Expression of Pertussis Toxin Adenosine Diphosphate-Ribosyltransferase in a T-Cell Hybridoma Reduces Metastatic Capacity

Mariëtte H.E. Driessens, Ellen A.M. van Rijthoven, Geertje La Rivière, and Ed Roos

T-cell hybridomas are highly metastatic, and their in vitro invasiveness correlates with metastatic capacity. Invasion is blocked by pertussis toxin (PT), which adenosine diphosphate (ADP)-ribosylates G-proteins; thus, adhesion to endothelial venules of the lymph node and out of the thymus were highly metastatic, particularly to the liver, but also activated T cells with the nonmetastatic T lymphoma BW5147 were limited, i.e., reduction of the tumor burden in the liver to 10% of the controls. We conclude that G-proteins play an important role in T-cell hybridoma metastasis. We propose that the reduction in metastasis is due to diminished entry of tumor cells from the blood into tissues.

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CIRCULATING BLOOD cells express integrins, but bind poorly or not at all to their ligands. Adhesion requires activation of the cells, e.g., by factors such as C5a, FMLP, interleukin-8 (IL-8), or platelet-activating factor (PAF), produced at sites of inflammation, which thus regulate influx of cells into these sites. Activation by these factors is often inhibited by pertussis toxin (PT), which adenosine diphosphate (ADP)-ribosylates G-proteins; thus, adhesion apparently requires signals transmitted by G-proteins. PT causes lymphocytes, indicating that, in noninflamed tissues, migration of lymphocytes requires similar signals, as has in fact been shown for migration through high endothelial venules of the lymph node and out of the thymus.

Metastasis formation by hematopoietic tumor cells starts with the invasion of blood-borne tumor cells into tissues. We have shown that metastatic lymphoma cells and activated normal T cells invade hepatocyte cultures, a model for in vivo invasion of the liver. This suggested that invasion mechanisms of normal and transformed blood cells are similar. Indeed, T-cell hybridomas generated by fusion of activated T cells with the nonmetastatic T lymphoma BW5147 were highly metastatic, particularly to the liver, but also to many other organs. We regard the T-cell hybridomas, therefore, as a model for both migration of normal activated T cells into noninflamed tissues and for metastasis formation by lymphoid tumors.

Previous results showed that, in TAM2D2 T-cell hybridoma cells, the integrin leukocyte function-associated antigen-1 (LFA-1: CD11a/CD18) is required for invasion of cells into hepatocyte and fibroblast monolayers in vitro and metastasis in vivo. Yet, the cells do not adhere to intercellular adhesion molecule-1 (ICAM-1), indicating that LFA-1 is not in the active state. PT treatment of TAM2D2 cells reduced invasion and metastasis. The divalent cation Mn²⁺ induced adhesion of the cells to the LFA-1 ligand ICAM-1, and we observed that PT-pretreated cells, which were virtually noninvasive, invaded extensively in the presence of Mn²⁺. Also, phorbol myristate acetate (PMA) stimulated LFA-1-mediated adhesion and reversed the inhibition of invasion by PT (La Rivière et al., unpublished results). We propose therefore that PT blocks a signal delivered by an extracellular factor that is required for activation of LFA-1-mediated adhesion and, hence, for invasion. This implies that metastasis in vivo is dependent on factors present in the metastatic target organs such as the liver, i.e., in noninflamed tissues. To investigate this, we have previously assessed metastasis formation of TAM2D2 T-cell hybridomas after PT-pretreatment. The results supported a role of G-protein signals in metastasis. However, the effect was limited, i.e., reduction of the tumor burden in the liver to 10% of the controls and no clear effect on metastasis to other sites such as spleen, kidneys, and ovaries. Therefore, either the role of G-proteins in metastasis is marginal or the limited effect was due to the restricted duration of the toxin effect. To distinguish between these two possibilities, the present study was performed.

