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Pertussis toxin inhibition of T-cell hybridoma invasion is reversed by manganese-induced activation of LFA-1

Geertje La Rivière, Jacqueline W. T. M. Klein Gebbinck, Mariëtte H. E. Driessens and Ed Roos*

Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

*Author for correspondence

SUMMARY

Pertussis toxin (PT) inhibits invasiveness of T-cell hybridomas in vitro and metastasis formation in vivo. We present evidence for the hypothesis that PT interferes with functional activation of LFA-1. Invasion by TAM2D2 T-cell hybridoma cells of fibroblast monolayers was completely blocked by PT pretreatment, but the cells regained invasiveness in the presence of Mn^{2+}, which activates LFA-1. This invasion was blocked by anti-LFA-1 mAb, and Mn^{2+} did not stimulate invasiveness of LFA-1-deficient TAM2D2 mutants. TAM2D2 cells did not adhere to surfaces coated with the LFA-1 counterstructure ICAM-1, but Mn^{2+} induced adhesion. Hence, LFA-1 on TAM2D2 cells requires activation before it can participate in the invasion process.

The hypothesis is further supported by the slightly different results obtained with the TAM8C4 T-cell hybridoma. PT inhibited invasion strongly but not completely. This reduced invasion was increased by Mn^{2+}. TAM8C4 cells did adhere to ICAM-1, but Mn^{2+} enhanced adhesion. Thus, part of LFA-1 on TAM8C4 cells is constitutively active, allowing for some PT-insensitive invasion, but further activation is required for optimal adhesion and invasion.

PT blocks G-protein-mediated signals, suggesting that an extracellular factor is involved. This is not a serum component or an autocrine motility factor, since the PT effect was serum-independent, and PT did not inhibit motility. Therefore, it is probably produced by the fibroblasts, and either secreted or associated with the cell surface. These results are in line with the hypothesis that a fibroblast constituent activates LFA-1 via a PT-sensitive G-protein and thus stimulates invasion of T-cell hybridomas into the fibroblast monolayer.

Key words: integrin, metastasis, G-protein

INTRODUCTION

Highly metastatic T-lymphoma cells invade hepatocyte cultures in vitro, a model for the invasion of the liver in vivo. Activated T-lymphocytes invade similarly, suggesting that T-lymphoma cells and normal T-lymphocytes use the same invasion mechanism (Roos et al., 1981; Roos and Van de Pavert, 1983). This notion is supported by the properties of T-cell hybridomas, produced by fusion of invasive T-cells with non-invasive BW5147 T-lymphoma cells. These hybrids, which are highly invasive in hepatocyte cultures and in monolayers of embryonal fibroblasts, metastasize widely (La Rivière et al., 1988; Roos et al., 1985). In contrast, the BW5147 fusion partner and a few T-cell hybridomas that had lost invasiveness, probably due to loss of the relevant chromosome, were not metastatic (La Rivière et al., 1988; Roos et al., 1985). This correlation of wide-spread metastasis with invasiveness in fibroblast cultures was also observed for (human × mouse) hybrids (Collard et al., 1987b), and for BW5147 cells in which invasiveness was induced by other means (Collard et al., 1987a; De Baetselier et al., 1988; Habets et al., 1990).

The β_2-integrin LFA-1 (Springer, 1990) is expressed by all invasive T-cell hybridomas (La Rivière et al., 1992b, 1993), and is essential for invasion and metastasis, as demonstrated by the inhibition of invasion by anti-LFA-1 antibodies (Roos and Roossien, 1987; La Rivière et al., 1993) and the strongly reduced invasive and metastatic potential of LFA-1-deficient mutants (Roossien et al., 1989). LFA-1 is expressed by most leukocytes and mediates their mutual adhesion as well as adhesion to endothelial cells and fibroblasts (Dustin et al., 1986; Dustin and Springer, 1988; Springer, 1990). LFA-1 on T-lymphocytes is in a low avidity state, but binding to its counterstructure ICAM-1 can be greatly enhanced by appropriate stimulation (Van Kooyk et al., 1989, 1991; Dustin and Springer, 1989; Dransfield et al., 1992a; Landis et al., 1993). This functional upregulation is due to a change in conformation that for human LFA-1 can be detected with conformation-specific monoclonal antibodies (Dransfield et al., 1992a; Landis et al., 1993). For many integrins, including LFA-1, the binding to counterstructures is enhanced in the presence of the divalent cation manganese (Sonnenberg et al., 1988; Gailit and Ruoslahti, 1988; Conforti et al., 1990; Kirchhofer et al., 1990; Altieri, 1991; Elices et al., 1991; Dransfield et al., 1992b), that apparently induces integrins to adopt the high avidity state.

Pertussis toxin (PT) causes lymphocytosis, which is an accumulation of lymphocytes in the blood, due to inhibition of lym-

phocyte entry into lymph nodes (Spangrude et al., 1985) and of migration into tissues such as the skin (Spangrude et al., 1984). In addition, PT inhibits infiltration of thymocytes into monolayers of thymic epithelial cells (Sugimoto et al., 1983). In transgenic mice expressing the catalytic subunit of PT in the thymus under the control of the *lck* promoter, T-cells do not leave the thymus (Chaffin et al., 1990). This is due to a defect in migration, since the transgenic thymocytes perform very poorly in homing assays in vivo, yet closely resemble normal mature peripheral T-lymphocytes with respect to cell surface phenotype and responses to allogenic spleen cells (Chaffin and Perlmutter, 1991). Thus, a PT-sensitive G-protein is required for lymphocyte migration.

This is also relevant for lymphoma invasion and metastasis, since PT strongly inhibits invasion by lymphomas, as well as T-cell hybridomas (Roos and Van de Pavert, 1987; La Rivière et al., 1988, 1992a; Verschueren et al., 1991). Invasiveness is blocked for several days, whereas proliferation is not affected at all, allowing the effect of PT to be tested in vivo. PT pretreatment of the cells before i.v. injection reduced liver metastasis formation to 10-25% of controls (Roos and Van de Pavert, 1987). Thus, a PT-sensitive signaling pathway is involved in invasion of both normal and malignant lymphoid cells in vitro and migration into tissues in vivo. G-proteins transmit signals delivered by binding of extracellular factors to certain cell surface receptors (Simon et al., 1991). Hence, the inhibition by PT indicates that efficient invasion depends on an extracellular invasion-enhancing factor.

The role of LFA-1 in T-cell hybridoma invasion suggested that it was continuously in the high avidity state, because otherwise it would not be able to bind to the relevant counterstructures on the fibroblasts. However, we show here that LFA-1 on TAM2D2 T-cell hybridoma cells does not bind to ICAM-1, whereas invasion of these cells, which is extensive within as little as five minutes after addition to fibroblast monolayers (La Rivière et al., 1990), is completely dependent on LFA-1 (Roos and Roossien, 1987; Roossien et al., 1989). This can only be explained by rapid activation of LFA-1 after addition of the cells to the fibroblasts. Here we present evidence supporting the hypothesis that pertussis toxin interferes with this induction of the high avidity state of LFA-1. Furthermore, we provide evidence suggesting that the PT-affected signal is delivered by a fibroblast constituent.

