hybridoma, obtained by dilution cloning. Table 1 refers to the publications containing the information regarding each line. All cells were cultured in a 1/1 mixture of RPMI1640 and DMEM medium with 10% fetal calf serum and antibiotics.

The metastatic behavior of all cell lines used in the present study had been characterized on earlier occasions. Table 1 therefore summarizes their in vivo malignancy, with ref-

Abbreviations: MIA, monolayer invasion assay.
Correspondence and reprint requests: Hendrik Verschueren, Pasteur Institut van Brabant, Engelandstraat 642, B-1180 Brussels, Belgium.
Received December 20, 1993; accepted December 28, 1993.
ence to earlier publications. Experimental metastasis had been assessed by i.v. injection of 10^6 cells in syngeneic animals. In most instances, there was a clear distinction between nonmetastatic and metastatic variants: either all animals survived disease-free or all animals died within a few weeks with massive tumor infiltrates in the spleen and the liver, and sometimes the kidneys. BW-O-Li3b is particular in that it formed lymph node metastases and killed some of the injected animals, yet in Table 1 it is reported as negative for metastasis because there were no macroscopic lesions in liver, spleen, or kidneys.

The in vitro invasiveness of the cell lines had also been tested earlier, at the same time as their in vivo metastatic competence (see references in Table 1). We have reassessed the invasiveness of each line in the MIA in parallel with the motility and F-actin assays presented below in order to ascertain that the cell lines had retained their reported malignancy. The MIA is based on the enumeration, in phase contrast microscopy of living cultures, of T lymphoma cells that have passed through a precultured monolayer of C3H-10T1/2-clone8 cells (an immortalized murine fibroblast-like line). A detailed description of the assay has been published [2]. 10^4 lymphoma cells/cm^2 were inoculated on confluent monolayers of 10T1/2 cells; 24 h later the cells lying on top of the monolayer were squinted off, and the underlying lymphoma cells were then counted in 10 microscope fields per culture dish. The average values in Table 1 were obtained from both present and past experiments. Each cell line was tested at least nine times in duplicate dishes. Within this collection of BW5147 variants, there was complete agreement between the capability of the cells to extravasate and form metastasis in visceral organs on one hand and their capacity to penetrate through the fibroblast monolayer in vitro on the other hand: all nonmetastatic variants, including BW-O-Li3b, were not invasive or were poorly invasive (less than 3%), whereas all metastatic variants were highly invasive (more than 20%).

We had previously analyzed the morphology of the cells involved in the present study: using the perimeter/area ratio as a measure of shape irregularity we had shown that noninvasive variants were round, whereas invasive variants had a more tortuous outline [6] (there was one exception: BW-O-Li3b cells were noninvasive but had irregular outlines). Although irregular shapes and the presence of surface extensions are indicative of cell movement, analyses of static pictures cannot yield much information about the dynamic aspects of the pseudopod activity, in particular, the speed at which the cell shapes change. We have devised two approaches to quantify shape changes of the lymphoma cells. A full description and discussion of the methods have recently been published [8]. In both cases, paired images were stored with a time interval of 1 min, and the two contours of at least 30 cells were analyzed using an IBAS image analyzer (Kontron, Munich). In the first approach, the contours of each cell were superimposed, and the nonoverlapping area was used to calculate an incongruence factor. The mean incongruence factor of a cell population is a measure of the extent of shape change in the given time interval, but does not contain information to describe the type of shape alterations. The second, more elaborate approach is based on Fourier analysis of the cell outlines: a function, describing the cell outline in polar coordinates, is decomposed in a number of sinusoidal waves of increasing frequency, the harmonics. The amplitudes of the lower frequencies describe alterations of the entire shape; high frequencies relate to bendings in smaller segments of the cell's outline. Whereas amplitude values describe static cell shape, the differences in amplitude between two images taken 1 min apart are used to describe the shape alterations.

When applied to the BW5147 variants mentioned in Table 1, static Fourier analysis of the cell outlines showed that all metastatic, invasive variants had an irregular shape with large pseudopods, as the amplitude of the lower frequencies were raised considerably above the background (Fig. 1a).