PT catalyzes the ADP-ribosylation of G-proteins, causing their uncoupling from the receptor and hence a blockade of signals transmitted on binding of receptor agonists. PT consists of five subunits, but only the S1 subunit is required for ADP-ribosylation. The others mediate binding to and translocation across the plasma membrane. G-proteins are heterotrimeric that are associated with membranes and are involved in multiple signal pathways. The guanosine diphosphate (GDP)-bound trimer binds to cell surface receptors, in most cases proteins with seven transmembrane domains. Interaction of agonists with these receptors triggers a change in G-protein conformation, resulting in GDP-GTP exchange and dissociation of the a-subunit from the bγ-subunits. Ga and bγ-subunits activate effectors such as adenyl cyclase, phospholipases, ion channels, tyrosine and serine-threonine kinases. The PT-sensitive G-proteins expressed by T lymphocytes are Ga12 and Ga13. Chaffin et al. generated transgenic mice, in which the S1 catalytic
subunit was expressed under the control of a thymus-specific promoter. They found that thymocytes developed normally, but that the mature T cells did not leave the thymus and, on transfer to other mice, were unable to leave the blood. This provided compelling evidence that PT-sensitive G-proteins are essential for T-lymphocyte migration.

We have used here a similar approach to investigate the role of PT-sensitive G-proteins in metastasis. We transfected the cDNA of the S1 ADP-ribosyltransferase subunit of PT into TAM2D2 T-cell hybridoma cells to inhibit G,-protein signaling permanently. We found that metastasis by a S1 transfectant, in which 95% of the G,-proteins was ADP-ribosylated, was strongly reduced, especially in the liver, but also in other organs. For a second transfectant, with 88% ADP-ribosylated G,-proteins, less metastases were also formed in the liver and the spleen. We conclude that G,-protein-mediated signals are essential for metastasis formation by T-cell hybridomas.

MATERIALS AND METHODS

Cells and culture conditions. The highly invasive T-cell hybridoma TAM2D2 and S1 transfectants were cultured in hybridoma medium as described.14 Embryo fibroblasts 208F (REF) cells were cultured in Dulbecco’s modified Eagle’s medium (DME) with 10% newborn calf serum (GIBCO BRL, Paisley, UK) and used for invasion assays between passages 5 and 15.

Generation of S1 cDNA expression construct. S1 cDNA was removed from vector p1011.7.1 by BamHI digestion. The BamHI site is located just 3’ of the initiation codon. To regenerate the initiation codon, the recessed termini were filled in and blunt ligated to the MFG-IacZ retroviral vector,15 which had been digested with NcoI and BamHI and also filled in. Subsequently, the resulting MFG-S1 construct was digested with NheI and XbaI and the 1.0-kb S1 cDNA fragment was cloned into the XbaI site of the expression vector pcDNAhyg, directing expression of the S1 cDNA from the cytomegalovirus (CMV) promoter. pcDNAhyg was made by cloning the hygromycin resistance gene under the control of the pgk promoter as a blunt BglII fragment into the ScaI site of pcDNA I (Invitrogen, San Diego, CA). The presence of the correct translation initiation sequence was confirmed by DNA sequencing.

Generation of S1 transfectants. TAM2D2 cells (2 × 10^6) in 0.8 mL RPMI medium were electroporated in the presence of 100 μg pcDNAhyg or pcDNAhyg plasmid (both linearized with NheI) using the Bio-Rad gene pulser (360 V, 960 μF; Bio-Rad, Richmond, CA). Cells were seeded in 48-well culture dishes at 10^4 cells per well in hybridoma medium for 45 minutes. Each well contained 50 μL HBSS supplemented with 20 μg/mL soybean trypsin inhibitor (Sigma, St Louis, MO), 2 μg/mL aprotinin (Bayer, Leverkusen, Germany), and 0.5 mmol/L pefabloc (Boehringer Mannheim, Indianapolis, IN). Cell lysate was collected after spinning down the nuclei for 10 minutes at 200g. Protein concentration was determined by BCA protein assay (Pierce, Rockford, IL). Ten micrograms of protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and immunoblotted in 10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.2% Tween-20 supplemented with 1% bovine serum albumin (BSA), and 3% nonfat dried milk. Ascites of murine monoclonal antibody (MoAb) 151C1 against PT S1 subunit (a gift from Dr J. Poolman; Netherlands Institute of Health, Biltoven, The Netherlands) was used at a dilution of 1:5,000 and the secondary reagent sheep antimouse IgG was conjugated to peroxidase (Amersham, Arlington Heights, IL) at a dilution of 1:10,000. Bound antibodies were detected using enhanced chemiluminescence (ECL) detection (Amersham).

