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α4β7 Integrin Expression Is Associated with the Leukemic Evolution of Human and Murine T-Cell Lymphoblastic Lymphomas

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We have previously shown that the* in vivo *coordinated expression of individual α4 and β7 integrin chains correlated with the leukemic potential displayed by cell lines derived from murine lymphoblastic T-cell lymphomas (T-LBLs) when transplanted subcutaneously into syngeneic AKR mice. In the present study, by using immunofluorescence and immunocytochemical analyses, we have confirmed that the* in vivo *up-regulation of the α4β7 heterodimeric complex is associated with the leukemic behavior of AKR T-LBLs. In addition, when compared with the parental, highly leukemic NQ22 cells, the variant cell line NQ22V exhibited a reduced leukemic potential that was invariably associated with a delayed α4β7 up-regulation* in vivo*. Moreover, the leukemic cell line SJ-1, derived from a spontaneous T-LBL of the SJL strain, also displayed high levels of α4β7 expression with a pattern of tissue distribution similar to that of NQ22 cells from leukemic AKR animals. Of note, in most of the tissues involved by murine T-LBL dissemination, and particularly in liver, kidney, and lung, α4β7-positive leukemic cells were always located around strongly VCAM-1-positive vascular spaces. These findings are consistent with a possible role of α4β7/VCAM-1 interactions in the extravasation and, consequently, in the leukemic dissemination of murine T-LBL cells. Immunocytochemical analysis carried out in 11 human T-LBLs showed that pathological lymph nodes from all 7 cases with bone marrow infiltration at presentation carried α4β7-positive cells, whereas all 4 aleukemic T-LBLs were repeatedly α4β7 negative, also in metachronous lesions. These findings suggest that α4β7-positive human T-LBLs may represent a distinct clinicopathological entity. In addition, α4β7 expression was significantly more prevalent in younger patients (<11 years; P = 0.02), further supporting such a hypothesis. Moreover, as in murine T-LBLs, the pattern of α4β7 positivity in involved lymph nodes was mainly focal, whereas nearly all neoplastic cells infiltrating bone marrow expressed this integrin, suggesting a possible role for α4β7 in the leukemic dissemination also of human T-LBLs. (Am J Pathol 1997, 150:1595–1605)

Lymphoblastic lymphoma (LBL) is a neoplasm mainly derived from immature T lymphocytes characterized by a high degree of biological and clinical heterogeneity.[1,2] In general, LBL patients present with progressively enlarging mediastinal masses, usually of thymic origin, which may be associated with peripheral lymphadenopathy.[1,2] Although a small proportion of cases remain aleukemic throughout the clinical course of the disease, more frequently, LBLs evolve to a leukemic phase morphologically indistinguishable from T-cell acute lymphoblastic leukemia (T-ALL).[1,2] Whether T-LBL and T-ALL are separate entities or represent different stages of a single disease is still unclear. This issue, in fact, has been addressed so far by only a few

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studies that have reported inconclusive results.[3–6] Furthermore, the mechanisms responsible for leukemic cell dissemination are largely unknown. Progress in the understanding of these aspects is hampered by the scarcity of appropriate animal models. In fact, after subcutaneous (s.c.) transplantation into immunodeficient mice, cells from both lymphomas and leukemias usually give rise to solid, localized tumors that only rarely spread to bone marrow or other secondary sites.[7–10] Such an *in vivo* growth pattern is unrepresentative of the leukemic evolution of human lymphomas and not suitable for the study of leukemic cell dissemination.

We have recently developed an experimental system, composed of a series of cell lines derived from MCF-247 murine leukemia virus-induced AKR T-cell lymphomas, in which the growth and spreading of neoplastic cells in the syngeneic host largely reproduce the behavior of human lymphomas with differential leukemic potential.[11] In particular, one of these lines (NQ22) displays a highly reproducible leukemic phenotype. These cells, in fact, rapidly spread from the s.c. site of transplantation, giving early peripheral blood invasion and widespread dissemination with a leukemic pattern of tissue involvement.[11] These biological characteristics displayed *in vivo* by NQ22 cells closely recall the leukemic evolution of human T-cell LBLs.

As the invasive and spreading abilities of leukemic cells may be dependent on the expression of cell surface molecules responsible for specific interactions with vascular endothelial cells and/or extracellular matrix, we have recently characterized our T-LBL lines with differential leukemic properties for the *in vivo* expression of several adhesion molecules.[12,13] The analysis revealed a correlation between the leukemic phenotype and the expression of individual $\alpha 4$ and $\beta 7$ integrin chains,[13] suggesting that $\alpha 4\beta 7$ integrin may play a role in leukemic cell dissemination. The known properties of this molecule[14] appear of potential relevance in this respect and particularly the capacity of $\alpha 4\beta 7$ to mediate lymphocyte interactions with fibronectin[15,16] and endothelial cell counter-receptors. These latter include the vascular cell adhesion molecule 1 (VCAM-1),[15,16] inducible by pro-inflammatory mediators,[17,18] and the mucosal addressin cell adhesion molecule-1 (MAdCAM-1),[19,20] a molecule mainly expressed by venules at mucosal sites such as Peyer's patches and mesenteric lymph nodes.[19–21]