MATERIALS AND METHODS

Cells and culture conditions

Mouse T-cell hybridomas were generated and cultured in Hybridoma medium as described (Roos et al., 1985; La Rivière et al., 1988). In this study, we have used clones of the highly invasive T-cell hybridomas TAM2D2 and TAM8C4 (La Rivière et al., 1988). Generation and selection of the LFA-1-deficient mutants, A1A4, 2B10 and 1C9, of the TAM2D2 hybridoma has been described (Roossien et al., 1989). Rat embryo fibroblast 208F cells were cultured in DME with 10% newborn calf serum (Sera Lab., Sussex, UK), and used for invasion assays between passages 5 and 15 (La Rivière et al., 1988). All cell lines were mycoplasma-free as determined by regular screening with a mycoplasma rapid detection system (Gen-Probe Inc., San Diego, CA).

Reagents and antibodies

Pertussis toxin (List Biol. Lab., Campbell, CA) was dissolved in 0.1 M sodium phosphate, 0.5 M NaCl, pH 7. Cells (10^6 per ml) were incubated with 200 ng PT per ml in Hybridoma medium (La Rivière et al., 1988) for 2 hours at 37°C in 5% CO_2, washed free of toxin and then used for invasion assays. The LFA-1 antibody used was rat mAb M17/4 (ATCC TIB 217), which is directed against the murine α_L-chain (CD11a), the VLA-6 antibody was rat mAb GoH3 (Sonnenberg et al., 1988), directed against the murine α_6-chain (CD49f), and the anti-mouse ICAM-1 antibody was rat mAb YN1/1.7.4 (Horley et al., 1989). Antibodies were affinity-purified and Fab fragments were prepared as described (Roos and Roossien, 1987), and used at saturating concentrations. Further reagents were from Sigma (St Louis, MO), unless indicated differently.

Invasion assay

Invasiveness in embryonal fibroblast monolayers was determined as described (La Rivière et al., 1988), except that the medium was Hanks' balanced salt solution (HBSS) containing 20 mM Hepes and 2% FCS (Flow, Irvine, UK), supplemented either with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (HF-Ca/Mg) or with 2 mM $MnCl_2$ (HF-Mn). T-cell hybridoma cells and confluent fibroblast monolayers in 2 or 10 cm^2 wells were washed three times with HBSS. Hybridoma cells were then resuspended at a concentration of 10^6 cells per ml, added to fibroblast monolayers, and incubated for 1 hour at 37°C. Non-infiltrated cells were removed by repeated washing and the monolayers were fixed. Infiltrated cells, visible as dark and flattened cells in the phase-contrast microscope, were counted in 10 randomly distributed fields of 0.27 mm^2. From this, the number of infiltrated cells per well was calculated and invasion was expressed as the percentage of added cells. In some experiments, invasion was quantitated using [^{3}H]uridine-labeled cells (see migration assays for labeling conditions) as described (La Rivière et al., 1992a).

Immunofluorescence analysis

Cells were washed three times in HBSS, resuspended in HF-Mg/Ca or HF-Mn (see invasion assay) at a concentration of 0.35×10^6 cells per ml, incubated for 1 hour at 37°C, cooled to 4°C, and washed. Viability, determined by trypan blue exclusion, was 98 to 100%. The cells were then used for FACScan analysis as described (La Rivière et al., 1988, 1992b).

Purification of sICAM-1

Soluble ICAM-1 (sICAM-1) was purified from the culture supernatant of 4A2 cells (Kuhlman et al., 1991). 4A2 is a clone of a CHO cell line transfected with a truncated murine ICAM-1 cDNA that encodes the first four and part of the fifth Ig-like extracellular domains of the ICAM-1 protein, but not the putative transmembrane and cytoplasmic domains, resulting in secretion of sICAM-1 of 50 kDa (Kuhlman et al., 1991). Cells were maintained in serum-free Ex-Cell 301 medium (JR Scientific, Woodland, CA), supplemented with 0.5 μg/ml leupeptin. The culture supernatant was harvested by centrifugation, and protease inhibitors were added: 0.5 μg/ml leupeptin, 2 mM PMSF, 20 μg/ml soy bean trypsin inhibitor and 50 Klett units/ml aprotonin. After filtration, the supernatant was applied to a column of purified anti-ICAM-1 mAb YN1/1.7.4 (Horley et al., 1989) coupled to CNBr-Sepharose (Pharmacia, Uppsala, Sweden), according to the instructions of the manufacturer. The column was washed with PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ (PBS-Ca/Mg) and the protease inhibitor mixture, and subsequently with PBS-Ca/Mg containing only 0.05 μg/ml leupeptin. sICAM-1 was eluted with 20 mM triethylamine, pH 11.3, followed by immediate neutralization with 1 M Tris, pH 7.5. Dialysis and concentration were performed using a Centricon 10 (Amicon, Beverly, MA), then 0.05 μg/ml leupeptin was added and the purified sICAM-1 protein was stored at a concentration of 300 μg/ml at −70°C and used within one month.

Cell binding to purified sICAM-1

Purified sICAM-1 was diluted in ice-cold, sterile PBS with 1 mM

$CaCl_2$ and 1 mM $MgCl_2$, and used to coat 96-well plates (Costar, Cambridge, MA) for 18 hours at 4°C. Wells were then incubated with PBS containing 1% BSA (Boehringer, Mannheim, Germany) for 2 hours at 20°C and washed 3 times with HBSS prior to use. Cells (6×10^6) were labeled with 25 μCi ^{51}Cr as described (Roos and Van de Pavert, 1987), washed three times, resuspended at 0.5×10^6 cells/ml in HBSS containing either 1 mM $CaCl_2$ + 1 mM $MgCl_2$ or 2 mM $MnCl_2$, and 100 μl was added per well. Plates were then centrifuged for 1 minute at 125 ***g***, incubated for 30 minutes in 5% CO_2 at 37°C, and washed 3 times with HBSS. Bound cells were lysed with 1 M NaOH and radioactivity was counted. All measurements were done in triplicate. Specific adhesion was expressed as the percentage of cells bound to sICAM-1 after subtraction of the percentage of cells bound to wells coated with BSA, but not with sICAM-1.