ADP-ribosylation assay. Cells were homogenized as described above. After centrifugation for 10 minutes at 200g, membranes were collected by centrifugation for 30 minutes at 160,000g and suspended in 20 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA, 1 mmol/L DTT. Protein concentration was determined by BCA protein assay (Pierce). PT substrates in transfected cells were assayed using a modification of the procedure described by Chaffin et al.17 Twenty micrograms of membrane protein was used per reaction. To test whether ADP-ribosylating activity was present in the lysate, 10 μg lysate was incubated with 20 μg TAM2D2 membrane protein. The reaction volume was 50 μL with final concentrations of 0.1 mmol/L Tris-HCl, pH 8.0, 10 mmol/L thymidine, 1 mmol/L adenosine triphosphate (ATP), 0.1 mmol/L guanosine triphosphate (GTP). 2.5 mmol/L MgCl2, 1 mmol/L EDTA, 10 mmol/L DTT, 10 μmol/L nicotinamide adenine dinucleotide (NAD), and 1 μg 32P-NAD (NEN, Dreieich, Germany). PT (List, Campbell, CA) was resuspended in water at 1 mg/mL and activated just before use by the addition of DTT to 0.1 mmol/L and incubation at 25°C for 10 minutes. Reactions were performed for 60 minutes at 30°C and stopped by the addition of trichloroacetic acid to 20%. After 15 minutes on ice, samples were centrifuged at maximum speed in a microfuge and the pellets were washed twice with 200 μL ethyl ether. Samples were dissolved in Laemmli sample buffer containing 0.1 mmol/L DTT and subjected to SDS-PAGE in a 10% gel. After electrophoresis, gels were stained with Coomassie blue, destained, dried, and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) overnight. Quantitation of the incorporation of 32P-ADP was performed by phosphoimagery analysis (Fuji; BAS2000).

Western blot of G-protein α and β subunits. Membranes were prepared as described for the ADP-ribosylation assay. Fifteen micrograms of membrane proteins were resolved on 10% SDS-polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes, and immunoblotted using the following affinity-purified antisera: AS 7, selective for α1; α2; EC, selective for α1; and QL, selective for α1 and α1. To study β subunit expression, we used antisera SW directed against the carboxy-terminal sequence GSWSFLKWA of the β subunit. All sera were kindly provided by Dr S. Hermouet and are described in a previously published study22 and references therein. Antibody-antigen complexes were detected by ECL (Amersham).

Adhesion of cells to soluble intercellular adhesion molecule-1 (sICAM-1). Cells secreting murine sICAM-1 in the culture supernatant were a gift of Dr F. Takai (Terry Fox Lab, B.C. Cancer Research Center, Vancouver, Canada). Purification of sICAM-1 was performed as described by Welder et al.13 Microwell plates (Costar, Cambridge, MA) were coated by incubating each well with 100 μL ICAM-1 (2 μg/mL in phosphate-buffered saline [PBS]), overnight at 4°C. Unbound sites were subsequently blocked with 0.5% ovalbumin in phosphate-buffered saline (HBSS; GIBCO) for 2 hours at room temperature. Cells were resuspended with 50 μL (Amersham International, Amersham, UK) per 10^4 cells in 100 μL culture medium for 45 minutes. Each well contained 5 × 10^4 cells in a final volume of 100 μL HBSS supplemented with either 1 mmol/L CaCl2 and 1 mmol/L MgCl2 or 2 mmol/L MnCl2. Cells were incubated with...
RESULTS