To better understand the possible contribution of $\alpha 4\beta 7$ integrin to the leukemic spreading of T-LBLs, we further characterized our AKR cell lines for the *in vivo* expression of the $\alpha 4\beta 7$ heterodimeric complex by using the recently developed monoclonal antibody (MAb) DATK32 recognizing a combinatorial epitope formed on association of $\alpha 4$ with $\beta 7$ chains.[20] The analysis was also carried out in a variant of the NQ22 cell line characterized by a reduced leukemic potential and in a highly leukemic cell line derived from a spontaneous thymic lymphoma of the SJL mouse strain. Furthermore, taking into account the marked ontogenetic and biological similarities between murine and human T-LBLs, we wished to assess whether the association between $\alpha 4\beta 7$ and the leukemic behavior of malignant cells holds true also for human T-LBLs. To this end, the expression of $\alpha 4\beta 7$ integrin was retrospectively analyzed in T-LBLs with and without evidence of bone marrow involvement.

Materials and Methods

Mice

AKR/J, SJL, and (C57BL/6 × AKR/J) F_1 2- to 4-month-old mice of both sexes, maintained in our animal facility, were used in this study.

Murine T-LBL-Derived Cell Lines and in Vivo Transplantation

Establishment, properties, and culture conditions of AKR T-LBL-derived cell lines NQ22, NQ29, and NQ36 have been previously described.[11,13] The NQ22 cell line displays the phenotype Thy1.1$^+$ CD4$^-$CD8$^-$CD11a/CD18$^+$CD26$^-$CD44$^-$CD54$^+$ CD62L$^-$, whereas the NQ29 cell line is Thy1.1$^+$ CD4$^+$CD8$^+$CD11a/CD18$^+$CD26$^+$CD44$^+$CD54$^+$ CD62L$^-$, and the NQ36 cell line is Thy1.1$^+$ CD4$^-$CD8$^-$CD11a/CD18$^+$CD26$^+$CD44$^+$CD54$^-$ CD62L$^+$.[11,13] The SJ-1 cell line was derived from a T-LBL spontaneously developed in the thymus of an SJL mouse. SJ-1 was maintained by serial s.c. inoculation in syngeneic animals and was used within the seventh transplantation generation. Viable cells (10^5) of each line or *in vivo*-derived tumor, resuspended in 0.1 ml of phosphate-buffered saline (PBS), were injected s.c. in mice of both sexes. Transplanted animals were examined regularly and tumors allowed to grow until death to assess the mortality rates of the different cell lines. The size of s.c. tumors was measured with calipers, and tumor volumes were calculated by using the formula (length × width^2)/2. Postmortem examination was carried out to determine the gross appearance of tumor growth and spread. For immunophenotypic analyses, single-cell suspensions were purified over Ficoll/Hypaque (Pharmacia,

Uppsala, Sweden) density gradients from s.c. masses and involved organs of mice with advanced tumors.

Patients

Biopsy material from 11 patients with T-LBL was retrieved from the surgical pathology files of the Istituto Nazionale Tumori of Milan between the years 1986 and 1996. The diagnosis of T-LBL was based on the criteria of the updated Kiel classification of malignant non-Hodgkin's lymphomas.[22] Stage was defined according to Murphy.[23] At diagnosis, bone marrow involvement was present in 7 of 11 T-LBLs, with less than 20% of infiltrating lymphoblasts. One biopsy sample from a patient with reactive lymphadenopathy was also included in the study as control.

Monoclonal Antibodies

The following MAbs to mouse antigens were used: anti-α4 (clone R1–2[24] and clone 9C10[20]), anti-$\alpha4_{IEL}$/$\alpha4_{M290}$ (clone M290[25] and clone 2E7[26]), anti-H2-K^b-FITC, anti-H2-K^k, anti-CD3, anti-CD31, and anti-CD106 (VCAM-1), obtained from PharMingen (Rome, Italy), and anti-CD4 (L3T4) and anti-CD8 (Lyt-2), purchased from Becton Dickinson (Milan, Italy). Anti-β7 (clone M298[27]), kindly provided by Dr. P. J. Kilshaw, anti-heat-stable antigen (HSA) (clone B2A2), a generous gift from Dr. H. R. MacDonald, and anti-α4β7 (clone DATK32[20]) were used as hybridoma supernatants. Immunophenotyping of human T-LBLs was performed using MAbs specific for CD2, CD3, CD4, CD5, CD7, CD8, CD19, CD20, and CD22, (obtained fom Becton Dickinson), terminal deoxynucleotidyl transferase (TdT), purchased from SERA-LAB (Sussex, UK), and Mib-1, from Immunotech (Marseille, France). Expression of human α4β7 was evaluated by using the ACT-1 antibody.[28,29]

