N-Cadherin Expressed on Malignant T Cell Lymphoma Cells is Functional, and Promotes Heterotypic Adhesion Between the Lymphoma Cells and Mesenchymal Cells Expressing N-Cadherin

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Cadherins are Ca^{2+}-dependent cell–cell adhesion molecules, and are involved in the formation and maintenance of the organocellular architecture. Using a combination of molecular biologic and biochemical methods, we analyzed cadherins expressed on cultured human malignant lymphoma cell lines (adult T cell lymphomas, human T cell leukemia virus type 1-negative T cell lines, and thymus-derived lymphoma cell lines), and obtained evidence that N-cadherin is the major cadherin expressed on these cells. These cells were found to form cell aggregates in a Ca^{2+}-dependent manner, and more importantly to coaggregate and adhere with cells expressing N-cadherin, suggesting that N-cadherin on lymphoma cells is functionally active. Therefore, N-cadherin expressed on lymphoma cells could underlie the frequent invasion of these cells into the mesenchymal tissue in the skin and the central nervous system. ***Key words: catenin/malignant lymphoma/lymphocyte. J Invest Dermatol 112:62–66, 1999***

Cadherins are calcium-dependent cell–cell adhesion molecules that constitute a large superfamily, and are involved in the formation and maintenance of the histo-architecture (Takeichi, 1988). In addition to their role in normal tissue formation, cadherins seem to play a crucial role in the cell–cell interaction of cancer cells in tumorigenesis, invasion, and metastasis (Behrens *et al*, 1989; Chen and Obrink, 1991; Navarro *et al*, 1991; Vleminckx *et al*, 1991; Hamaguchi *et al*, 1993).

The cytoplasmic domain of cadherins interacts with either β-catenin or γ-catenin (plakoglobin), which in turn binds to α-catenin, and the resultant complex seems to be associated with cortical actin filaments (Ozawa *et al*, 1989; Knudsen and Wheelock, 1992). This interaction between cadherins and catenins is essential for cadherin-mediated adhesion and association of the complex with the cytoskeleton (Nagafuchi and Takeichi, 1988; Ozawa *et al*, 1990; Hirano *et al*, 1992).

Because cadherins are known to be morphoregulatory molecules during embryonic development and are important in the maintenance of tissue integrity, there have been a few reports on their expression and involvement in cells of hematopoietic lineage (Tang *et al*, 1993; Lee *et al*, 1994; Armeanu *et al*, 1995). We previously reported that at least two types of cadherins were expressed in certain malignant lymphoma cell lines but not in normal lymphocytes, and one of them was N-cadherin (Tsutsui *et al*, 1996). In that study we could not show that N-cadherin expressed on the cell surface is functionally active. Furthermore, the nature of the other cadherin has yet to be determined. To identify other cadherins expressed in malignant lymphoma cell lines, we used a combination of molecular biologic and biochemical methods, and obtained evidence that N-cadherin is the major cadherin expressed on these cells. To demonstrate that N-cadherin expressed on the cell surface is active, we changed the conditions for the aggregation assay and found that cell-expressing N-cadherin forms aggregates in a Ca^{2+}-dependent manner, and more importantly coaggregates with and adheres with cells expressing N-cadherin, suggesting that N-cadherin on lymphoma cells is functionally active.

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Abbreviations: DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchorate; EK cells, K562 cells expressing E-cadherin; NK cells, K562 cells expressing N-cadherin.

MATERIALS AND METHODS

Cell lines The human transitional carcinoma cell line, BOY (Matsusako *et al*, 1992), and human osteosarcoma cell line, HOS (McAllister *et al*, 1971), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum under a 10% CO_2 atmosphere at 37°C. The cloned human T cell leukemia virus type 1-infected cell lines, Oh13T, F6T, and K3T, have been described previously (Arima *et al*, 1991). These cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 0.5 Urecombinant IL-2 (Takeda, Osaka, Japan) per ml, and 2 μg phytohemagglutinin P (Wako, Osaka, Japan) per ml under a 5% CO_2 atmosphere at 37°C. The human T cell leukemia virus type 1-negative T cell line, Jurkat, and the human thymus-derived T cell line, Molt-4F, were cultured as described previously (Tsutsui *et al*, 1996). K562 cells expressing E-cadherin (EK cells) were described previously (Ozawa and Kemler, 1998). K562 cells expressing N-cadherin (NK cells) were established by transfecting cells with an expression vector, pCAGGS neo (Niwa *et al*, 1991), containing N-cadherin cDNA as described (Ozawa and Kemler, 1998).