Migration assay

Migration of T-cell hybridoma cells through polycarbonate filters with 8 μm pores was assessed using Transwell cell culture chamber inserts (Costar). Filters were pretreated with sterile PBS containing 5% BSA for 24 hours at 37°C, and washed prior to use. Hybridoma cells (0.25×10^6 per ml) were labeled with 1 μCi 5-[^{3}H]uridine (specific activity 29 Ci/mmol; Amersham Int., Amersham, UK) per ml of Hybridoma medium (La Rivière et al., 1988) for 24 hours at 37°C in 5% CO_2, chased for 16 to 24 hours, washed and resuspended in RPMI 1640 medium containing 2% SF-1 serum supplement (Costar). For migration assays, 600 μl RPMI 1640 with 2% SF-1 was added to a 2 cm^2 well, on top of which the Transwell insert with 2×10^5 to 5×10^5 cells in 100 μl was placed. After incubation at 37°C in 5% CO_2, filters were rinsed and the contents of the bottom chambers were transferred to tubes, centrifuged, and both filters and cells were mixed with scintillation fluid (Ultima Gold; Canberra Packard Int., Zurich, Switzerland). Radioactivity was determined by liquid scintillation counting. All measurements were done in triplicate. Migration was expressed as the percentage of cells that had migrated to the bottom chamber. In some experiments the number of migrated cells was determined with a Coulter counter. For this, Transwell inserts for 6-well culture plates were used, and 2.6 ml medium and 1.5 ml cell suspension were added to the lower and upper compartments, respectively.

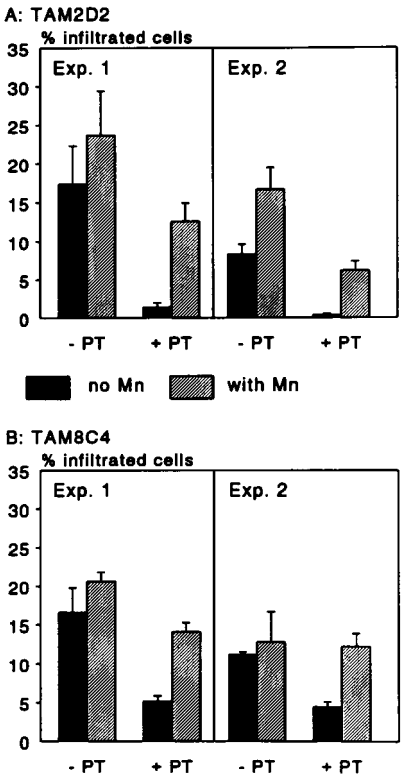

Fig. 1. Effects of pertussis toxin and Mn^{2+} on invasion of fibroblast monolayers. (A) TAM2D2; (B) TAM8C4 T-cell hybridoma. –PT, untreated cells (control); +PT, cells pretreated for 2 hours with 200 ng/ml pertussis toxin. no Mn, invasion in HBSS containing 1 mM Ca^{2+} and 1 mM Mg^{2+}; with Mn, invasion in HBSS containing 2 mM Mn^{2+}. Shown are the results of two separate experiments for each T-cell hybridoma, out of nine experiments with similar results. Invasion is given as percentage of added cells that have infiltrated after 1 hour. Standard deviations represent the variability between counted fields.

RESULTS

Effects of pertussis toxin and manganese on invasion

For the present study, we used clones of two independently obtained highly invasive T-cell hybridomas, TAM2D2 (Roos et al., 1985) and TAM8C4 (La Rivière et al., 1988). As previously reported (Roos and Van de Pavert, 1987; La Rivière et al., 1988, 1992a), pretreatment with 200 ng per ml pertussis toxin (PT) strongly inhibited invasion by these cells of rat embryo fibroblast monolayers. The level of control invasion varied between the nine experiments performed, so that the results could not easily be combined. Therefore, we show results of individual experiments (Fig. 1; more examples are presented in Figs 2, 6, 7). In most cases, invasion of TAM2D2 cells was virtually completely blocked, whereas invasion of PT-pretreated TAM8C4 cells was usually not less than 20% of control values.

To test whether the PT inhibition could be due to interference with LFA-1 activation, we added manganese. This activates integrins, including LFA-1 (Dransfield et al., 1992b), by an effect on the integrin protein. It should act distally to the PT-affected step, and therefore revert inhibition by PT. As shown in Figs 1 and 2, invasion was in fact stimulated by 2 mM Mn^{2+}. The most striking effect was on PT-pretreated TAM2D2 cells: invasion was strongly enhanced, up to 15-fold when PT had reduced invasion to less than 1% of added cells. In several experiments, the level of Mn^{2+}-stimulated invasion of PT-pretreated TAM2D2 cells was similar to that of control cells. Mn^{2+} enhanced invasion of PT-pretreated TAM8C4 cells two- to threefold, again close to control values. Mn^{2+} also stimulated invasion of untreated cells, but less strikingly: up to twofold for TAM2D2 cells, and not or only marginally for TAM8C4 cells. The effect of Mn^{2+} on invasion was dose-dependent (not shown), and had not yet leveled off at 2 mM. However, higher concentrations were deleterious to the fibroblasts and were therefore not used.

Involvement of LFA-1 in manganese-stimulated invasion

If the effect of Mn^{2+} on invasion was due to activation of LFA-1, Mn^{2+}-stimulated invasion should be blocked by anti-LFA-1 antibodies. Anti-LFA-1 mAb M17/4 inhibits invasion of T-cell hybridomas (Roossien et al., 1989). This was confirmed in the present set of experiments: the mAb inhibited invasion of TAM2D2 cells, and also of TAM8C4 cells, on average to 12% of control values. As shown in Fig. 2, anti-LFA-1 mAb M17/4

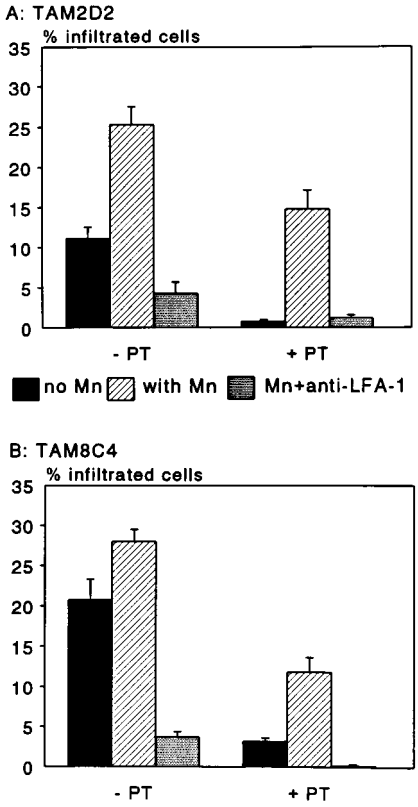

Fig. 2. Contribution of LFA-1 to Mn^{2+}-stimulated invasion. (A) TAM2D2; (B) TAM84 cells. –PT, untreated control cells; +PT, cells pretreated with 200 ng/ml PT for 2 hours. no Mn, invasion in HBSS containing 1 mM Ca^{2+} and 1 mM Mg^{2+}; with Mn, invasion in the presence of HBSS containing 2 mM Mn^{2+}. +anti-LFA-1, cells pretreated with a saturating concentration of anti-LFA-1 mAb (5 μg/ml) for 30 minutes at 20°C and then added to the fibroblast monolayer, in the continued presence of the mAb.

similarly inhibited the Mn^{2+}-stimulated invasion of both PT-treated and untreated cells. Fab fragments had a comparable effect (not shown). As a control, we used anti-VLA-6 mAb GoH3 (Sonnenberg et al., 1988), and found that it had no effect on invasion either with or without Mn^{2+}. The reduced invasion of PT-treated TAM8C4 cells was further inhibited by anti-LFA-1 mAb, suggesting that this PT-insensitive invasion was also mediated by LFA-1.