Immunofluorescence Analysis

Immunophenotypic features of *in vitro* cultured T-LBL lines were investigated by single-color indirect immunofluorescence, as previously described.[13] Briefly, after preincubation for 30 minutes at 4°C in PBS supplemented with 10% rabbit serum, 100 μl of cell suspension (10^6 cells) were incubated with saturating concentrations of the primary antibody for 30 to 45 minutes at 4°C. After three washes in PBS, cells were incubated for an additional 30 to 45 minutes at 4°C with optimal dilutions of phycoerythrin (PE)-conjugated second-step antibody. Cells were then washed three times in PBS, fixed in 1% buffered paraformaldehyde, and analyzed (10,000 events in each sample) with a FACScan flow cytometer (Becton Dickinson) gated to exclude nonviable elements. Each sample was also analyzed for nonspecific binding of PE-antibody. To distinguish the *in vivo* phenotypic pattern of transplanted AKR T-LBL lines ($H2^k$) from that of host cells, we used as recipient animals (C57BL/6 × AKR/J)F_1 mice ($H2^{kb}$). Single-cell suspensions, obtained from s.c. tumor masses, peripheral blood, lymph nodes, and spleen of animals with overt leukemia, were analyzed by two-color fluorescence using as a first-step antibody a fluorescein-isothiocyanate-conjugated anti-H2-K^b as described above. After washing, cells were incubated separately with an optimal dilution of purified primary antibody and counterstained with anti-rat-PE antibody. Negative controls consisted of cells incubated with fluorescein-conjugated anti-mouse Ig and anti-rat Ig-PE antibodies only. The number of positive cells and the fluorescence intensity of the cell-surface molecules tested were evaluated considering only the H2-K^b-negative cell populations, thus eliminating signals derived from contaminating cells of the host.

Immunocytochemistry

Blocks of fresh mouse tissue samples were embedded in OCT compound (Miles Laboratory, Naperville, IL), snap frozen in liquid nitrogen-cooled isopentane, and stored at −70°C until sectioning. Cryostat sections 5 μm thick were cut, air dried, and fixed in acetone for 10 minutes. After washing in PBS, the rat anti-mouse primary antibodies M298 (β7), DATK32 (α4β7), CD31, and VCAM-1 were applied at a dilution of 1:1000, 1:200, 1:2000, and 1:1000, respectively. Biotinylated anti-rat IgG (Vector Laboratories, Burlingame, CA) was followed by peroxidase ABC-Elite complex (Vector). In double-immunolabeling experiments, a peroxidase-ABC method developed in black with diaminobenzidine-nickel was used in the first reaction, followed by an alkaline phosphatase/biotin/streptavidin method developed in red with Vector-Red (Vector).

Human T-LBLs were analyzed with a panel of MAbs used on frozen tissue (CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD19, CD20, and CD22) and on paraffin sections (CD3, CD20, TdT, and Mib-1). Two-micron frozen and formalin-fixed, paraffin-embedded sections were cut and mounted on slides coated with poly-L-lysine (Sigma Chemical Co., St. Louis, MO). Frozen sections were air dried overnight and fixed in cold acetone. Paraffin sections were deparaffinized in xylene, rehydrated in graded alcohols,

Table 1. In Vivo *Expression of α4 and β7 Chains and the Heterodimeric α4β7 Complex in NQ22 and NQ22V Cells*

	s.c. tumor			Spleen			Peripheral blood		
	α4 (R1–2)	β7 (M298)	α4β7 (DATK32)	α4 (R1–2)	β7 (M298)	α4β7 (DATK32)	α4 (R1–2)	β7 (M298)	α4β7 (DATK32)
NQ22	53 ± 18	58 ± 19	29 ± 7	86 ± 14	86 ± 6	71 ± 14	82 ± 6	86 ± 9	58 ± 16
NQ22V	15 ± 2 (p = 0.03)	14 ± 10 (p = 0.01)	4 ± 3 (p = 0.006)	86 ± 10	86 ± 9	76 ± 11	83 ± 11	87 ± 10	69 ± 5

Results are expressed as the percentage of cells that stained positive in flow cytometric analysis with the specific mAbs. Values represent means and standard deviations of five independent experiments. The analysis was carried out in AKR mice with end-stage leukemic cell dissemination after s.c. inoculation with NQ22 or NQ22V cells.

