Involvement of LFA-1 in Lymphoma Invasion and Metastasis Demonstrated with LFA-1-deficient Mutants

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Abstract. Lymphocyte function-associated antigen-1 (LFA-1) is a leukocyte and lymphoma cell surface protein that promotes intercellular adhesion. We have previously shown that the invasion of hepatocyte cultures by lymphoma cells is inhibited by anti-LFA-1 antibodies (Roos, E., and F. F. Roossien. 1987. J. Cell Biol. 105:553–559). In addition, we now report that LFA-1 is also involved in invasion of lymphoma cells into fibroblast monolayers.

To investigate the role of LFA-1 in metastasis of these lymphoma cells, we have generated mutants that are deficient in LFA-1 cell surface expression because of impaired synthesis of either the α or β subunit precursor of LFA-1. We identified at least three distinct mutant clones. The invasive potential of the mutant cells in vitro, in both hepatocyte and fibroblast cultures, was considerably lower than that of parental cells.

The metastatic potential of the mutants was much reduced, indicating that LFA-1 expression is required for efficient metastasis formation by certain lymphoma cells.

When metastatic tumor cells invade tissues from the blood they have to adhere to both extracellular matrix and cells in those tissues. Since extracellular matrix is scarce in the liver, adhesion to cells is probably the most important. Certain lymphomas readily metastasize to the liver and invade extensively between hepatocytes. Such lymphoma cells are highly invasive in cultures of isolated hepatocytes (Roos et al., 1981). We have recently shown this invasion to be inhibited by an antibody against LFA-1, apparently because of its interference with adhesion to the upper surface of the hepatocytes (Roos and Roossien, 1987), suggesting that LFA-1 is important for lymphoma metastasis.

LFA-1 is expressed by leukocytes but not by nonhematopoietic cells (Kürzinger et al., 1981). It was discovered with a monoclonal antibody that inhibited killing by cytotoxic T cells and natural killer cells by interfering with adhesion to the target cell, a step that precedes and is necessary for lethal hit delivery (for review see Martz, 1987). LFA-1 is now known to be involved in many adhesion-related phenomena associated with immune response (Springer et al., 1987). In addition to interleukocyte adhesion, LFA-1 partially mediates binding of activated tumoricidal macrophages to tumor cells (Strassman et al., 1986), and of lymphocytes to endothelial cells (Mentzer et al., 1986), fibroblasts (Dustin et al., 1986), and high endothelium of lymph node postcapillary venules (Pals et al., 1988).

LFA-1 belongs to a family of three leukocyte adhesion proteins, including Mac-1 and p150,95. These are heterodimeric glycoproteins consisting of a unique α subunit (180, 170, and 150 kD, respectively) noncovalently associated with a common 95-kD β subunit. Both α and β subunits of this family show considerable homology with those of the integrins—proteins that mediate adhesion to extracellular matrix components (Hynes, 1987; Kishimoto et al., 1987a; Law et al., 1987). The importance of the LFA-1/Mac-1/p150,95 family of proteins is demonstrated in patients suffering from leukocyte adhesion deficiency, a life-threatening genetic disease (for review see Anderson and Springer, 1987). The leukocytes of these patients are defective in several adhesion-related functions as a result of a deficiency in surface expression of LFA-1, Mac-1, and p150,95. This deficiency originates primarily from defects in the common β-chain (Kishimoto et al., 1987b).

The role of LFA-1 in metastasis might be tested by in vivo administration of anti-LFA-1 antibody. However, such treatment would not only block adhesion of the lymphoma cells but also multiple LFA-1-dependent processes important for both specific and nonspecific host resistance to the tumor cells. In particular, macrophage and natural killer function would be affected. To circumvent this problem, we have generated a set of LFA-1-deficient lymphoma mutant cell lines, and now show that these cells exhibit reduced invasive potential in vitro, as well as attenuated metastatic capacity in vivo.