Manganese does not stimulate invasion of LFA-1-deficient mutants

Previously, we have generated three independent LFA-1-deficient mutants of the invasive TAM2D2 T-cell hybridoma: A1A4, 2B10 and 1C9. The first does not synthesize the β-chain, whereas the other two do not produce the α-chain of the LFA-1 heterodimer. The invasive capacity as well as the metastatic potential of these mutants was very low, compared with that of the parental cells (Roossien et al., 1989).

To assess further whether the effect of Mn^{2+} on invasion required LFA-1, we tested whether Mn^{2+} was able to induce invasion of the three LFA-1-deficient mutants; 2 mM Mn^{2+} did not stimulate invasion of these mutants, whereas invasion of the parental TAM2D2 cells was stimulated twofold (not shown). This provides further evidence that the effect of Mn^{2+} on invasion is not due to activation of LFA-1-independent adhesion mechanisms.

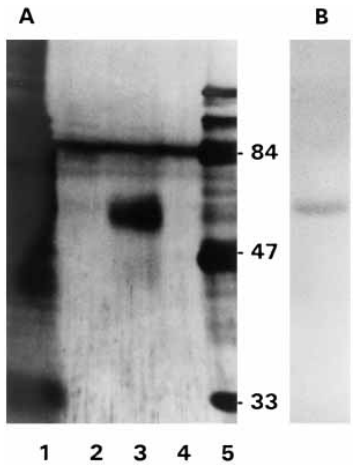

Fig. 3. Purification of sICAM-1. (A) Silver staining of SDS-PAGE gel. Lane 1, supernatant of 4A2 cells; lane 2, flow-through of Centricon 10 concentrator after concentration of eluate from immobilized anti-ICAM-1 mAb; lane 3, concentrated purified sICAM-1 (5 μg); lane 4, buffer only; lane 5, molecular mass markers (in kDa). Note aspecific bands that are also stained in the lane loaded with buffer only. (B) Western blot of the same gel, stained with YN1/1.7.4 anti-mouse ICAM-1 mAb.

Manganese induces adhesion to ICAM-1

To assess the activation state of LFA-1 on the two T-cell hybridomas, we measured adhesion to surfaces coated with ICAM-1. For this we used soluble murine ICAM-1 (sICAM-1), produced by a CHO cell line transfected with a cDNA encoding a truncated ICAM-1 protein. This contains most of the extracellular part but lacks the transmembrane and cytoplasmic domains (Kuhlman et al., 1991). Fig. 3 shows that virtually pure sICAM-1 was obtained from the supernatant of these cells by affinity chromatography on immobilized anti-mouse ICAM-1 mAb.

We determined the adhesion of cells to wells coated with purified sICAM-1, and then blocked with BSA. Fig. 4 shows the results, given as the percentage of specific adhesion over the background value of adhesion to BSA, which varied between 30 and 40% of added cells. The results were similar in multiple experiments. However, the magnitude of the effect of Mn^{2+} was somewhat variable, and therefore we show two representative experiments rather than combined data. Another example is given in Fig. 5. Quite strikingly, we consistently observed no, or very little, specific adhesion of TAM2D2 cells to surfaces coated with up to 100 ng/well sICAM-1. This clearly shows that LFA-1 on these cells is not constitutively active. However, in the presence of 2 mM Mn^{2+}, these cells did adhere, in a dose-dependent fashion. Maximal specific adhesion of approximately 40% of added cells was observed at 70-100 ng/well.

The results obtained with TAM8C4 cells were somewhat different. These cells did adhere spontaneously to sICAM-1, with maximal specific adhesion varying between 20 and 30% of added cells. However, Mn^{2+} further enhanced this adhesion approximately twofold. Thus, in contrast to TAM2D2 cells, part of LFA-1 on TAM8C4 cells is in an active state, but this

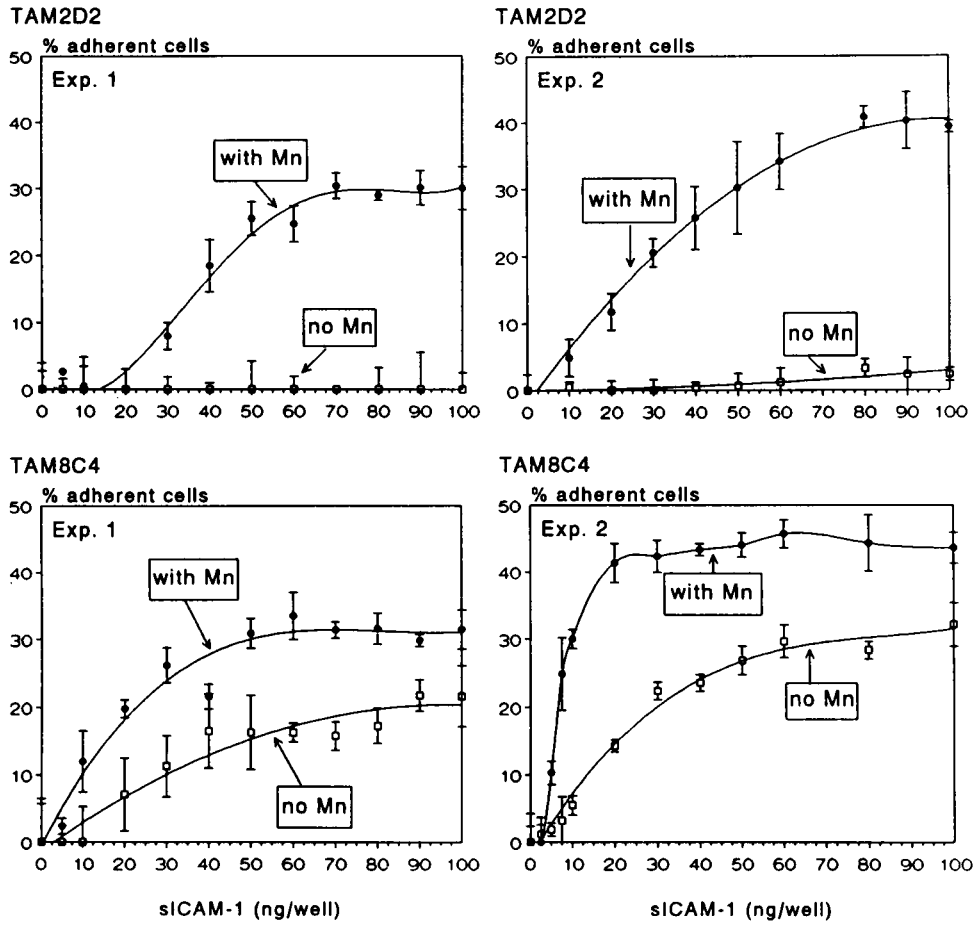

Fig. 4. Adhesion of T-cell hybridomas to ICAM-1. Wells in a 96-well plate were coated with the indicated amounts of sICAM-1. Given are the results of two separate experiments for both cell lines: TAM2D2 (upper panels) and TAM8C4 (lower panels). Adhesion is expressed as the percentage of added cells that adheres, after subtraction of the percentage of cells adhering to wells that were coated with BSA only. no Mn, adhesion in HBSS containing 1 mM Ca^{2+} and 1 mM Mg^{2+}; with Mn, adhesion in HBSS containing 2 mM Mn^{2+}.

is not enough for optimal adhesion to the LFA-1 counterstructure. The level of active LFA-1 is further increased by manganese.