and after three washes in PBS, immersed in 10 mmol/L citrate buffer (in distilled water, pH 6) and subjected to microwave irradiation for two cycles of 8 and 5 minutes at 700 W, separated by 5 minutes of pause. After cooling to room temperature (30 minutes) and three washings in PBS for 15 minutes, tissues were treated with 3% H_2O_2 in methanol for 30 minutes and 1 hour, respectively, to block the endogenous peroxidase. After three rinses, sections were treated for 30 minutes with normal goat serum (DAKO, Glostrup, Denmark; 1:20) and then incubated overnight with primary antibodies ACT-1 (1:200), CD2 (1:20), CD3 (1:40), CD4 (1:20), CD5 (1:40), CD7 (1:40), CD8 (1:40), CD19 (1:100), CD20 (1:100), CD22 (1:100), TdT (1:200), and Mib-1 (1:100) at 4°C. After several brief washes, sections were overlaid with a 1:100 dilution of biotinylated goat anti-mouse IgG (DAKO), and final incubation was for 30 minutes with 1:350 streptavidin conjugated with horseradish peroxidase (DAKO). Omission of primary antibodies was used as negative control. Color was routinely developed using aminoethyl carbazole for 10 minutes, followed by a light hematoxylin counterstain (Carazzi's hematoxylin). Immunostaining was assessed twice by two different pathologists in a double-blind fashion.

Statistical Evaluation

The significance of immunophenotypic changes was evaluated by the Student's *t*-test. Correlation between α4β7 integrin expression and clinicopathological parameters was statistically evaluated by using the two-tailed Fisher's exact test.

Results

α4β7 Integrin Expression Correlates with the Leukemic Behavior of AKR T-LBL Lines

Although the nonleukemic lines NQ29 and NQ36 were α4β7 negative both *in vitro* and *in vivo* (data not shown), the highly leukemic NQ22 cells displayed a coordinated up-regulation of α4 and β7 integrin chains in transplanted animals[13] (Table 1). The analysis with the DATK32 antibody confirmed that the α4β7 heterodimeric complex was markedly induced on NQ22 cells *in vivo* (Table 1 and Figure 1). Moreover, the NQ22 cell line was repeatedly negative for the expression of $\alpha4_{IEL}/\alpha4_{M290}$ (not shown), the other α-chain known to bind to β7,[25,27] indicating that in these cells virtually all β7 chains are probably associated with the α4 subunit. As shown in Table 1, the highest numbers of α4β7-positive NQ22 cells were observed in the peripheral blood and spleen of AKR mice with widespread leukemic dissemination. α4β7 integrin was also strongly expressed by NQ22 cells infiltrating lymph nodes contralateral to the site of inoculation as well as mesenteric and abdominal lymph nodes, which are usually enlarged in animals

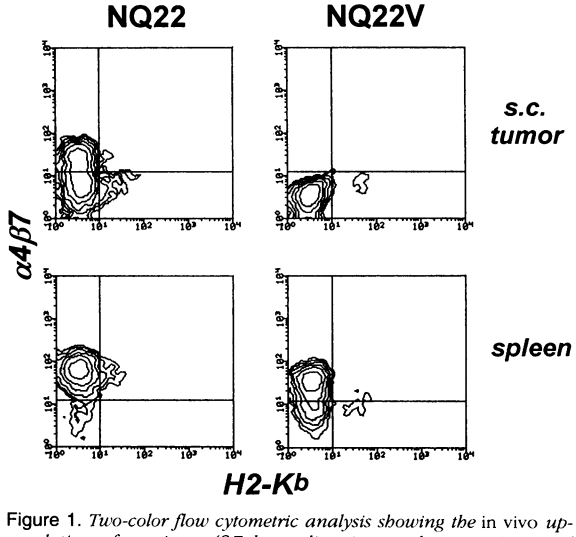

Figure 1. *Two-color flow cytometric analysis showing the* in vivo *up-regulation of murine α4β7 heterodimeric complex on NQ22 and NQ22V cells. When compared with the parental NQ22 cells, significantly fewer α4β7-positive cells were detected in subcutaneous tumors induced by the variant cell line NQ22V characterized by a reduced leukemic potential. Conversely, the large majority of lymphoblasts of both cell lines infiltrating the spleen of leukemic animals express high levels of the α4β7 heterodimeric complex. Most of gated cells are H2-K^b negative, thus assuring that the observed staining intensity was actually that of leukemic cells. Lines used to define positive* versus *negative cells were set against the outermost contour of controls where the primary antibodies were omitted. Representative results of one of five experiments are shown.*