Materials and Methods

Cells and Culture Conditions

Generation and properties of mouse T cell hybridomas were reported elsewhere (Roos et al., 1985). Hybridomas were cultured in RPMI 1640 medium supplemented with 10 mM Hepes, 10% NCTC 135 (Flow Laboratories, Ayeshire, UK), 35 mM NaHCO3, 2 mM glutamine, 0.5 mM 2-mer-
Biosynthetic Labeling

Cells (5 × 10⁶) were pulse-labeled for 60 min at 37°C with 50 μCi of [35S]methionine in methionine-free MEM (modified) with Earle's salts (EMEM; Flow Laboratories) supplemented with 10% FCS (dialyzed against PBS). Half of the cells were harvested and stored as a pellet at −70°C. The remaining cells were diluted with 2 vol of EMEM containing 80 μg/ml methionine, chased for 15 h, and stored as a frozen pellet.

Immunoprecipitations

Labeled cells (10⁶) were lysed in 0.25 ml lysis buffer. After 15 min on ice, cell debris was removed by a 10 min centrifugation in a minifuge. Lysates were precleared by subsequent incubations with 1 μl normal rabbit serum (overnight) and 2 μl packed beads of protein A-Sepharose (Pharmacia Fine Chemicals; 30 min). After a 2 h incubation with 2 μl rabbit anti-LFA-1 antibody, the immune complexes were precipitated with 4 μl Protein A-Sepharose (30 min). The precipitates were washed five times with wash buffer, after which the immune complexes were eluted with 10% Triton X-100 in 0.1 M glycine-HCl, pH 2.5, neutralized with 1 M Tris, 1:1 diluted with twice-concentrated solubilization buffer (Laemmli, 1970), denatured for 5 min at 100°C, and subjected to SDS-PAGE on 7.5% polyacrylamide gels.

Invasion Assay in Hepatocyte Cultures

Adhesion and invasion were quantitated by light microscopy, as described previously (Roos and Van de Pavert, 1982). 5 × 10⁵ tumor cells in 500 μl MEM were added per hepatocyte culture in a 16-mm well. When anti-LFA-1 antibodies were used (final concentration: 1 μg/ml), they were preincubated with the tumor cells for 30 min at 37°C and then present during incubation with hepatocytes. The cultures were incubated for 4 h at 37°C. Washed once with PBS, fixed with glutaraldehyde, dehydrated, and scraped from the dish. The culture fragments were pelleted and embedded in Epon. 1-μm-thick sections were stained and observed with a light microscope. Hepatocyte nuclei and infiltrated tumor cells were counted. As a measure of invasion, the infiltration index was calculated (infiltrated tumor cells/hepatocyte nucleus). Measurements were performed at least three times.

Invasion Assay in Fibroblast Cultures

Invasiveness in fibroblast cultures was quantitated essentially according to the method of Verschueren et al. (1987) and Collard et al. (1987). Tumor cells were washed with PBS, suspended in 25 ml of MEM supplemented with 10 mM Hepes, and incubated, with or without anti-LFA-1 monoclonal antibodies (final concentration: 1 μg/ml), for 30 min at 37°C. The cell suspension was then added to confluent rat embryo fibroblast monolayers in 10-cm² wells and incubated for 4 h at 37°C and 5% CO². Subsequently, noninfiltrated tumor cells were removed by repeated washing, and the monolayers were fixed with 2% paraformaldehyde in PBS. Infiltrated cells were easily recognized in the phase-contrast microscope as dark and flattened cells. The number of infiltrated cells was counted in 10 different 0.27-mm² areas, distributed at random over the monolayers. From this, the number of infiltrated cells per well was calculated, and invasiveness was expressed as the percentage of added cells.

DNA Analysis

Approximately 10⁷ cells were fixed with 70% ethanol, treated with 0.1% RNase and 0.1% pepsin (Collard and Smets, 1974), stained with a mixture of ethidium bromide (10 μg/ml) and Hoechst 33258 (4 μg/ml), and analyzed in a Phrye ICP-11 flow cytometer. Trout red blood cells were included as an internal standard. DNA contents were expressed as a percentage of that of BW5147 cells.