Specific adhesion to sICAM-1 was fully blocked by anti-LFA-1 mAb (not shown). FACS analysis showed that Mn^{2+} had no effect on LFA-1 surface levels (not shown). If manganese acts distally to the PT-affected signal, PT should not affect adhesion to sICAM-1. Fig. 5 shows that this was in fact found: Mn^{2+} stimulated adhesion of PT-treated and control cells to sICAM-1 equally well.

Inhibition of invasion by pertussis toxin is not influenced by serum

The above results show that, at least on TAM2D2 cells, LFA-1 requires activation before it can participate in the invasion process, and are consistent with the hypothesis that an extracellular factor, which delivers a signal via a PT-sensitive G-protein, is responsible for this activation. A possible source of such a factor is serum. Invasion of these T-cell hybridomas is in fact somewhat reduced in serum-free medium. However, PT pretreatment inhibited invasion both in the presence and in the absence of serum. To exclude the possibility that a serum factor had remained bound to the cell surface, T-cell hybridoma cells were washed three times, cultured for 24 hours in serum-free medium (Hybridoma medium with 2% SF-1), and then used for invasion assays in the same medium. As shown in Fig. 6, PT inhibited invasion to a similar extent as it did in the presence of serum. Thus, the putative factor is not a serum component.

Pertussis toxin does not affect spontaneous migration

We have considered an alternative hypothesis, that the effect of PT on invasion was due to interference with motility. This would be in line with observations that metastatic tumor cells produce autocrine motility factors (AMF) to stimulate their own motility, and that the activity of an AMF is PT-sensitive (Evans et al., 1991; Liotta et al., 1986; Nabi et al., 1990; Silletti et al., 1991; Stracke et al., 1987; Watanabe et al., 1991). We tested the effect of PT on spontaneous migration of T-cell hybridoma cells through polycarbonate filters with 8 μm pores, since for melanoma cells such migration has been shown to be stimulated by an autocrine motility factor (Liotta et al., 1986).

Migration increased with time, and after 24 hours 30 to 35% of the cells had moved through the filter (not shown). [^{3}H]uridine-labeled cells were used to quantitate migration, but similar values were obtained when migrated cells were counted with a Coulter counter. For example, in a 6 hour assay 15.6±0.4% and 15.0±3.0% of added TAM8C4 cells had migrated to the lower compartment as determined using labeled cells and the Coulter counter, respectively. We never observed inhibition by PT. Fig. 7 shows an example of an experiment in which the effect of PT was tested on migration (6 hours) and on invasion (1 hour) of the same cells. Both

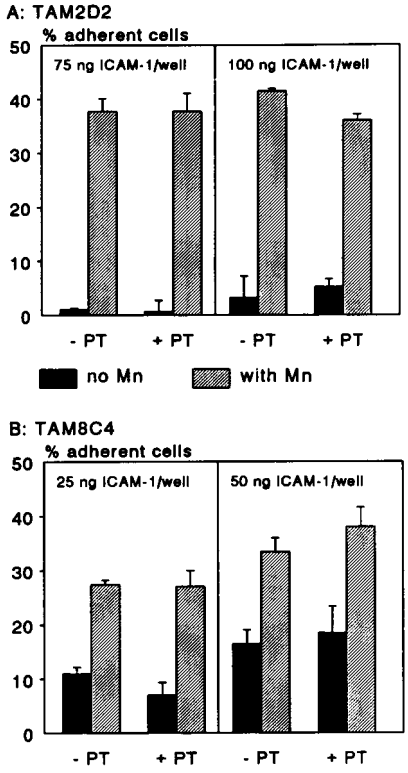

Fig. 5. Adhesion of PT-pretreated cells to ICAM-1. Shown is the percentage of adhering cells, after subtraction of background adhesion to BSA only. (A) TAM2D2; (B) TAM8C4. –PT, without pretreatment (control); +PT, after pretreatment for 2 hours with 200 ng/ml pertussis toxin. no Mn, adhesion in HBSS containing 1 mM Ca^{2+} and 1 mM Mg^{2+}; with Mn, adhesion in HBSS containing 2 mM Mn^{2+}.

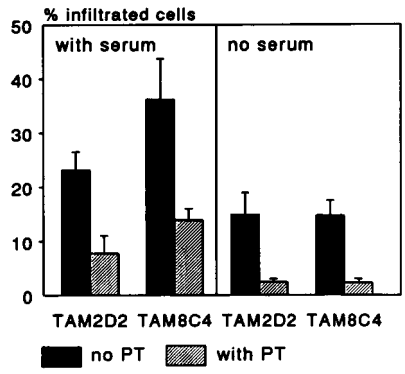

Fig. 6. The effect of PT on invasion in the presence or absence of serum. TAM2D2 and TAM8C4 cells were washed three times, cultured for 24 hours in Hybridoma medium containing 10% FCS (with serum) or 2% SF-1 serum supplement (no serum), and then used for invasion assays in the same medium. no PT, untreated cells (control); with PT, cells pretreated for 2 hours with 200 ng/ml pertussis toxin.

migration and invasion were measured by counting the radioactivity of [^{3}H]uridine-labeled cells. Whereas PT inhibited invasion as usual, it had no effect on migration of TAM8C4 cells and in fact consistently stimulated migration of

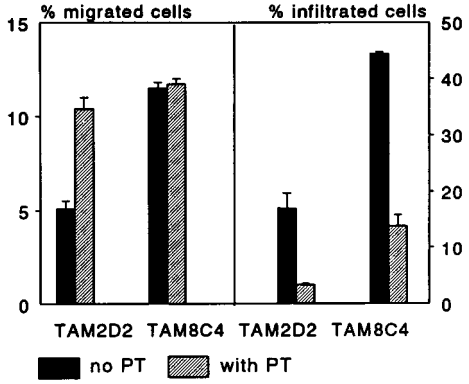

Fig. 7. Effect of PT on spontaneous migration through polycarbonate filters with 8 μm pores. Left panel: migration of TAM2D2 and TAM8C4 cells after 6 hours in medium (no PT) or in medium containing 200 ng PT per ml (with PT). Right panel: invasion of the same cells preincubated with or without PT for 5.5 hours, and then used for a 1 hour invasion assay. Migration is expressed as the percentage of migrated cells, and invasion as the percentage of infiltrated [^{3}H]uridine-labeled cells. Averages of two experiments, which are representative of six, are given.

TAM2D2 cells. Thus, the motility of these T-cell hybridomas is not dependent on a PT-sensitive AMF.