cDNA cloning cDNA clones were isolated by means of reverse transcription-polymerase chain reaction from the cDNA of lymphoma cell lines (Oh13T and Molt-4F) as described previously (Ozawa *et al*, 1995). Two highly conserved amino acid sequences in the cytoplasmic domain were chosen, and corresponding oligonucleotides, 5′ GAATTCACNGCNCC-NCCNTAYGA (primer 1) and 5′ GAATTCTCNGCNARYTTYTTRAA (primer 2) (Suzuki *et al*, 1991), were synthesized and used as polymerase chain reaction primers. The reaction mixture was subjected to 36 cycles of denaturation (94°C, 1 min), annealing (46°C, 2 min), and extension (72°C, 3 min). The polymerase chain reaction products (about 130 bp in size) were isolated and subcloned into the *Sma* I site of the Bluescript KS(+) vector (Stratagene). Twenty-three and 68 clones obtained from Oh13T and Molt-4F cells, respectively, were analyzed by DNA sequencing using Thermo SequenaseTM+ Cycle sequencing kit (Amersham, Bucks, U.K.). Homology was searched for using the EMBL database.

Antibodies Antibodies against N-cadherin were purified by affinity chromatography on Sepharose coupled with the fusion proteins as described previously (Tsutsui *et al*, 1996). Antibodies against R-cadherin were a kind gift of Dr. T. Yanagida (Department of Ophthalmology, Kagoshima University). The monoclonal antibodies against E- and P-cadherin, and α- and β-catenin, were obtained from Transduction Laboratories.

Northern blotting Total RNA was prepared from T cell lymphoma cell lines using ISOGEN (Nippon Gene, Tokyo, Japan). Poly(A)$^+$-RNA enriched with oligo(dT)-cellulose (Takara, Kusatsu, Japan) was used to produce cDNA using MMTV reverse transcriptase (Gibco/BRL, Gaithersburg, MD) and a random-hexamer primer according to the method described previously (Ozawa *et al*, 1995). Poly(A)$^+$-RNA (5 μg) was fractionated by electrophoresis on a 1% agarose gel, and then blotted onto a nylon membrane (Amersham), and probed with cDNA fragments for N-, E-, P-, R-, and T1-like cadherin, and cadherin-11, which had been radiolabeled by the random priming method (Feinberg and Vogelstein, 1983). The hybridization and washing conditions were as described previously (Ozawa and Muramatsu, 1993).

Western blotting and immunoprecipitation Immunoblotting and immunoprecipitation were carried out as described previously (Ozawa *et al*, 1989) except that an ECL kit (Amersham) was used in the immunoblot experiment. In the immunodepletion experiments, Oh13T cells (1×10^7) were lyzed with 1 ml of phosphate-buffered saline containing 1% Triton X-100, 1% Nonidet p-40, 1 mM $CaCl_2$, and 1 mM phenylmethlysulfonyl fluoride. After centrifugation, a part of the supernatant, trichloroacetic acid (final 15%), was added to precipitate proteins and used as total cell lysates. Another part was subjected to immunoprecipitaion using rabbit anti-N-cadherin antibodies (10 μg) or rabbit anti-human IgG antibodies (10 μg), as an irrelevant control. After 2 h incubation, the immun-complexes were collected using protein A-Sepharose. The unabsorbed proteins were precipitated by the addition of trichloroactetic acid, and used as the immuno-depleted materials.

Cell aggregation assay and mixing experiments Aggregation assays were performed as described (Ozawa *et al*, 1990), with the following modifications. To obtain single cells, cells were passed through Pasteur pipettes several times (Ozawa and Kemler, 1998). Thus, trypsin treatment in the presence of Ca^{2+}, a method commonly used to obtain single cells for a cadherin-mediated aggregation assay (Takeichi, 1988), was not used in this study. After dissociation, the cells were washed with and suspended in a 1:1 mixture of HEPES-buffered saline containing 2 mM $CaCl_2$ or 5 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid and RPMI 1640 containing 5% fetal calf serum. The cells were then allowed to aggregate for 30 min at 37°C with constant rotation at 30 r.p.m. The extent of cell aggregation was calculated according to Nagafuchi and Takeichi (1988) as the index (No – Nt)/No, where Nt is the total particle number after the incubation (30 min) and No is the total particle number at the start of the incubation.