Immunofluorescence Analysis

Cells were washed and resuspended in 50 μl PBS containing saturating concentrations of monoclonal antibody. After a 30-min incubation on ice, cells were washed twice with 0.5% BSA in PBS and resuspended in the same buffer containing optimal concentrations of FITC-labeled rabbit anti-rat immunoglobulin (Nordic Immunological Laboratories, Tilburg, The Netherlands). After 30 min on ice, cells were washed twice with 0.5% BSA in PBS and resuspended in 0.5 ml of this buffer. Fluorescence was analyzed with a FACS IV flow sorter (Becton-Dickinson & Co., Sunnyvale, CA).

Metastatic Potential and Tumorigenicity

Metastatic potential was assessed by injection of 10⁵ cells into the tail vein of 6-10-wk-old syngeneic AKR mice. Animals were killed when moribund or after 10 d and examined for the presence of metastases. Tumorigenicity
Hybridoma Cells and LFA-1-deficient Mutants

Table I. Cell Surface Antigens of TAM2D2 T Cell Hybridoma Cells and LFA-1-deficient Mutants

| Cell line | Monoclonal antibody | Anti-LFA-1 | Anti-T200 | Anti-Thy.1 |
|-----------|---------------------|------------|-----------|------------|
| TAM2D2    |                     | 103.4 (2.0)| 313.2     | 698.2      |
| 2B10      |                     | 3.2 (2.2)  | 283.9     | 202.8      |
| 1C9       |                     | 3.0 (2.0)  | 284.4     | 2.2        |
| 3D1       |                     | 2.7 (1.9)  | 177.1     | 1.9        |
| 1A4       |                     | 2.3 (2.0)  | 146.4     | 359.4      |
| 3C12      |                     | 2.4 (2.1)  | 127.8     | 372.6      |

Cells were stained with monoclonal antibodies specific for different cell surface antigens, as described. Fluorescein-coupled rabbit anti-rat immunoglobulin was used as second reagent. Data are expressed in arbitrary units and represent the mean fluorescence detected in the presence of the corresponding monoclonal antibody in a representative experiment. Values in parentheses were obtained with negative control samples by omitting the incubation with monoclonal antibody. The monoclonal antibodies M17/4, M1/9.3, and M5/49 were directed against the mouse antigens LFA-1 (α-chain), T-200, and Thy.1, respectively.

was tested by subcutaneous injection of 10⁶ cells in the neck region of AKR mice.

Results

Generation of LFA-1-deficient Mutants

Mutation of TAM2D2 T cell hybridoma cells was accomplished by treatment of the cells with the alkylating reagent ethyl methanesulfonate (0.4 mg/ml, 24 h). Initial cell death upon this treatment amounted to 70-90%. Viable cells were expanded and LFA-1-deficient variants were selected on culture plates coated with anti-LFA-1 monoclonal antibody (panning). Nonadhered cells were collected, expanded, and analyzed by immunofluorescence for LFA-1 surface levels; the panning procedure was then repeated. After 6-10 rounds of selection, at least 50% of the cells exhibited substantially reduced LFA-1 levels. After cloning by limiting dilution, we obtained five cell lines on which virtually no LFA-1 was present: 1C9, 2B10, 3D1, 1A4, and 3C12 (Table I). The mutants were screened for T200 and Thy.1 levels; T200 and Thy.1 are two surface antigens which are abundant on parental TAM2D2 cells. Table I shows that the introduced mutations did not result in major changes in the T200 levels. All five mutants exhibited high T200 levels, although somewhat lower than TAM2D2 cells, but 1C9 and 3D1 cells were Thy.1 deficient. The growth rates and DNA contents of all mutants were essentially equal to those of TAM2D2 cells (Table II). As will be discussed later, the mutants were derived from at least three distinct mutant clones (Table III): type I (2B10), type II (1C9 and 3D1), and type III (1A4 and 3C12).