DISCUSSION

LFA-1 is essential for invasion and metastasis of the TAM2D2 T-cell hybridoma, as we demonstrated by antibody blocking and with LFA-1-deficient mutants (Roos and Roossien, 1987; Roossien et al., 1989; La Rivière et al., 1993). Yet, we show here that LFA-1 is not in an active state, since the cells did not bind to surfaces coated with the LFA-1 counterstructure ICAM-1, even at high densities. Hence, LFA-1 must be activated after addition of the cells to the fibroblast monolayer. Because substantial invasion can be seen within as little as five minutes (La Rivière et al., 1990), this activation must be a very rapid process. In this study, we have obtained evidence for the hypothesis that activation depends on a signal that can be blocked by pertussis toxin, indicating that PT affects invasion and metastasis by interference with the activation of LFA-1. Mn^{2+} induces the high avidity state of many integrins, including LFA-1 (Sonnenberg et al., 1988; Gailit and Ruoslahti, 1988; Conforti et al., 1990; Kirchhofer et al., 1990; Altieri, 1991; Elices et al., 1991; Dransfield et al., 1992b), and in fact we observed that Mn^{2+} induced adhesion of TAM2D2 cells to ICAM-1. Pertussis toxin blocked invasiveness of TAM2D2 cells virtually completely, but in the presence of Mn^{2+} this inhibition was reversed and a substantial number of PT-pretreated TAM2D2 cells did invade the fibroblast monolayers. Mn^{2+}-stimulated invasion was almost completely abolished by anti-LFA-1 antibodies, so that stimulation is mainly due to an effect on LFA-1-mediated adhesion and not on other independent adhesion pathways. This notion is further substantiated by the finding that Mn^{2+} did not stimulate invasion of three independent non-invasive LFA-1-deficient TAM2D2 mutants (Roossien et al., 1989).

The hypothesis that PT inhibits invasion by interference with LFA-1 activation is further supported by the somewhat

different results obtained with TAM8C4, another highly invasive T-cell hybridoma. These cells did adhere to some extent to ICAM-1, so that at least part of LFA-1 was in the active state. However, the levels of active LFA-1 were suboptimal, since adhesion to ICAM-1 was enhanced approximately twofold by Mn^{2+}. Whereas PT blocked invasion of TAM2D2 cells in many experiments virtually completely, PT inhibition was never complete for TAM8C4 cells. The remaining invasion of PT-treated TAM8C4 cells was increased two- to threefold by Mn^{2+}. This can be readily explained by our hypothesis: the level of constitutively active LFA-1 on TAM8C4 is sufficient for some PT-insensitive invasion. However, optimal LFA-1 activity requires further activation by a PT-sensitive signal, the effect of which can be mimicked to some extent by Mn^{2+}. Also the difference between the two cell lines in the effect of Mn^{2+} on invasion of untreated cells can thus be understood. For TAM8C4 cells, constitutive plus fibroblast-induced activation together provide for (almost) sufficient levels of active LFA-1 to be no longer rate-limiting, so that Mn^{2+} has no, or only a marginal, effect on invasion. In contrast, TAM2D2 cells are completely dependent on only the PT-sensitive activation induced by the fibroblasts, and the level of active LFA-1 thus obtained is still rate-limiting. Therefore, invasion can be stimulated further by Mn^{2+}.

Mn^{2+} or the PT-affected signal may also activate other integrins, which are possibly involved in invasion. However, of the potential candidates, Mac-1, VLA-4 and VLA-5 are not present on these T-cell hybridoma cells (Roos and Roossien, 1987; La Rivière et al., 1992b, and unpublished results). VLA-6 is expressed (La Rivière et al., 1992b) and can be activated by Mn^{2+}, since Mn^{2+} induced VLA-6-dependent adhesion and spreading on laminin (unpublished results). However, anti-VLA-6 antibodies (Sonnenberg et al., 1988) affected neither invasion of untreated cells nor invasion in the presence of Mn^{2+}, indicating that VLA-6 is not required for invasion of fibroblast monolayers (La Rivière et al., 1993). This is in line with our finding that these embryonal fibroblasts produce very little of the VLA-6 counterstructure laminin (La Rivière et al., unpublished results).

The reversion of PT inhibition was variable and usually not complete. One obvious reason might be that Mn^{2+} is less effective than a physiological activation signal. Furthermore, the concentration used (2 mM) may be suboptimal, but we could not test higher concentrations because of their adverse effect on the fibroblasts. An alternative explanation is that the PT-affected signal may also stimulate other components of the invasion machinery that are not susceptible to Mn^{2+}. However, the substantial invasion seen in the presence of Mn^{2+} shows that the activation of LFA-1 is essential, whereas stimulation of these other components is not.

The putative invasion-enhancing factor may be derived from three sources: serum, the hybridoma cells or the fibroblasts. PT inhibited invasion by serum-free cultured cells in serum-free medium, showing that a serum component is not, or not the only factor involved. We have also considered the possibility that T-cell hybridoma cells produce a substance similar to the autocrine motility factors (AMF) secreted by certain types of metastatic tumor cells (Liotta et al., 1986; Evans et al., 1991; Silletti et al., 1991; Watanabe et al., 1991). The activity of a 55 kDa AMF produced by melanomas and fibrosarcomas that binds to a 78 kDa cell surface receptor is inhibited by PT (Stracke et al., 1987; Nabi et al., 1990). Since this AMF stimulates spontaneous migration through Nucleopore filters (Liotta et al., 1986), we have tested whether PT affects such spontaneous migration by T-cell hybridomas, and observed no inhibition. Therefore, the effect of PT on invasion is not due to interference by an AMF-like factor. The adhesion-enhancing factor is unlikely to be produced by the hybridoma cells themselves, at least not by TAM2D2 cells, since this should cause the constitutive activation of LFA-1. This leaves the fibroblasts as the only possible source. Since PT also inhibits invasion into hepatocyte cultures (Roos and Van de Pavert, 1987), hepatocytes must also produce this factor, or a different substance that has a similar effect.

The proposed mechanism is not without precedent. Lorant et al. (1991) showed that platelet activating factor (PAF), which is produced by activated endothelium and remains associated with the membrane, is an adhesion-enhancing factor. Polymorphonuclear leukocytes (PMN) interact with PAF, resulting in a signal that causes activation of the β_2-integrins LFA-1 and Mac-1. This induces adhesion to their counterstructures on the endothelium (Lorant et al., 1991). PAF is a small phospholipid that binds to a G-protein-coupled receptor, and PAF-induced GTPase activity in PMN membranes is inhibited by PT (Prescott et al., 1990). PMN adhesion can be enhanced by several other agents as well, including interleukin-8, *N*-formylMet-Leu-Phe (fMLP) and C5a, all known to bind to receptors with seven transmembrane domains, characteristic of G-protein-coupled receptors (Thomas et al., 1990; Gerard and Gerard, 1991; Holmes et al., 1991; Honda et al., 1991). As reviewed by Butcher (1991), this is likely to be a quite general mechanism for regulating adhesiveness of blood cells. Recently, Tanaka et al. (1993) showed that MIP-1β is able to enhance binding of the β_1 integrin VLA-4 on T-cells to its counterstructure VCAM-1, but only when MIP-1β was bound to a proteoglycan, coated with VCAM-1 on the same surface. This implies that, like PAF, adhesion-enhancing peptides act locally because they are immobilized on the cell surface. Similarly, the putative fibroblast factor could be associated with the fibroblast surface, as suggested by our observation that invasion was not inhibited by thorough washing of the fibroblasts, which should have removed all secreted factors.