*S1 transfectants of TAM2D2 cells express S1 mRNA.* TAM2D2 T-cell hybridoma cells were transfected with the pcDNAhygS1 expression vector or the negative control pcDNAhyg. Inversion of transfectants into REF monolayers was determined. Of 60 transfectants, 6 that in an initial screening test appeared to be low-invasive were selected for further analysis, together with 6 others. The cells were screened for S1 expression by Western blotting, but expression was not detected. Northern blot analysis showed that 2 of the 12 transfectants, S05 and S09, expressed a high level of S1 mRNA (Fig 1). A prominent band of 1.6 kb was observed and a minor band of 1.8 kb. Clones S04 and S01 expressed intermediate amounts of S1 mRNA and the other transfectants very little or no S1 mRNA.

**PT ADP-ribosylation substrates in S1 transfectants.**

Four clones were selected for further analysis: the two high expressors S05 and S09; S04, an intermediate expressor; and S03, a low expressor. To determine the presence of functional S1 ADP-ribosyltransferase, we examined the ADP-ribosylation of Gαi-subunits by PT in vitro. Labeling by PT should not occur if the Gαi-proteins had already been ADP-ribosylated by the endogenously expressed S1 enzyme. More importantly, the amount of unmodified, still functional Gαi-protein present in the transfectants can be determined in this way. The results are shown in Fig 2. In Table 1, the incorporation of label into the Gαi-subunits is shown and compared with the parental TAM2D2 cells. In the TAM2D2 cells, the control transfectant H11 and the S03 cell line, which had a low level of S1 transcripts, a high incorporation of label in 40-41-kD proteins was observed. These 40- and 41-kD proteins probably represent Gαi2 and Gαi3 subunits, respectively. In contrast, in the S09 and S05 cells, labeling was only 5% and 12% of that in TAM2D2 cells, respectively. In the S04 transfectant, which expressed an intermediate level of S1 mRNA, this was 41%. These results indicated that the three latter transfectants express S1 mono-ADP-ribosyltransferase activity. We also assayed whole cell lysates of these transfectants for ADP-ribosyltransferase activity towards TAM2D2 plasma membranes, but even after 24 hours of incubation, no incorporation of label in the Gαi-subunits was observed.

To verify that the pcDNAhygS1 construct did encode a functional S1 mono-ADP-ribosyltransferase protein, it was

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**Table 1. Percentage of ADP-Ribosylation and Invasion of S1 Transfectants, TAM2D2 Cells, and the Control Transfectant H11**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>% ADP-Ribosylation</th>
<th>Invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAM2D2</td>
<td>100</td>
<td>++ +</td>
</tr>
<tr>
<td>TAM2D2 + PT</td>
<td>ND</td>
<td>+/-</td>
</tr>
<tr>
<td>H11</td>
<td>81</td>
<td>++ +</td>
</tr>
<tr>
<td>S03</td>
<td>85</td>
<td>+++</td>
</tr>
<tr>
<td>S04</td>
<td>41</td>
<td>+</td>
</tr>
<tr>
<td>S05</td>
<td>12</td>
<td>+/-</td>
</tr>
<tr>
<td>S09</td>
<td>5</td>
<td>+/-</td>
</tr>
</tbody>
</table>

The incorporation of 32P in Gαi-proteins in ADP-ribosylation experiments, as described in Fig 2, was quantitated by phosphoimaging analysis. The value of TAM2D2 was set at 100% and mean values of two experiments are shown. Invasion of cells was estimated and data out of four experiments with similar results are shown. Abbreviation: ND, not determined.
G-proteins involved in T-cell hybridoma metastasis 3119