### Table 1. Metastatic Capability, in vitro Invasiveness, Motility and F-actin Content of BW5147 Derived Cell Lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Metastasis^5</th>
<th>Invasion^5</th>
<th>Motility^5</th>
<th>F-actin content^5</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW-O</td>
<td>Parental BW5147</td>
<td>-</td>
<td>0.2 ± 0.2</td>
<td>0.108 ± 0.036</td>
<td>766 ± 212</td>
</tr>
<tr>
<td>BW-6T6</td>
<td>In vitro variant, karyotypic differences[1]</td>
<td>-</td>
<td>0.01 ± 0.01</td>
<td>0.091 ± 0.038</td>
<td>978 ± 258</td>
</tr>
<tr>
<td>BW-O-Li3b</td>
<td>In vivo fusion with host T-cell, and chromosome segregation in culture[1]</td>
<td>-</td>
<td>0.04 ± 0.07</td>
<td>0.193 ± 0.062</td>
<td>1357 ± 453</td>
</tr>
<tr>
<td>BW-O-Li1</td>
<td>In vivo fusion with host T-cell[5]</td>
<td>+</td>
<td>60 ± 18</td>
<td>0.228 ± 0.079</td>
<td>1736 ± 317</td>
</tr>
<tr>
<td>TAS 5C4</td>
<td>In vitro fusion with spleen T-cell[6]</td>
<td>-</td>
<td>0.07 ± 0.06</td>
<td>0.105 ± 0.041</td>
<td>1152 ± 317</td>
</tr>
<tr>
<td>TAM 4D1.2</td>
<td>In vitro fusion with activated spleen T-cell[6]</td>
<td>+</td>
<td>20 ± 8</td>
<td>0.212 ± 0.062</td>
<td>1621 ± 150</td>
</tr>
<tr>
<td>TAM 4D6.2</td>
<td>In vitro fusion with activated spleen T-cell[6]</td>
<td>+</td>
<td>24 ± 15</td>
<td>0.232 ± 0.063</td>
<td>1973 ± 130</td>
</tr>
<tr>
<td>DCH10</td>
<td>In vitro fusion with macrophage-like cell[7]</td>
<td>-</td>
<td>2.0 ± 1.8</td>
<td>0.088 ± 0.033</td>
<td>1024 ± 386</td>
</tr>
<tr>
<td>DCH10-Sp</td>
<td>In vivo fusion of DCH10 with host T-cell[8]</td>
<td>+</td>
<td>27 ± 8</td>
<td>0.225 ± 0.084</td>
<td>1277 ± 291</td>
</tr>
<tr>
<td>BW-19</td>
<td>In vitro fusion with CTL-D T-cell line[9]</td>
<td>+</td>
<td>56 ± 33</td>
<td>0.162 ± 0.072</td>
<td>1267 ± 183</td>
</tr>
<tr>
<td>BW-19cll</td>
<td>Subclone of BW-19[9]</td>
<td>-</td>
<td>2.8 ± 2.3</td>
<td>0.119 ± 0.066</td>
<td>809 ± 356</td>
</tr>
</tbody>
</table>

^5Metastasis formation after i.v. injection.
- : all animals survive for at least 40 days without macroscopic lesions (except BW-O-Li3b; see \[j\]).
+ : at least 4 of 5 animals die within 40 days with macroscopic lesions in liver and other organs.
^5Ratio invading cells/inoculated cells in a 24-h MIA; mean and SD for 9 experiments.
^5Mean and SD for 30 cells.
^6Flow cytometry of FITC-phalloidin stained cells; mean and SD of 4 experiments, 10^6 cells measured per experiment.
^16[16].
^17[17].
^9A gift from E. Roos, Amsterdam [18, 19].
^20[20].
^18[18, 21].
^22of 5 animals died with metastases in lymph nodes, no liver involvement.
On the contrary, five of the six noninvasive variants had a more roundish morphology, as shown by the low amplitudes for all frequencies. As already mentioned, BW-O-Li3b forms an exception: these cells were noninvasive but had irregular outlines. The dynamic Fourier analysis of cell outlines over a 1 min time interval showed that the irregular-shaped variants did perform rapid and gross shape changes, as the amplitudes of the lower frequencies were actually changing within this short time period (Fig. 1b). The spherical-shaped cell lines did not show any modification of their Fourier amplitudes. The amplitude changes prove that the variants with an irregular, locomotor shape were actually protruding and retracting large pseudopodia. For the BW-O-Li3b line in particular, this analysis proves that the cells were not fixed in their polarized configuration but indeed were actively moving. Thus, the profiles of the graphs in Fig. 1b show a clear distinction between motile and nonmotile variants. In addition, the similarity of the profiles for the motile variants indicate that they all display a similar type of shape change, brought about by the extension and withdrawal of large pseudopodia in multiple directions.

In addition to the Fourier analysis, we also determined the simple incongruence factor. Although this factor does not contain information about the type of shape alterations, it also allowed discrimination between the motile and the nonmotile variants (Table 1), and as a single factor it is more suitable than the multiple Fourier coefficients for analysis of correlation with other parameters (see below).