The above-mentioned adhesion-enhancing factors are all produced in inflamed tissues. However, the putative substance that activates LFA-1 on metastasizing T-cell hybridomas should be produced in normal, non-inflamed tissues, in which metastases are formed. Memory T-cells move from blood to lymph through various tissues rather than through lymph nodes (Mackay et al., 1990). Previously we have provided evidence showing that metastasizing T-cell hybridomas use similar pathways (La Rivière et al., 1993). We propose that constituents of the non-inflamed tissues, through which the memory T-cells migrate, induce invasion of these cells by activating LFA-1. The involved factors are probably different from the ones produced in inflamed tissues. These same factors may allow T-cell hybridomas and other malignant lymphomas to migrate from the blood into the same tissues, thus allowing for the formation of metastases.

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REFERENCES

Altieri, D. C. (1991). Occupancy of CD11b/CD18 (Mac-1) divalent ion binding site(s) induces leukocyte adhesion. *J. Immunol.* **147**, 1891-1898.

Butcher, E. C (1991). Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell* **67**, 1033-1036.

Chaffin, K. E., Beals, C. R., Wilkie, T. M., Forbush, K. A., Simon, M. I. and Perlmutter, R. M. (1990). Dissection of thymocyte signaling pathways by in vivo expression of pertussis toxin ADP-ribosyltransferase. *EMBO J.* **9**, 3821-3829.

Chaffin, K. E. and Perlmutter, R. M. (1991). A pertussis toxin-sensitive process controls thymocyte emigration. *Eur. J. Immunol.* **21**, 2565-2573.

Collard, J. G., Schijven, J. F. and Roos, E. (1987a). Invasive and metastatic potential induced by ras-transfection into mouse BW5147 T-lymphoma cells. *Cancer Res.* **47**, 754-759.

Collard, J. G., Van de Poll, M., Scheffer, A., Roos, E., Hopman, A. H. M., Geurts van Kessel, A. H. M. and Van Dongen, J. J. M. (1987b). Location of genes involved in invasion and metastasis on human chromosome 7. *Cancer Res.* **47**, 6666-6670.

Conforti, G., Zanetti, A., Pasquali-Ronchetti, I., Quaglino, D., Neyroz, P. and Dejana, E. (1990). Modulation of vitronectin receptor binding by membrane lipid composition. *J. Biol. Chem.* **265**, 4011-4019.

De Baetselier, P., Roos, E., Van Hecke, D., Verschaeve, L., Brys, L. and Verschueren, H. (1988). Syngeneic in vivo passage of the murine BW5147 lymphoma results in the expression of a stable metastatic phenotype. *Int. J. Cancer* **41**, 720-726.

Dransfield, I., Cabañas, C., Barrett, J. and Hogg, N. (1992a). Interaction of leukocyte integrins with ligand is necessary but not sufficient for function. *J. Cell Biol.* **116**, 1527-1535.

Dransfield, I., Cabañas, C., Craig, A. and Hogg, N. (1992b). Divalent cation regulation of the function of the leukocyte integrin LFA-1. *J. Cell Biol.* **116**, 219-226.

Dustin, M. L., Rothlein, R., Bhan, A. K., Dinarello, C. A. and Springer, T. A. (1986). Induction by IL 1 and interferon-gamma: Tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). *J. Immunol.* **137**, 245-254.

Dustin, M. L. and Springer, T. A. (1988). Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. *J. Cell Biol.* **107**, 321-331.

Dustin, M. L. and Springer, T. A. (1989). T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature* **341**, 619-624.

Elices, M. J., Urry, L. A. and Hemler, M. E. (1991). Receptor functions for the integrin VLA-3: Fibronectin, collagen, and laminin binding are differentially influenced by ARG-GLY-ASP peptide and by divalent cations. *J. Cell Biol.* **112**, 169-181.

Evans, C. P., Walsh, D. S. and Kohn, E. C. (1991). An autocrine motility factor secreted by the dunning R-3327 rat prostatic adenocarcinoma cell subtype AT2. 1. *Int. J. Cancer* **49**, 109-113.

Gailit, J. and Ruoslahti, E. (1988). Regulation of the fibronectin receptor affinity by divalent cations. *J. Biol. Chem.* **263**, 12927-12932.

Gerard, N. P. and Gerard, C. (1991). The chemotactic receptor for human C5a anaphylatoxin. *Nature* **349**, 614-617.

Habets, G. G. M., Van der Kammen, R. A., Scholtes, E. H. M. and Collard, J. G. (1990). Induction of invasive and metastatic potential in mouse T-lymphoma cells (BW5147) by treatment with 5-azacytidine. *Clin. Exp. Metast.* **8**, 567-577.

Holmes, W. E., Lee, J., Kuang, W., Rice, G. C. and Wood, W. I. (1991). Structure and functional expression of a human interleukin-8 receptor. *Science* **253**, 1278-1280.

Honda, Z., Nakamura, M., Miki, I., Minami, M., Watanabe, T., Seyama, Y., Okado, H., Toh, H., Ito, K., Miyamoto, T. and Shimizu, T. (1991). Cloning by functional expression of platelet-activating factor receptor from guinea-pig lung. *Nature* **349**, 342-346.

Horley, K., Carpenito, C., Baker, B. and Takei, F. (1989). Molecular cloning of murine intercellular adhesion molecule (ICAM-1). *EMBO J.* **8**, 2889-2896.

Kirchhofer, D., Gailit, J., Ruoslahti, E., Grzesiak, J. and Pierschbacher, M. D. (1990). Cation-dependent changes in the binding specificity of the platelet receptor GPIIb/IIIa. *J. Biol. Chem.* **265**, 18525-18530.

Kuhlman, P., Moy, V. T., Lollo, B. A. and Brian, A. A. (1991). The accessory function of murine intercellular adhesion molecule-1 in T lymphocyte activation. *J. Immunol.* **146**, 1773-1782.

La Rivière, G., Schipper, C. A., Collard, J. G. and Roos, E. (1988). Invasiveness in hepatocyte and fibroblast monolayers and metastatic potential of T-cell hybridomas in mice. *Cancer Res.* **48**, 3405-3410.

La Rivière, G., Roossien, F. F. and Roos, E. (1990). In *Genetic Mechanisms in Carcinogenesis and Tumor Progression.* UCLA Symposia on Molecular and Cellular Biology, New series, vol. 114 (ed. C. C. Harris and L. A. Liotta), pp. 189-202. New York: Alan R. Liss.