Mixing experiments were performed as follows. F6T cells were added to an equal number of NK or EK cells, which had been labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchorate (DiI) (Honig and Hume, 1986). The cells were labeled by adding 2 μl of a 2.5 mg per ml solution of DiI in ethanol per ml of cells. Incubation was performed for 15 min at 37°C, at which time the cells were collected by centrifugation and washed extensively with phosphate-buffered saline. Immunofluorescence microscopic examination revealed that almost all the cells, if not all, were labeled with the dye. Labeled cells (3×10^5 per ml) were added to an equal number of F6T cells and then allowed to aggregate for 30 min at 37°C with constant rotation at 30 r.p.m. The cell aggregates were fixed in 3% paraformaldehyde and then examined by phase-contrast or fluorescence microscopy.

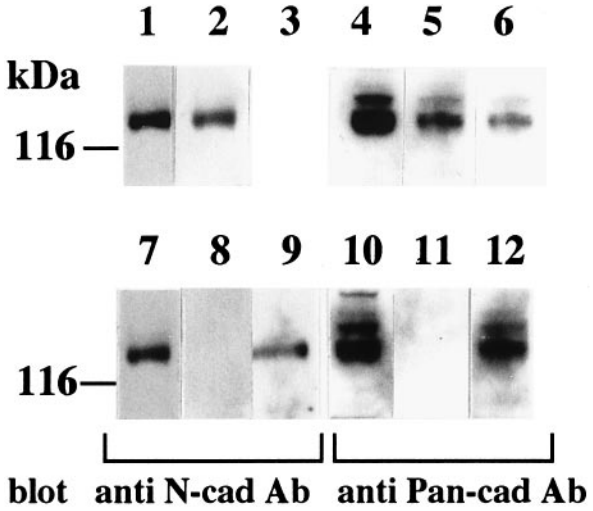

Figure 1. T cell lymphoma cell lines expressed other cadherins in addition to N-cadherin. N-cadherin was removed from cell lysates of Oh13T cells by immunoprecipitation with N-cadherin antibodies as described under *Materials and Methods*. Total cell lysates (*lanes 1*, *4*, *7*, and *10*), the materials collected on immunoprecipitation with N-cadherin antibodies (*lanes 2* and *5*) or irrelevant control antibodies (*lanes 8* and *11*), and cell lysates depleted with N-cadherin antibodies (*lanes 3* and *6*) or control antibodies (*lanes 9* and *12*), were subjected to immunoblot analysis with N-cadherin (*lanes 1–3*, *7–9*) or pan-cadherin (*lanes 4–6*, *10–12*) antibodies. The signal obtained with pan-cadherin antibodies in *lane 5* is stronger than that in *lane 6*, suggesting that N-cadherin is the major cadherin in the cells.

Adhesion to L fibroblasts Molt-4F cells labeled with DiI as described above were added to L fibroblasts expressing either N-cadherin or E-cadherin, which had been allowed to spread on cover slips to 50% confluency, and then incubated for 24 h at 37°C. After gentle washing three times with phosphate-buffered saline, cells were examined as described above.

RESULTS AND DISCUSSION

Previously, we found that the N-cadherin-catenin complex is expressed on certain T cell lymphoma cells (Tsutsui *et al*, 1996). To examine the possible expression of cadherins other than N-cadherin in T cell lymphoma cell lines, we first removed N-cadherin from Oh13T cell lysates by immunoprecipitation with N-cadherin antibodies, then the cell lysates were subjected to immunoblot analysis with pan-cadherin antibodies (**Fig 1**). As shown in **Fig 1**, the lysates depleted of N-cadherin (**Fig 1**, *lane 3*) still contained materials reacting with pan-cadherin antibodies (**Fig 1**, *lane 6*), but not with N-cadherin antibodies (**Fig 1**, *lane 3*). Similar results were obtained using Jurkat and Molt-4F cells (data not shown, but see below). These results demonstrate that in addition to N-cadherin, another member(s) of the cadherin family is expressed in T cell lymphoma cell lines. Quantitative analysis revealed, however, that N-cadherin is the major cadherin expressed in these cells.