Impaired Synthesis of Either α or β Subunit Precursor in Different Mutants

LFA-1 α and β chain synthesis was assayed in pulse-chase experiments using [35S]methionine. For immunoprecipitations a rabbit antiserum was raised against affinity-purified LFA-1. The antiserum immunoprecipitated the α/β complex from NP-40 lysates of 125I-labeled TAM2D2 cells and the individual α and β subunits from SDS-denatured cell lysates, showing that the antiserum reacted with both subunits (not shown). TAM2D2 parental or mutant cells were pulse-labeled with [35S]methionine for 1 h and then chased with unlabeled methionine for 15 h. The α and β chains were immunoprecipitated from NP-40 cell lysates with the rabbit antibody against the LFA-1 α chain immunoprecipitated exclusively the α chain precursor from pulse-labeled cells, whereas the α/β complex was precipitated from chased cells (not shown). In agreement with observations by others (Ho and Springer, 1983; Springer et al., 1984), this demonstrates that the association of the LFA-1 α and β chains occurs upon processing of the precursor forms.

Pulse–chase studies with the obtained set of mutants revealed two different types of mutations leading to LFA-1 deficiency. Mutant cells 1C9, 2B10, and 3D1 did not synthesize detectable amounts of α chain precursor (Fig. 1 A, lanes 3, 5, and 9, respectively). Although the β chain precursor was produced, the absence of the α chain precursor prevented its processing to the higher molecular mass mature form, and during the 15-h chase it was degraded (Fig. 1 A, lanes 4, 6, and 8). An opposite type of mutation was found in mutants 1A4 (Fig. 1 B) and 3C12 (not shown); these mutants did not synthesize the β chain precursor although they did synthesize the α chain precursor (Fig. 1 B, lane 3) which degraded during chase (Fig. 1 B, lane 4).

Table II. Growth Rates and DNA Contents of TAM2D2 T Cell Hybridoma Cells and LFA-1-deficient Mutant Cells

| Cell line | Doubling time | DNA content* |
|-----------|---------------|--------------|
| TAM2D2    | 11.7 ± 0.6    | 182          |
| 2B10      | 10.9 ± 2.6    | 186          |
| 1C9       | ND            | 189          |
| 1A4       | 10.1 ± 0.7    | 187          |
| 3C12      | 10.7 ± 1.2    | 192          |

The doubling times were calculated from the growth rates of exponentially growing cells. The DNA contents of cells were measured as described. Shown are the data of a representative experiment.

* DNA contents are expressed as a percentage of that of BW5417 cells.

Table III. Classification of TAM2D2 Mutant Cell Lines

| Type of mutation | Cell line | Thy.1 surface level | α Subunit | β Subunit |
|------------------|-----------|---------------------|-----------|-----------|
| Type I           | 2B10      | high                | absent    | normal    |
| Type II          | 1C9, 3D1  | absent              | absent    | normal    |
| Type III         | 1A4, 3C12 | high                | absent    | absent    |

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Classification of LFA-1-deficient Mutants

Fluorescence analysis (Table I) and pulse-chase studies (Fig. 1) enabled us to divide the obtained set of mutants into three distinct types (Table III). Type I and Type II mutants synthesize the LFA-1 β chain precursor, but do not produce the α chain precursor. Type I mutants have a high Thy.1 expression; type II mutants are Thy.1 deficient. Type III mutants produce the LFA-1 α chain precursor, but do not synthesize the β chain precursor.

Invasiveness of the Mutants in Hepatocyte Cultures

The invasive potential of parental and mutant cells was assayed in vitro in hepatocyte monolayers, as described before (Roos and Van de Pavert, 1982). $5 \times 10^6$ tumor cells were added to hepatocyte monolayers which had been cultured for 24 h in 16-mm wells. After a 4 h incubation at 37°C, invasion was terminated by glutaraldehyde fixation. The monolayers were dehydrated, scraped off the culture dishes, and embedded in Epon. Sections were cut and stained, and the number of tumor cells infiltrated in the monolayers was counted. Results were expressed as infiltration index, a measure for invasion (for further details, see Materials and Methods; Roos and Van de Pavert, 1982). TAM2D2 cell infiltration of hepatocyte monolayers was inhibited by anti-LFA-1 monoclonal antibody to ~30% of controls (Table IV; Roos and Roossien, 1987). The invasive potential of each of the three classes of LFA-1-deficient mutants was considerably lower than that of parental cells (Table IV) and comparable to that of parental cells in the presence of an excess of anti-LFA-1 antibody. As expected, this limited invasiveness of the mutants was not further inhibited by the addition of anti-LFA-1 monoclonal antibody.