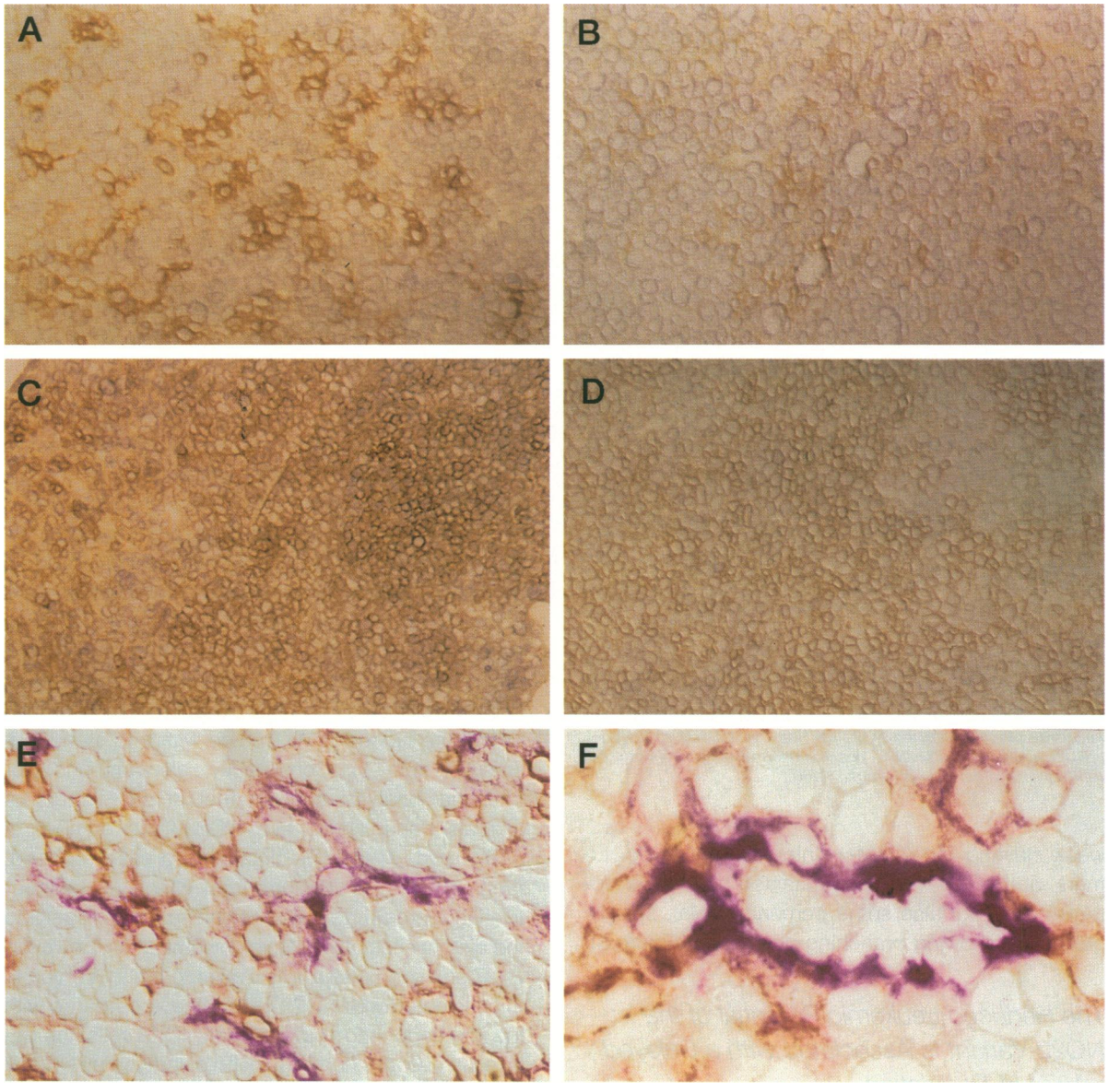

Figure 2. A: *Immunohistochemical staining of NQ22 cells in s.c. tumors induced in AKR mice using the DATK32 antibody. Original magnification, ×200.* **B:** *Positive staining for murine α4β7 integrin in a s.c. tumor induced by the variant cell line NQ22V; the number of α4β7-positive cells is significantly reduced when compared with that detected in s.c. masses induced by the parental cell line NQ22. Original magnification, ×200.* **C:** *Immunostaining with the DATK32 antibody of NQ22 cells infiltrating the kidney of an AKR mouse.* **D:** *Mesenteric lymph node from a leukemic SJL mouse infiltrated by SJ-1 cells showing a strong expression of α4β7 integrin. Original magnification, ×200. In all distant sites of leukemic cell dissemination, the large majority of lymphoblasts is α4β7 positive.* **E:** *α4β7/CD31 double immunolabeling of s.c. tumors from mice transplanted with NQ22 cells showing that α4β7-positive blasts have a prevalent perivascular location (α4β7 is in brown, CD31 in red). Magnification, ×400 and ×1000.*

with overt leukemia (data not shown). These findings were confirmed by immunocytochemical analysis that showed that in s.c. tumors the DATK32 antibody labeled the membrane of approximately 15 to 25% of NQ22 cells, whereas in all distant sites of leukemic dissemination (peripheral and abdominal lymph nodes, spleen, liver, kidney, and lung), remarkably higher numbers of α4β7-positive cells (50 to 80%) were detected (Figure 2). When compared with DATK32, M298 antibody usually decorated a larger percentage of NQ22 cells (data not shown). The difference was more evident in the s.c. tumors where β7-positive cells ranged from 45 to 60% of neoplastic cells. Interestingly, α4β7-positive cells were mainly located near vascular spaces delineated by CD31 immunostaining (Figure 2). Of note, in all sites examined, leukemic cell infiltrates were always centered by intensely VCAM-1-positive vascular spaces (Figure 3), whereas endothelial cells of uninvolved parenchymal areas almost invariably showed only