transfected with pcDNAhygS1 contain ADP-ribosyltransferase activity in the lysates in vitro, we performed in the presence of lysates of cells transfected with pcDNAhygS1. To establish that very low levels of S1 protein are sufficient to ADP-ribosylate G-proteins, we observed considerably reduced labeling of G-proteins in S1 transfected COS-7 cells. In lysates, we did detect three bands of S1 protein with molecular weights of approximately 30, 28, and 23 kD by Western blot analysis (Fig 3A). The detection limit was approximately 10 pg S1 protein. Furthermore, as can be seen in Fig 3B, Gαi-proteins of TAM2D2 cells were labeled when ADP-ribosyltransferase reactions were performed in the presence of lysates of S1-transfected cells (lane 3) or PT (lane 2), but not in the presence of lysates of mock-transfected cells (lane 1). Hence, S1-transfected COS cells contained a functional S1 protein. Because we observed considerably reduced labeling of G-proteins in S1 transfected, whereas we detected neither S1 protein nor ADP-ribosyltransferase activity in the lysates in vitro, we conclude that very low levels of S1 protein are sufficient to ADP-ribosylate G-proteins.

To establish that the reduced labeling of Gαi-subunits of S05 and S09 cell lines by exogenous PT was due to ADP-ribosylation by the endogenous (transfected) S1 enzyme and not due to lower amounts of Gαi-subunits in these clones, we determined Gαi expression by Northern and Western blot analysis. Gαi- and Gαi3 mRNAs were detected, whereas Gαi4 and Gαs, the other PT-sensitive G-proteins, were not expressed (data not shown). As shown in Fig 4, Western blot analysis of plasma membranes showed similar amounts of Gαi3 and Gαs in TAM2D2 and S09 and S05 cells. The slower migration in SDS-PAGE of Gαi3 and Gαs subunits, in S09 and S05 cells, and in TAM2D2 cells that had been cultured in the presence of 200 ng/mL PT for 6 days. This slower migration is characteristic for ADP-ribosylated G-proteins and thus provides independent evidence that most of the Gαi3 and Gαs proteins in S05 and S09 cells are ADP-ribosylated. A doublet band of Gαi3 was observed. This may be due to phosphorylation of Gαi3. All cells expressed similar amounts of Gβ5- and Gαi3-subunits.

**Adhesion molecule expression and adhesive and invasive capacity of the transfecants.** The expression levels of adhesion molecules potentially relevant for metastasis were determined by flow cytometry. As mentioned before, the LFA-1 integrin is required for metastasis of the TAM2D2 cells. CD44 has also been implicated in lymphoma metastasis, but for MDAY-D2 lymphoma cells we have shown that CD44 is not required for cells to metastasize to the liver and spleen. The α6β1 integrin may be involved in melanoma metastasis to the lungs. L-selectin expression on the cells was very low as had been determined previously. CD44 and LFA-1 were present at high levels and the α6β1 integrin at low levels: the level of expression was not different on the transfecants compared with the parental cells (results not shown).

Of the 12 transfecants shown in Fig 1, invasion of S05 and S09 cells into rat embryo fibroblast monolayers was low and comparable to that of PT-pretreated TAM2D2 cells (Table 1). Invasion by the S04 cells was reduced compared with that of parental TAM2D2 cells, but was higher than
invasion by PT-pretreated TAM2D2 cells. Other transfectants (data not shown), including the H11 control cells, were highly invasive. Also, the three other transfectants that had been selected because of reduced invasiveness in the initial screening were highly invasive in subsequent tests. Of those 12 transfectants, only in S04, S05, and S09 plasma membranes was a substantial reduction in the amount of G-proteins ADP-ribosylated by exogenous PT detected. Thus, invasive capacity correlated with the amount of functional Ga1-protein present in the cells.

Previously, it was established that inhibition of invasion of TAM2D2 cells by PT was reversed by Mn2+, probably because Mn2+ stimulates adhesion mediated by LFA-1. In Fig 5, it is shown that both Mn2+ and the phorbol ester PMA, another activator of LFA-1-mediated adhesion, stimulate adhesion to ICAM-1. TAM2D2 T-cell hybridoma cells and the S05 and S09 cells were allowed to adhere to wells coated with murine ICAM-1. Very few cells adhered without stimulation (Fig 5). Mn2+ induced adhesion of approximately 35% of the cells at 2 mmol/L, at which concentration adhesion was maximal, in the absence of other divalent cations. Also ~30% of cells treated with PMA (100 ng/mL in medium with 1 mmol/L Mg2+ and 1 mmol/L Ca2+) adhered to ICAM-1.