Because the pan-cadherin antibodies were raised against the conserved carboxy-terminal amino acid residues of N-cadherin, it is possible that some cadherins are poorly recognized by the antibodies. Therefore, we next isolated cDNA from the T cell lymphoma cell lines with the aid of polymerase chain reaction. This method comprises polymerase chain reaction with degenerate primers based on well-conserved amino acid sequences of the intracellular domains of cadherin molecules and sequencing of the products obtained, and has been successfully used to isolate cDNA for new cadherins expressed in nervous tissues (Suzuki *et al*, 1991). In addition to cDNA for N-cadherin, cDNA encoding R- and P-cadherins were obtained from Molt-4F cells, and cDNA for

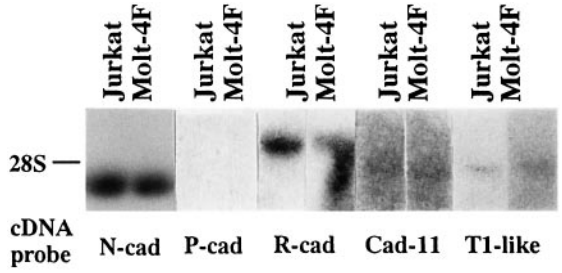

Figure 2. Northern blot analysis revealed the expression of mRNA for N-cadherin, R-cadherin, cadherin-11, and T1-like cadherin. Poly(A)$^+$ RNA (5 μg) from Jurkat cells and Molt-4F cells were run on an 1% agarose gel and then transferred to a nylon membrane. The membrane was probed with the respective cDNA labeled with ^{32}P. Single transcripts of ≈4.3–4.8 kb were detected.

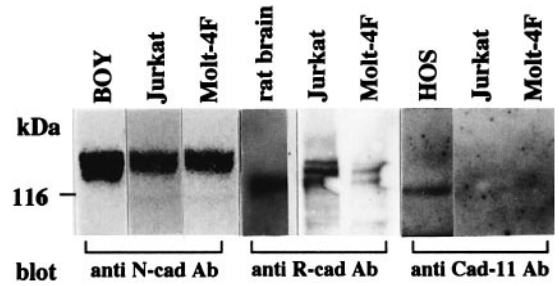

Figure 3. N- and R-cadherin, but not cadherin-11, were expressed in human T cell lymphoma cell lines. Jurkat cells and Molt-4F cells were subjected to immunoblot analysis with anti-N-cadherin, R-cadherin, and cadherin-11 antibodies. As positive controls for N-cadherin, R-cadherin, and cadherin-11, BOY, a human transitional carcinoma cell line, rat brain, and HOS, a human osteosarcoma cell line, respectively, were used.

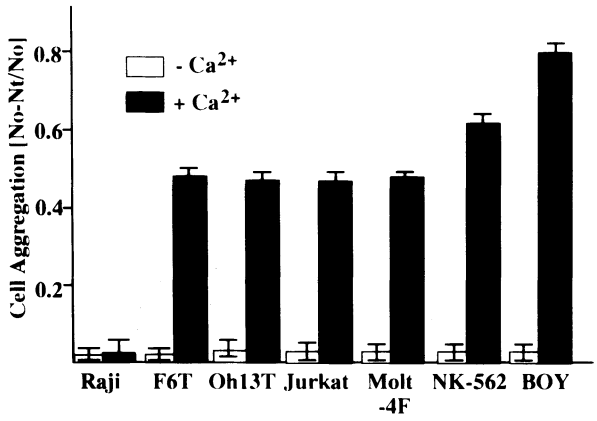

Figure 4. Ca^{2+}-dependent aggregation of lymphoma cells. Cells were allowed to aggregate for 30 min in the presence of 2 mM $CaCl_2$ (closed bars) or 5 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (open bars) as described under *Materials and Methods*. As positive controls, BOY cells expressing N-cadherin and K562 cells transfected with N-cadherin cDNA, and as a negative control, Raji cells, which are negative for N-cadherin expression, were used.

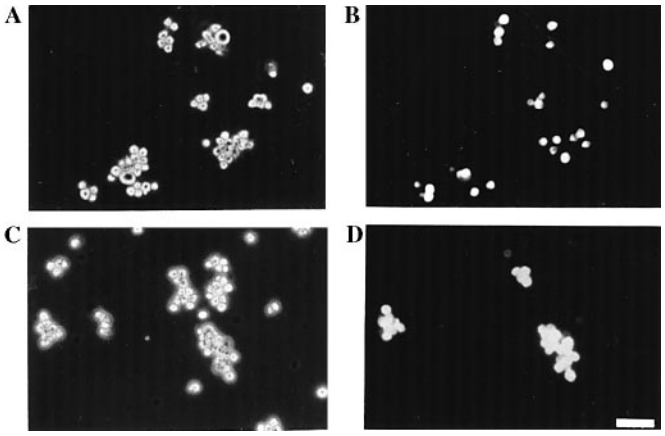

Figure 5. T cell lymphoma cell lines showed N-cadherin-mediated aggregation under suspension conditions. F6T cells were added to an equal number of K562 cells expressing N-cadherin (*A*, *B*), or K562 cells expressing E-cadherin (*C*, *D*), that had been labeled with DiI, and then were allowed to aggregate for 30 min. The cell aggregates were examined by phase-contrast (*A*, *C*) or fluorescence microscopy (*B*, *D*). *Scale bar*: 50 μm.