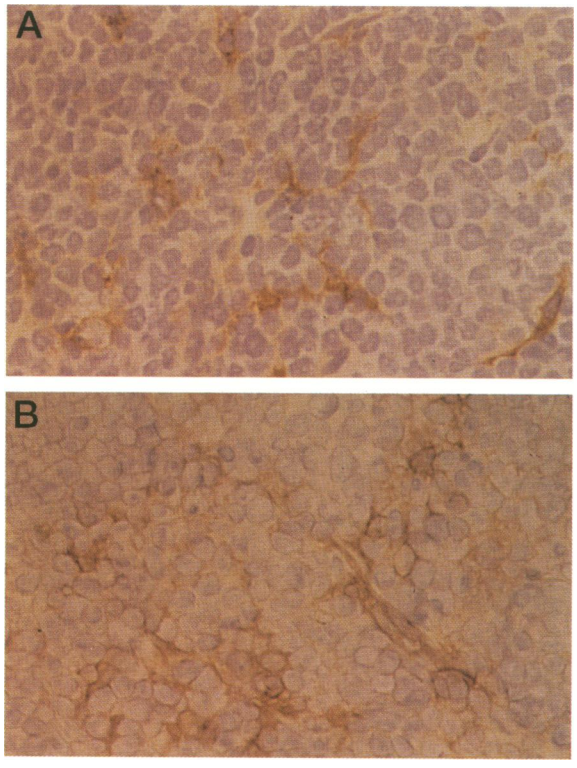

Figure 3. *Subcutaneous tumor* (**A**) *and kidney* (**B**) *from leukemic AKR mice transplanted with the NQ22 cell line. VCAM-1 is strongly expressed by endothelial cells of vessels surrounded by neoplastic cell infiltrates. Original magnification,* ×*400.*

weak staining for VCAM-1. Interestingly, in s.c. tumors, anti-VCAM-1 antibody stained not only endothelial cells but also some peri-endothelial, spindle cells often showing branching processes (Figure 3).

The Reduced Leukemic Potential of the NQ22 Variant Is Associated with a Delayed α4β7 Up-Regulation

The NQ22 variant (NQ22V) appeared spontaneously after more than 6 months of continuous *in vitro* culture. When compared with the parental NQ22 cell line, NQ22V cells showed a less rounded, irregular shape and a more pronounced tendency to grow in large sheets with a markedly reduced number of single elements. The immunophenotypic profile of cultured NQ22V was largely superimposable to that of the parental cell line (Thy1.1$^+$CD4$^-$CD8$^-$H2-K^{k+}CD11a/CD18$^+$CD26$^-$CD44$^-$CD54$^+$CD62L$^-$). Interestingly, the animals injected s.c. with NQ22V cells displayed a significantly delayed mortality in comparison with the parental cell line (Figure 4). Moreover, whereas NQ22 formed very small s.c. tumors (usually <80 to 100 mm^3), if any, NQ22V cells developed larger masses at the site of inoculation,

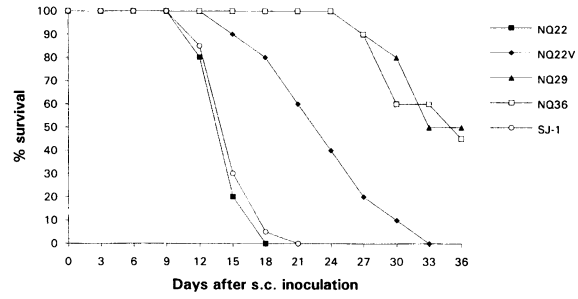

Figure 4. *Survival curve of AKR mice inoculated s.c. with the NQ22, NQ22V, NQ29, and NQ36 cell lines. Survival rate of SJL mice transplanted s.c. with the SJ-1 cell line are also shown.*

almost invariably greater than 200 to 300 mm^3. Nevertheless, all of the animals injected with NQ22V cells ultimately developed a fatal widespread leukemic picture almost indistinguishable from that of mice transplanted with NQ22 cells. When compared with the parental NQ22 cell line, a significantly reduced number of NQ22V cells expressing individual α4 and β7 chains and the α4β7 heterodimer were present in s.c. tumors (Table 1 and Figure 1). Conversely, as for NQ22 cells, high levels of α4β7 expression were detected in the large majority of NQ22V cells present in the peripheral blood or infiltrating the spleen of AKR mice with end-stage leukemia (Table 1). Consistently, also immunocytochemical analysis showed that in s.c. tumors of animals transplanted with NQ22V the number of α4β7- and β7-positive cells was greatly reduced, ranging from 5 to 10% and from 10 to 20%, respectively (Figure 2 and data not shown). With regard to the distant sites of leukemic dissemination, however, α4β7 staining pattern was similar to that observed in mice injected with NQ22 cells. When compared with the immunophenotypic profile of NQ22 cells, the delayed *in vivo* α4β7 up-regulation displayed by NQ22V cells was the only relevant change observed among the differentiation antigens (Thy1.1, CD4, CD8, and H2-K^k) and the other adhesion molecules (CD11a/CD18, CD26, CD44, CD54, and CD62L) investigated. Like the parental cell line, α4β7-positive NQ22V cells also displayed a prevalent perivascular location. In addition, the vascular structures surrounded by NQ22V cell infiltrates also constantly expressed high levels of VCAM-1.