Invasion of S05 and S09 cells was low (only 1%), but in the presence of 2 mmol/L Mn2+ or 100 ng/mL PMA, both S05 and S09 cell lines did invade REF to a level similar to that of PT-pretreated TAM2D2 cells in the presence of Mn2+ or PMA (Fig 6). This finding further indicates that the lack of invasion is due to the ADP-ribosylation of G-proteins and not to clonal variation in other parameters that influence invasiveness.

Metastatic capacity of S1 transfectants. In the first experiment, 5 x 10^5 cells were injected into a tail vein of syngeneic mice. Mice were autopsied when moribund or after 28 days, and the presence of metastases was determined both macroscopically and microscopically. The results of this experiment are shown in Table 2A. TAM2D2 cells formed metastases in the liver, spleen, kidneys, lymph nodes, and ovaries. The liver was either diffusely invaded or contained multiple nodules. In some cases, tumor was also found in the retroperitoneal fat and muscle tissue. In contrast, S09 cells formed only in one of five animals metastases in the liver (only 1 nodule) and in the spleen, whereas metastases in the lymph nodes and in the retroperitoneal tissue were found in most of the mice. S05 cells metastasized to the same organs as TAM2D2 parental cells, but metastasis was reduced: two of five animals had considerable tumor growth in the liver, whereas three animals had only a few nodules. Spleen tumors occurred only in three of five animals, but at the other sites considerable metastasis was observed.

In a second experiment using a lower dose of tumor cells (10^4), we also tested the control transfectant H11. Animals were killed when moribund or after 28 days. Liver and spleen weights were determined as a measure for the amount of tumor. The results are shown in Table 2B. All mice injected with TAM2D2 cells and almost all animals injected with the control transfectant H11 developed metastases in liver, spleen, and kidneys. Tumor burden in liver and spleen was similar in these two groups. In this experiment, with the lower cell dose, the tumor burden in S05-injected mice was much lower (and now significantly different from TAM2D2- and H11-injected mice), but again the reduction in metastasis...
G-PROTEINS INVOLVED IN T-CELL HYBRIDOMA METASTASIS

Table 2. Metastasis of TAM2D2 T-Cell Hybridoma Cells, H11 Control Transfectant, and S05 and S09 S1 Transfectants

<table>
<thead>
<tr>
<th></th>
<th>TAM2D2</th>
<th>H11</th>
<th>S05</th>
<th>S09</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>5/5</td>
<td>4/5</td>
<td>1/5</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>5/5</td>
<td>2/5</td>
<td>1/5</td>
<td></td>
</tr>
<tr>
<td>Ovaries</td>
<td>5/5</td>
<td>4/5</td>
<td>1/5</td>
<td></td>
</tr>
<tr>
<td>Kidneys</td>
<td>5/5</td>
<td>4/5</td>
<td>2/5</td>
<td></td>
</tr>
<tr>
<td>Lymph nodes</td>
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<td>4/5</td>
<td>4/5</td>
<td></td>
</tr>
<tr>
<td>Retroperitoneum</td>
<td>4/5</td>
<td>3/5</td>
<td>4/5</td>
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<table>
<thead>
<tr>
<th></th>
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<td>6/12</td>
<td>2/9</td>
<td>6/10</td>
<td>4/12</td>
</tr>
</tbody>
</table>

* Number of female mice injected.