E-cadherin and cadherin-11 were obtained from Oh13T cells (data not shown). A cDNA encoding an amino acid sequence that showed similarity (90% identity) to mouse T1-cadherin (Munro and Blaschuk, 1996; Munro *et al*, 1996) was also isolated from Oh13T cells. To determine whether or not mRNA for these cadherins were really expressed in these cells, northern blot analysis were performed using the respective cDNA probes. The results shown in **Fig 2** reveal that the mRNA for N-cadherin, R-cadherin, cadherin-11, and T1-like cadherin were expressed in these cells, but the mRNA for P-cadherin and E-cadherin (data not shown) were not.

Immunoblot analysis revealed that a large amount of N-cadherin and a small amount of R-cadherin, but no cadherin-11 protein, were expressed in Molt-4F and Jurkat cells (**Fig 3**). Consistent with the results of northern blot analysis, no E-cadherin or P-cadherin protein was detected in any of the cell lines examined (data not shown). Human T cell leukemia virus type 1-infected T cell lines (Oh13T and F6T cells) expressed N-cadherin but not R-cadherin (data not shown).

The results described above demonstrate that the major cadherin expressed in leukemia cells is N-cadherin. In the previous experiments (Tsutsui *et al*, 1996), we found that N-cadherin is expressed on the cell surface and is associated with catenins. With the standard cell aggregation assay employed, however, we could not detect the cell adhesive activity of N-cadherin expressed on the leukemia cells. In the assay, intercellular collisions were generated through rotational forces. Generally, the number of collisions between cells in suspension is proportional to the rotational speed imposed upon the suspension; however, as the speed increases, the shear forces that disrupt new aggregates also become greater. Thus, it may be expected that only the effects of surface molecules that mediate relatively strong adhesive interactions will be detected because of the relatively high shearing forces (Pizzey *et al*, 1989). Although N-cadherin is the major cadherin expressed on leukemia cells, its relative amount is small as compared with those expressed on other types of cells such as fibroblasts. Because the adhesive strength depends on the amount of cell adhesion molecules (Friedlander *et al*, 1989), we changed the conditions for the aggregation assay, i.e., the rotational force of 70 r.p.m. was changed to one of 30 r.p.m. Under these conditions malignant lymphoma cells formed cell aggregates in a calcium-dependent manner, i.e., cell aggregates were formed only in the presence of $CaCl_2$ but not in its absence (**Fig 4**). These results demonstrate that these cells exibit calcium-dependent cell aggregation. The lack of function-inhibiting antibodies to N-cadherin, however, prevented us from demonstrating that the aggregation of these cells is N-cadherin dependent.

To determine whether N-cadherin expressed on these cells is functional or not, we performed mixing experiments. F6T cells were added to an equal number of K562 cells expressing N-cadherin (NK cells) or E-cadherin (EK cells), both of which had been labeled with the fluorescent dye, DiI, and then allowed to aggregate. Almost all NK cells or EK cells were labeled with DiI under the conditions used, and labeling with the dye had no effect on the aggregating activity of the cells (data not shown). As shown in **Fig 5**, F6T cells were found in aggregates of NK cells but not in