Characterization of a Spontaneous SJL T-LBL with High Leukemic Properties

The SJ-1 lymphoma developed spontaneously in the thymus of a 10-month-old SJL mouse that also showed a macroscopic involvement of peripheral and abdominal lymph nodes and a remarkable en-

Table 2. *Differentiation Antigen Profile and α4β7 Integrin Expression of Neoplastic Cells from Patients with T-LBL*

Patient	Sex Age (years)	Source of cells	Stage	BM	α4β7 (%)*	Mib-1	TdT	CD2	CD3	CD5	CD7	CD4	CD8
1	M 5	Cervical LN	IV	+	+(>50%)	>75%	+	+	+	−	+	−	−
2	M 6	Supraclavicular LN	IV	+	+(25–50%)	>50%	+	+	+	+	+	+	+
3	F 11	Supraclavicular LN	IV	+	+(>50%)	>50%	+	+	+	+	+	+	+
4	M 3	Cervical LN	IV	+	+(25–50%)	>75%	+	+	+	−	+	−	−
5	M 7	Cervical LN	IV	+	+(25–50%)	>90%	+	+	+	−	+	−	−
6	M 6	Supraclavicular LN	IV	+	+(25–50%)	>50%	+	+	+	+	+	+	+
7	M 11	Cervical LN	IV	+	+(25–50%)	>90%	+	+	+	−	+	−	−
8	M 13	Cervical LN	III	−	−	>75%	+	+	+	−	+	−	−
9	F 12	Axillary LN	III	−	−	>75%	+	+	+	+	+	−	−
10	M 11	Supraclavicular LN	III	−	−	>75%	+	+	+	+	+	+	+
11	M 20	Inguinal LN	II	−	−	>90%	+	+	+	+	+	−	−

BM, bone marrow involvement at the time of diagnosis (in all cases with bone marrow involvement, the number of T blasts was <20%); M, Male; F, female; LN, lymph node.
*Percentage of α4β7-positive cells observed in involved lymph nodes.

largement of the spleen. The differentiation antigen profile of SJ-1 cells ($CD3^+CD4^+CD8^-HSA^+$) was consistent with the derivation from an immature lymphoid T cell. SJ-1 T-LBL was easily transplantable in syngeneic mice and showed an *in vivo* behavior very similar to that of the highly leukemic AKR cell line NQ22. In fact, mice injected s.c. with SJ-1 cells showed a very short survival (<20 days), with mortality rates close to those of animals transplanted with NQ22 cells (Figure 4). In addition, like NQ22 cells, SJ-1 T-LBL gave a very limited (<100 mm^3) or no development of tumors at the site of inoculation and a rapid and widespread involvement of lymphoid and nonlymphoid organs. At histological examination, all lymphoid areas were widely infiltrated by lymphomatous cells, whereas both liver and kidneys displayed a nodular and/or interstitial dissemination pattern. Aspects of perivascular infiltration by clusters of neoplastic cells were relatively frequent. Immunofluorescence (not shown) and immunohistochemical analyses (Figure 2D) showed that SJ-1 cells were also intensely α4β7 positive, with an *in vivo* staining pattern of DATK32 and M298 antibodies similar to that described for the NQ22 cells.

Histological and Immunophenotypic Characterization of Human T-LBLs

Clinical data of T-LBL patients, including age, sex, site of biopsied lymphadenopathy, stage, and bone marrow status at presentation, are reported in Table 2. Involved lymph nodes showed in all cases a distortion of the architecture due to heavy infiltration by neoplastic cells that entirely replaced the lymphoid tissue (Figure 5A). Lymphoma cells were relatively small or medium sized, with round or convoluted nuclei, finely dispersed chromatin, inconspicuous nucleoli, and scanty, basophilic cytoplasm. Tumor cells were not cohesive, and several mitotic figures were evident (Figure 5B). In some cases, numerous large macrophages displaying aspects of tumor cell phagocytosis were seen. At the time of diagnosis, bone marrow involvement was present in 7 of 11 cases (Table 2), with percentages of lymphoblasts ranging from 5 to 20% of the total cell population. Neoplastic cells infiltrating bone marrow looked like immature lymphoid elements with a high nuclear/cytoplasmic ratio and a basophilic cytoplasm without granules. Immunophenotypic analyses showed that in all cases T-LBL cells were TdT positive and expressed the T-cell-associated antigens CD2, CD3, and CD7; expression of other T-cell markers (CD4, CD5, and CD8) was variable (Table 2). Blast cell infiltrates were negative for CD10 and did not express the B-cell-associated antigens CD19, CD20, and CD22 (data not shown). The proliferative activity, assessed as nuclear positivity with the MAb Mib-1, ranged from 50 to 90% of the neoplastic cells.