In experiment 1, 5 x 10^6 cells were injected intravenously. Animals were killed when moribund or after 28 days. Shown are the number of animals that had metastases in the indicated organ of the number of mice injected. In experiment 2, 10^6 cells were injected intravenously. Animals were killed when moribund or after 28 days.

was most evident in the animals injected with S09 cells (Fig 7). Only 4 of the 12 mice had metastases, the other 8 were tumor-free after 28 days, when the experiment was terminated. The metastases in these 4 mice were mainly located in the lymph nodes and in retroperitoneal fat and muscles. Only 1 of the animals had a tumor nodule in the liver. Tumor cells were also present in the spleens of the 4 mice, but the amount of tumor (i.e., spleen weight minus the weight of control spleens) was reduced: 54 mg as compared with 172 and 115 mg in the TAM2D2 and H11 groups, respectively (Fig 7B). The S05 group had a tumor burden in the spleen of 58 mg.

To determine whether metastases were formed by cells with similar S1 levels as the injected cells or perhaps by variants with reduced S1 expression, we isolated cells from randomly selected metastases of S05 and S09 cells. S1 mRNA expression in the metastases was similar as in the injected cell population, showing that S1 expression was stable during the in vivo experiment (data not shown).

Occasionally, metastases occurred in the submandibular lymph nodes, which obstructed the esophagus. These animals could not eat, their body weight was reduced, and they died prematurely (19 days). The frequency was comparable in all groups: TAM2D2, 1 of 13; H11, 2 of 11; S05, 2 of 12; and S09, 1 of 13. These mice were excluded from the analysis.

To establish that all cell lines were able to grow in vivo, 10^6 cells were injected intraperitoneally into six syngeneic AKR mice. On day 14, all animals had developed ascitic fluid. The total number of tumor cells present in the ascitic fluid was, on average, 23 x 10^6 cells/mouse for TAM2D2, 46 x 10^6 for S05, 82 x 10^6 for H11, and 155 x 10^6 for S09 cells. We conclude that the reduced metastatic capacity of S05 and S09 cells cannot be attributed to impaired in vivo growth.

DISCUSSION

To assess the role of G-proteins in metastasis of T-cell hybridomas, we generated transfectants in which G-proteins are uncoupled from receptors by continuous expression of the ADP-ribosyltransferase S1 subunit of PT. We report here a substantial reduction in the metastatic capacity of two S1 transfectants, in which 88% or 95% of the G-proteins was ADP-ribosylated and thereby unable to transduce signals from the receptor. Two-thirds of the mice injected with the S09 transfectant with the highest amount of ADP-ribosylated G-proteins were tumor-free. The reduction was particularly striking for the liver, which was tumor-free in virtually all mice, quite in contrast to the extensive tumor burden in control mice. Metastasis in spleen and kidneys was also strongly reduced. This reduction in metastatic capacity is much larger than that observed for PT-pretreated cells in previous experiments, in which liver metastasis was reduced but not prevented, and metastasis to kidneys, ovaries, and lymph nodes was seemingly not affected. Thus, permanent expression of S1, rather than transient activity after PT-pretreatment, greatly enhances its effect. The S05 transfectant with 88% ADP-ribosylated G-proteins metastasized to all sites, but tumor burden in the liver and spleen was much reduced when 100,000 tumor cells were injected. These results support the notion that functional G-proteins are re-
required for efficient metastasis formation of TAM2D2 T-cell hybridoma cells.

The reduced invasiveness of the transfectants was not due to clonal variation in expression of adhesion molecules or another property required, because invasion was restored by stimulation of LFA-1 by Mn²⁺ or the phorbol ester PMA, similar to what occurs in PT-pretreated cells. Furthermore, reduced metastasis was not due to impaired proliferation, because transfectants and parental cells grew equally well, both in vitro and in vivo.

S05 cells formed more metastases than S09 cells and in all animals, whereas two-thirds of the mice in the S09 group was tumor-free. S05 cells contain approximately twofold more functional G₁-proteins than S09 cells (12% vs 5%). Therefore, the level of G₁-protein is apparently more critical in vivo than in short-term in vitro invasion assays, in which S05 and S09 cells performed equally poorly, and even the S04 cells, with 40% functional G₁-proteins, showed reduced invasiveness. We assume that the capability of S05 cells to still form some metastases in the liver and spleen is due to the extended time period allowed for in vivo invasiveness. This may also explain the limited effect of PT-pretreatment: the cells may have invaded after several days when the effect of the toxin had dwindled.