As the diacylglycerol-induced transition from a round to an irregular, so-called locomotor morphology is accompa-
Fig. 3. Synoptic presentation of the characteristics of 11 BW5147 cell lines. Metastatic capability is represented by a downward (nonmetastatic) or an upward (metastatic) arrow near the abscissa. Circles represent invasiveness as measured in the MIA. Diamonds represent motility expressed as the incongruence factor. Squares represent F-actin content as determined by flow cytometry after staining with labeled phalloidin. The lines connect the values for closely matched cell lines: 6T6 is a simple in vitro variant of BW-O, whereas BW-O-Lii and BW-O-Lii3b are in vivo-derived hybridomas from BW-O; TASSC4, TAM4Di.2, and TAM4Di.2M are in vitro-derived hybridomas from BW-O and normal spleen cells; DCH105p is an in vivo-derived hybridoma from DCH10; BW-19ri is a subclone of BW-19.

The established cell lines derived from BW5147 have either low or high F-actin contents constitutively, and therefore they behave either like unstimulated resting lymphocytes or like stimulated motile lymphocytes, respectively.

There is also a fair correlation between the motility and the invasion parameters (Fig. 4b), in agreement with our assertion that active cell deformation is an essential contribution to the invasive capability of the variants. Only BW-O-Lii3b is odd, as it is nonmetastatic and noninvasive, yet motile. A link between motility and invasiveness was not unexpected. Upon activation by various stimuli, lymphocytes start to extend pseudopodia and acquire a polarized shape [10]. By virtue of this locomotor morphology, activated lymphocytes become capable of crawling into protein gels or under precultured cell monolayers (reviewed in ref. 4). In addition, it is a common paradigm that active cell movement contributes to invasion and metastasis of most neoplastic cells (for a comprehensive review of experimental evidence, see ref. 11). Yet we know of only one example of a tumor model, the Dunning R-3327 rat prostatic carcinoma, for which a quantitative analysis of cell motility was combined with statistical evaluation of the correlation between the motility parameters and malignant behavior of 11 variants [12]. Our study is the first to provide statistical evidence in favor of cell motility as a determinant of invasive and metastatic behavior of lymphoma cells.

Finally, there is a weak yet significant correlation between F-actin content and invasiveness (Fig. 4c). This could reflect an indirect causal relationship: actin polymerization drives motility, motility in turn leads to invasion.

A study using artifacts such as established cell lines, in vitro invasion assays, and i.v. injection of tumor cells has only limited relevance for understanding cell behavior in natural malignancies. Metastasis formation is a multistep process, the outcome of which depends on multiple factors, and therefore it would be naive to conclude from our study that cell motility or F-actin content might have any predictable value for the malignancy of human lymphomas. In fact, we have emphasized the odd data with the BW-O-Lii3b line to show that high F-actin level and motility do not always lead to invasion and metastasis. What can be concluded from the present study is that actin polymerization and the ensuing cell movements contribute to tissue infiltration by malignant lymphoma cells. An important corollary of this is that the regulation of actin polymerization is a possible target for therapeutical intervention, aimed at moderating the spread of malignant lymphomas. Therefore a better understanding of the regulation of actin polymerization in normal and malignant lymphocytes is imperative. In the last few years there has been a major breakthrough in understanding the control of actin dynamics in fibroblasts, with the discovery that the small ras-related GTP-binding proteins rac and rho, downstream of ras itself, control membrane ruffling and stress fiber formation, respectively [13]. On the other hand, it has been shown that transfection of activated ras in BW5147 cells can induce invasive and metastatic competence.

Fig. 4. Correlation analysis of the parameters in Table 1 and Fig. 3. a) F-actin content (determined by flow cytometry after staining with labeled phalloidin) vs. motility (expressed as the incongruence factor). b) Motility vs. invasiveness (% invasion measured in the MIA). c) F-actin content vs. invasiveness. Linear regression (full lines), 95% confidence intervals (dotted lines), correlation coefficients (R values), and significance (P values) were computed with Inplot software (Graphpad, San Diego, CA).
via unknown mechanisms [14]. Together with our demonstration that actin polymerization is a determinant of lymphoma cell invasion, those findings suggest that the ras gene product could control metastatic behavior of lymphoma cells via an effect on the actin cytoskeleton, possibly mediated by rac and/or rho proteins. The fact that the ber gene product, which is affected by the translocation leading to the formation of the Philadelphia chromosome in acute lymphoblastic leukemia, is a GTPase-activating protein for rac [15] is an additional argument to urge investigation of the role of ras-related GTPases in the cytoskeletal dynamics of lymphoma cells.

REFERENCES