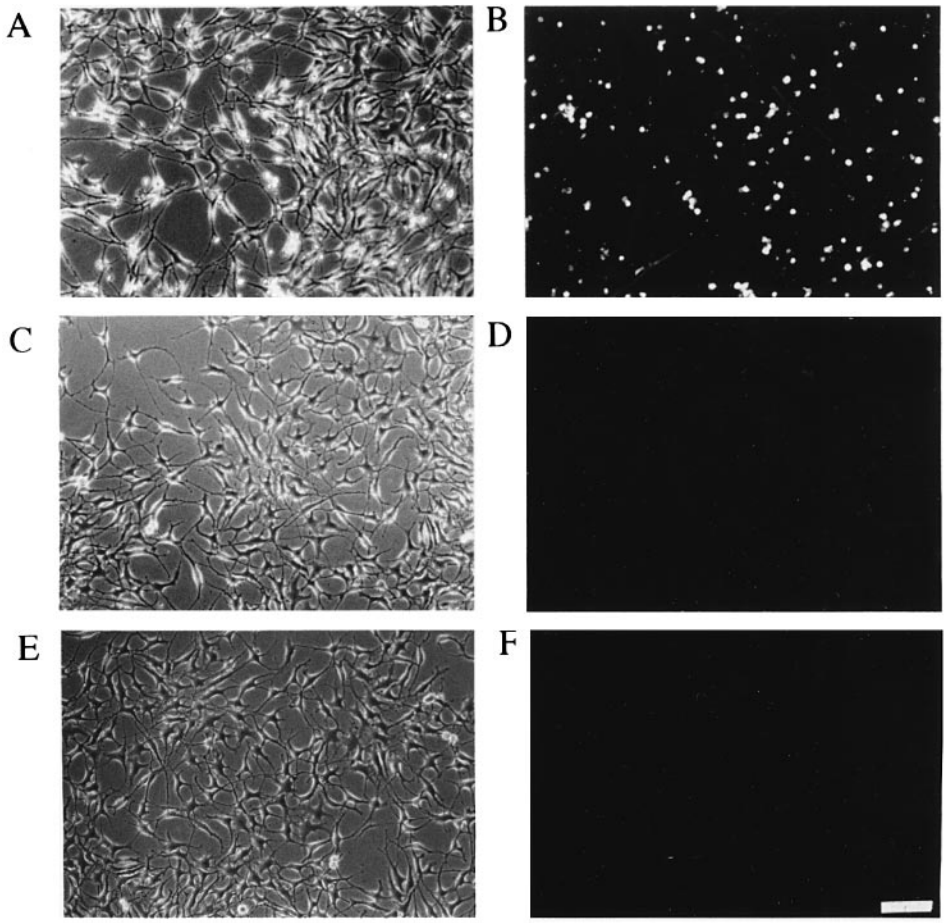

Figure 6. Molt-4F cells adhere to L fibroblasts expressing N-cadherin but not to those expressing E-cadherin. Molt-4F cells labeled with DiI were allowed to adhere to L fibroblasts expressing either N-cadherin (*A*, *B*), E-cadherin (*C*, *D*), or untransfected L cells (*E*, *F*) for 24 h as described in *Materials and Methods*. The cells adhered to L fibroblasts were examined by phase-contrast (*A*, *C*, *E*) or fluorescence microscopy (*B*, *D*, *F*). *Scale bar*: 50 μm.

ones of EK cells. Thus, N-cadherin expressed on F6T cells is functional. Analysis with Oh13T, Molt-4F, and Jurkat cells gave the same results (data not shown). Furthermore, Molt-4F cells were found to adhere to L fibroblasts expressing N-cadherin but not to those expressing E-cadherin (**Fig 6**).

In this study, we have obtained evidence that although a small amount of R-cadherin is expressed in some malignant T cell lymphoma cell lines, N-cadherin is the major cadherin expressed in lymphoma cells. Cadherins are involved in the formation and maintenance of the histo-architecture (Takeichi, 1988). In that sense, our finding that the cadherin–catenin complex is expressed on T cell lymphoma cell lines is rather unexpected because lymphocytes do not form a tissue structure. The physiologic meaning of the expression of the cadherin–catenin complex remains to be determined. The frequent invasion of lymphoma cells into the central nervous system has been reported (Jellinger and Radaszkiewicz, 1976). Therefore, the expression of N-cadherin could be related to the ability of certain lymphomas to invade or metastasize. The expression of N-cadherin is not restricted to neural tissues; instead, N-cadherin is widely expressed in various tissues. Some squamous cell carcinomas were found to express N-cadherin *in situ* and these tumors expressing N-cadherin were invasive (Islam *et al*, 1996). The possible involvement of N-cadherin expressed on breast cancer cells in facilitating invasion and metastasis by promoting their interaction with the surrounding stromal cells was recently proposed (Hazan *et al*, 1997).

Finally, if the malignant transformation of lymphocytes induces the expression of certain cadherins, cadherins could be useful markers for the diagnosis of and therapy for malignant lymphomas. Further studies are needed to examine the possible use of cadherins as markers for identifying certain types of lymphoma cells.

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