PT causes lymphocytosis due to inhibition of lymphocyte migration from the blood into tissues or across lymph node endothelium. Furthermore, in transgenic mice expressing the S1 catalytic subunit under the control of a thymus-specific promoter, thymocytes develop normally, but the mature T cells do not leave the thymus and, on transfer to other mice, are unable to leave the blood. PT prevents the activation-dependent firm adhesion of lymphocytes to high endothelial venules, which is required for lymphocyte arrest and subsequent migration. We propose that, in tumor cells in which G₁-proteins are ADP-ribosylated, the transduction of the recruitment signal is impaired. Therefore, entry of tumor cells from the blood into tissues is prevented, leading to reduced metastasis formation.

Metastasis of the transfectants was not completely prevented in all mice, particularly not to spleen, kidneys, lymph nodes, and retroperitoneal tissue. Invasion at these sites may have been induced via other signal pathways. For example, tumor necrosis factor α can stimulate invasion of TAM2D2 cells by a PT-insensitive mechanism. Furthermore, LFA-1-independent mechanisms may play a role in metastasis to these sites, because LFA-1-deficient mutants of TAM2D2 cells occasionally formed metastases in retroperitoneal tissue.

G-proteins are heterotrimeric that bind to cell surface receptors, in most cases proteins with seven transmembrane domains. The factors that activate adhesion, invasion, and metastasis of T-cell hybridoma cells are not known, but the most obvious candidates are members of the chemokine family. Macrophage chemotactic protein-1 (MCP-1), MCP-2, MCP-3, macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and interferon-γ-inducible protein have been described to be major chemoattractants for T lymphocytes. It is conceivable that some of these factors induce metastasis formation, even in the absence of inflammation, and observations supporting this notion have been reported. For instance, MCP-1 is expressed in human symptom-free arteries and veins in vivo and in human liver fat-storing cells, and the murine chemokine KC is constitutively present in liver and spleen. Because the TAM2D2 T-cell hybridoma, similar to many other lymphomas, metastasizes extensively to these organs, this chemokine is an interesting candidate. Alternatively, the relevant factor may not be involved in inflammation, but may be an as yet unknown chemokine regulating the normal recirculation of leukocyte subsets. The concept that chemokines are involved in this, was recently shown; in mice lacking the IL-8B receptor, recirculation of neutrophils was impaired. However, there are also other candidates that do not bind to seven transmembrane receptors, but instead to tyrosine kinase receptors, because certain signals induced by granulocyte-macrophage colony-stimulating factor (GM-CSF), epidermal growth factor, hepatocyte growth factor (HGF), and basic fibroblast growth factor have been shown to be sensitive to PT. In fact, HGF induces memory T-cell adhesion and migration. Identification of the involved factors may suggest ways to interfere with metastasis using antagonists or inhibitors.

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REFERENCES


22. Hermouet S, Murakami T, Spiegel AM: Stable changes in expression or activation of G protein αi or αq subunits affect the expression of both β1 and β2 subunits. FEBS Lett 327:183, 1993


33. La Rivière G, Klein Gebbinck JW, Schipper CA, Roos E: Tumor necrosis factor-α stimulates invasiveness of T-cell hybridomas and cytotoxic T-cell clones by a pertussis toxin-insensitive mechanism. Immunology 75:269, 1992

34. Loetscher P, Seitz M, Clark ST, Baggioni M, Miser B: Monocyte chemotactic proteins MCP-1, MCP-2, and MCP-3 are major attractants for human CD4+ and CD8+ T lymphocytes. FASEB J 8:1055, 1994


Expression of pertussis toxin adenosine diphosphate-ribosyltransferase in a T-cell hybridoma reduces metastatic capacity

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