| Cell line          | Anti-LFA-1 monoclonal antibody |
|--------------------|--------------------------------|
|                   | -                              |
| TAM2D2             | 0.79                           |
| 2B10 (type I)*     | 0.26                           |
| 1C9 (type II)      | 0.36                           |
| 1A4 (type III)     | 0.19                           |
| 3C12 (type III)    | 0.20                           |

TAM2D2 and mutant cell lines, preincubated with M7/4 anti-LFA-1 monoclonal antibody (1 μg/ml) where indicated, were incubated for 4 h with hepatocyte monolayers. When cells were pretreated with anti-LFA-1 antibody, the antibody remained present during the incubation with hepatocytes. The infiltration index was determined by counting cells in sections of embedded cultures, as described. Shown are the data for one of several experiments with similar results.

* Classification of mutants according to Table III.
Invasiveness of the Mutants in Fibroblast Monolayers

Recently it was shown that a number of T cell hybridomas, which were invasive in hepatocyte cultures, invade fibroblast monolayers as well (Collard et al., 1987; La Riviére et al., 1988). Invasiveness is easily quantitated in the phase-contrast microscope by counting the number of tumor cells that have infiltrated into the fibroblast monolayers. Parental TAM2D2 cells are highly invasive in these monolayers, and this process is inhibited to ~50% by anti-LFA-1 monoclonal antibody (Table V). In comparison with parental cells, the invasiveness of the LFA-1-deficient mutants was very low. Similar results were obtained with each of the three distinct types of mutants.

Metastatic Potential of the Mutants

The metastatic potential of TAM2D2 parental and mutant cell lines was tested by intravenous injection of the cells (Table VI). In agreement with earlier results, all animals injected with TAM2D2 cells developed extensive metastasis (Roos et al., 1985). Livers were heavily colonized and enlarged. Extrahepatic metastases were always found in ovaries and kidneys and frequently in lymph nodes, spleen, mesentery, and in the peritoneal cavity. In contrast, the metastatic potential of the LFA-1-deficient mutants was considerably lower. From each of the three distinct types of mutants, one cell line was tested (Table VI). Only ~10% of the injected animals developed metastases. In most cases a tumor mass was found in the peritoneal cavity behind the kidneys; in one animal small metastases were found in the liver, kidneys, ovaries, and lymphoid organs. The remaining animals survived for at least 100 d, after which no macroscopic metastases were detected. The few metastases originating from the mutant cell lines developed more slowly than those of parental cells (after 37-46 d instead of 17-22). All mutant cell lines were tumorigenic upon subcutaneous injection of cells. Parental cells developed large tumors in all animals after ~12-14 d. Tumors of similar size developed somewhat slower in all animals injected with the mutant cells (i.e., after 18-22 d).

Discussion

To elucidate invasion mechanisms of metastatic lymphoma cells, we used hepatocyte monolayers as an in vitro model. Lymphoma and T cell hybridoma cells that readily metastasize to the liver extensively invade such monolayers. Recently we found that invasion of hepatocyte monolayers was inhibited by a monoclonal antibody against LFA-1 (Roos and Roossien, 1987). To demonstrate the direct involvement of LFA-1 in metastasis, we generated a set of LFA-1-deficient T cell hybridoma mutants. The reduced invasive and metastatic potential of these mutants provide strong evidence that LFA-1 has an important role in lymphoma metastasis.

The cell line chosen to be mutated was the TAM2D2 T cell hybridoma that exhibits high LFA-1 surface levels and is highly invasive and metastatic (Roos et al., 1985; Roos and Roossien, 1987). Treatment of TAM2D2 cells with the alkylating reagent ethyl methanesulfonate, selection by panning, and subsequent cloning yielded a number of LFA-1-deficient mutants, among which three distinct types could be distinguished (Table III). Type I and II mutants synthesize the precursor of the β chain but not the α chain of LFA-1. In type III mutants the reverse was observed; cells synthesize the precursor of the α subunit but not of the β subunit. Type I and type II mutants differ in Thy.1 expression, which is similar to parental cells on type I but absent on type II mutant cells. Thus, the cell lines obtained were derived from at least three distinct mutant clones.