La Rivière, G., Klein Gebbinck, J. W. T. M., Schipper, C. A. and Roos, E. (1992a). Tumour necrosis factor-a stimulates invasiveness of T-cell hybridomas and cytotoxic T-cell clones by a pertussis toxin-insensitive mechanism. *Immunology* **75**, 269-274.

La Rivière, G., Schipper, C. A., Klein Gebbinck, J. W. T. M., Koch, G., Kuhn, L., Lefkovits, I. and Roos, E. (1992b). Putative invasion-specific proteins in mouse T-cell hybridomas that differ in invasive and metastatic potential. *Int. J. Cancer* **51**, 745-753.

La Rivière, G., Klein Gebbinck, J. W. T. M., Schipper, C. A., Mooi, W. J. and Roos, E. (1993). In vitro invasiveness of CTL clones and in vivo dissemination of CTL hybridomas. *J. Leuk. Biol.* **53**, 381-389.

Landis, R. C., Bennett, R. I. and Hogg, N. (1993). A novel LFA-1 activation epitope maps to the I domain. *J. Cell Biol.* **120**, 1519-1527.

Liotta, L. A., Mandler, R., Murano, G., Katz, D. A., Gordon, R. K., Chiang, P. K. and Schiffmann, E. (1986). Tumor cell autocrine motility factor. *Proc. Nat. Acad. Sci. USA* **83**, 3302-3306.

Lorant, D. E., Patel, K. D., McIntyre, T. M., McEver, R. P., Prescott, S. M. and Zimmerman, G. A. (1991). Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: A juxtacrine system for adhesion and activation of neutrophils. *J. Cell Biol.* **115**, 223-234.

Mackay, C. R., Marston, W. L. and Dudler, L. (1990). Naive and memory T cells show distinct pathways of lymphocyte recirculation. *J. Exp. Med.* **171**, 801-817.

Nabi, I. R., Watanabe, H. and Raz, A. (1990). Identification of B16-F1 melanoma autocrine motility-like factor receptor. *Cancer Res.* **50**, 409-414.

Prescott, S. M., Zimmerman, G. A. and McIntyre, T. M. (1990). Platelet-activating factor. *J. Biol. Chem.* **265**, 17381-17384.

Roos, E. and Van de Pavert, I. V. (1987). Inhibition of lymphoma invasion and liver metastasis formation by pertussis toxin. *Cancer Res.* **47**, 5439-5444.

Roos, E., Van de Pavert, I. V. and Middelkoop, O. P. (1981). Infiltration of tumour cells into cultures of isolated hepatocytes. *J. Cell Sci.* **47**, 385-397.

Roos, E. and Van de Pavert, I. V. (1983). Antigen-activated T lymphocytes infiltrate hepatocyte cultures in a manner comparable to liver-colonizing lymphosarcoma cells. *Clin. Exp. Metast.* **2**, 173-180.

Roos, E., La Rivière, G., Collard, J. G., Stukart, M. J. and De Baetselier, P. (1985). Invasiveness of T-cell hybridomas in vitro and their metastatic potential in vivo. *Cancer Res.* **45**, 6238-6243.

Roos, E. and Roossien, F. F. (1987). Involvement of leukocyte function-associated antigen-1 (LFA-1) in the invasion of hepatocyte cultures by lymphoma and T-cell hybridoma cells. *J. Cell Biol.* **105**, 553-559.

Roossien, F. F., De Rijk, D., Bikker, A. and Roos, E. (1989). Involvement of LFA-1 in lymphoma invasion and metastasis demonstrated with LFA-1-deficient mutants. *J. Cell Biol.* **108**, 1979-1985.

Silletti, S., Watanabe, H., Hogan, V., Nabi, I. R. and Raz, A. (1991). Purification of B16-F1 melanoma autocrine motility factor and its receptor. *Cancer Res.* **51**, 3507-3511.

Simon, M. I., Strathmann, M. P. and Gautam, N. (1991). Diversity of G proteins in signal transduction. *Science* **252**, 802-808.

Sonnenberg, A., Modderman, P. W. and Hogervorst, F. (1988). Laminin receptor on platelets is the integrin VLA-6. *Nature* **336**, 487-489.

Spangrude, G. J., Braaten, B. A. and Daynes, R. A. (1984). Molecular mechanisms of lymphocyte extravasation. *J. Immunol.* **132**, 354-362.

Spangrude, G. J., Araneo, B. A. and Daynes, R. A. (1985). Site-selective homing of antigen-primed lymphocyte populations can play a crucial role in the efferent limb of cell-mediated immune responses in vivo. *J. Immunol.* **134**, 2900-2907.

Springer, T. A. (1990). Adhesion receptors of the immune system. *Nature* **346**, 425-434.

Stracke, M. L., Guirguis, R., Liotta, L. A. and Schiffmann, E. (1987). Pertussis toxin inhibits stimulated motility independently of the adenylate cyclase pathway in human melanoma cells. *Biochem. Biophys. Res. Commun.* **146**, 339-345.

Sugimoto, M., Nakanishi, Y., Otokawa, M., Uchida, N., Yasuda, T., Sato, H. and Sato, Y. (1983). Effect of Bordetella pertussis leukocytosis (lymphocytosis)-promoting factor (LPF) on the physical lymphoepithelial-cell association studied with the use of an in vitro model of mouse thymus. *J. Immunol.* **130**, 2767-2774.

Tanaka, Y., Adams, D. H., Hubscher, S., Hirano, H., Siebenlist, U. and Shaw, S. (1993). T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-1β. *Nature* **361**, 79-82.

Thomas, K. M., Pyun, H. Y. and Navarro, J. (1990). Molecular cloning of the fMet-Leu-Phe receptor from neutrophils. *J. Biol. Chem.* **265**, 20061-20064.

Van Kooyk, Y., Van de Wiel-van Kemenade, P., Weder, P., Kuijpers, T. W. and Figdor, C. G. (1989). Enhancement of LFA-1-mediated cell adhesion by triggering through CD2 or CD3 on T lymphocytes. *Nature* **342**, 811-813.

Van Kooyk, Y., Weder, P., Hogervorst, F., Verhoeven, A. J., Van Seventer, G., Te Velde, A. A., Borst, J., Keizer, G. D. and Figdor, C. G. (1991). Activation of LFA-1 through a Ca2+-dependent epitope stimulates lymphocyte adhesion. *J. Cell Biol.* **112**, 345-354.

Verschueren, H., Dewit, J., Van der Wegen, A., De Braekeleer, J., Van Heule, G., Dekegel, D. and De Baetselier, P. (1991). The lymphocytosis promoting action of pertussis toxin can be mimicked in vitro. Holotoxin but not the B subunit inhibits invasion of human T lymphoma cells through fibroblast monolayers. *J. Immunol. Methods* **144**, 231-240.

Watanabe, H., Carmi, P., Hogan, V., Raz, T., Silletti, S., Nabi, I. R. and Raz, A. (1991). Purification of human tumor cell autocrine motility factor and molecular cloning of its receptor. *J. Biol. Chem.* **266**, 13442-13448.

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