The invasive potential of parental TAM2D2 cells, quantitated by their infiltration index, was reduced to ~30% in the presence of anti-LFA-1 monoclonal antibody. Since the invasiveness of the mutant cell lines is comparable, this effect of the antibody was apparently not due to steric hindrance or induction of a negative signal (Martz et al., 1983). The reduced but substantial infiltration of the mutants and of parental cells in the presence of anti-LFA-1 antibody shows that LFA-1 is not the only adhesion molecule involved. Inhibition by anti-LFA-1 antibody was previously found to result from a reduction in the number of tumor cells interacting with the hepatocyte monolayers, since the fraction of the reduced number of interacting cells that infiltrated the monolayers during the 4-h test was not diminished (Roos and Roossien, 1987). This indicates that LFA-1 is involved in the initial step of invasion (i.e., in adhesion to the exposed hepatocyte sur-

Table V. Infiltration of TAM2D2 T Cell Hybridoma Cells and LFA-1-deficient Mutants into Fibroblast Monolayers

| Cell line | Anti-LFA-1 monoclonal antibody |
|-----------|---------------------------------|
|           | ~                              | +                              |
| TAM2D2   | 34 ± 6                          | 16 ± 2                          |
| 2B10 (type I)† | 2 ± 1                          | 1 ± 1                          |
| 1C9 (type II) | 3 ± 1                          | 1 ± 1                          |
| IA4 (type III) | 4 ± 2                          | 2 ± 1                          |
| 3C12 (type III) | 4 ± 2                          | 2 ± 1                          |

Table VI. Metastatic Potential of TAM2D2 T Cell Hybridoma Cells and LFA-1-deficient Mutants

| Cell line | Number of mice injected | Moribund animals killed at day | Number of animals that developed metastases* | Location of metastases† |
|-----------|-------------------------|-------------------------------|---------------------------------------------|-------------------------|
| TAM2D2    | 11                      | 17-22                         | 11 (100) L,K,S,O,R                           |
| 2B10 (type I)† | 19                          | 37-43                         | 2 (11) R (L,K,O)†                            |
| 1C9 (type II) | 22                          | 37-46                         | 2 (9) R                                    |
| IA4 (type III) | 10                          | 0                             | 0 (0)                                       |

AKR mice were injected with 10⁶ cells in the tail vein. Moribund animals were killed after the indicated number of days and analyzed for metastases. At day 100 the experiment was terminated.

* Values in parentheses indicate the percentage of injected animals that developed metastases during the 100-d examination period.
† L, liver; K, kidney; S, spleen; O, ovaries; R, retroperitoneal (in the peritoneal cavity behind the kidneys).
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animals, mainly at an extrahepatic site. Taken together, this group, and they caused metastases in a limited number of animals, mainly at an extrahepatic site. Taken together, this suggests that a certain threshold level of invasiveness is required for widespread metastasis of this type of tumor cell.

Probably the mutant cell lines described harbor more mutations than the one affecting LFA-1 synthesis. This is evident for type II mutants which are also deficient in Thy-1. For two reasons, it is unlikely that the change in invasive and metastatic potential is caused by such additional mutations. First, because the reduction is similar for three distinct mutants that probably do not carry identical additional mutations. Second, because all mutants, irrespective of class, infiltrate hepatocyte monolayers to the same extent as parental cells in the presence of anti-LFA-1 antibody.

Recently, cloning of the human LFA-1 β gene has been described (Kishimoto et al., 1987a; Law et al., 1987). We are presently attempting to transfect this gene into a type III LFA-1-deficient mutant, in which β chain precursor synthesis is absent. This should result in a transfected that produces LFA-1 α and β chain precursors from mouse and human origin, respectively. It has been demonstrated that such precursors are able to associate and mature to a human/mouse interspecies LFA-1 that is expressed on the cell surface (Marlin et al., 1986). If this LFA-1 species is functional, the behavior of such a transfecant will hopefully provide definite proof that the reduced invasive potential in vitro and metastatic capacity in vivo of the mutants due to LFA-1 deficiency.

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