α4β7 Integrin Expression in Human T-LBLs

The lymph node from the case of reactive follicular and paracortical hyperplasia showed scattered α4β7-positive lymphoid cells mainly located in the follicular mantle zone. Lymphoblasts from 7 of the 11 T-LBLs investigated stained positively for the ACT-1 antibody (Table 2). In involved lymph nodes, the ACT-1 antibody showed a diffuse pattern of immunoreactivity (>50% of positive cells) in two cases, whereas α4β7 staining was focal (between 25 and 50% of positive lymphoblasts) in the other five cases (Table 2 and Figure 5C). ACT-1 immunoreactivity was not clearly related to vascularization. Interestingly, α4β7 integrin expression was closely correlated with bone marrow involvement at diagnosis (Table 2). Moreover, all T-LBLs α4β7 positive in

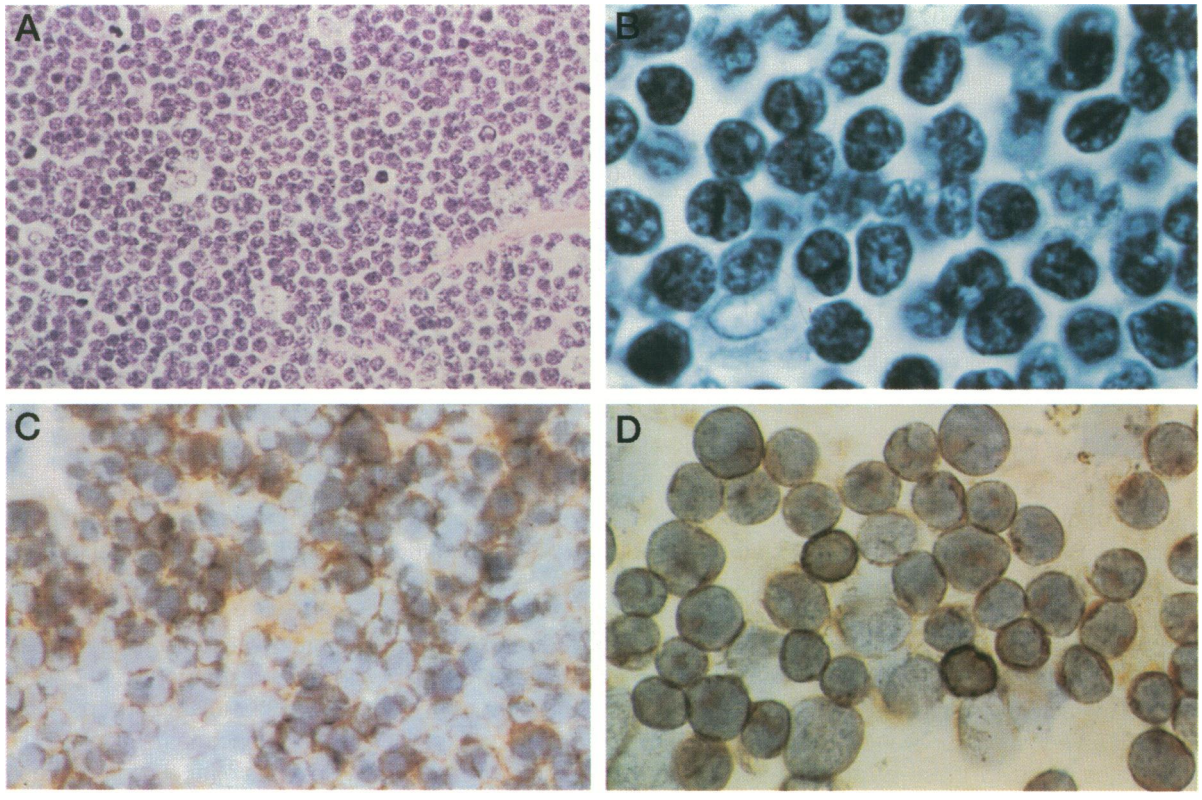

Figure 5. A: *Human T-LBL in lymph node. Neoplastic cells are separated from one another and often show mitotic figures. There are some large macrophages. H&E; original magnification, ×275.* B: *Lymphoma cells were relatively small or medium sized, with round or convoluted nuclei, finely dispersed chromatin, inconspicuous nucleoli, and scanty, basophilic cytoplasm. Giemsa; original magnification, ×1000).* C: *ACT-1 showing a diffuse pattern of immunoreactivity in a pathological lymph node from a T-LBL patient. Original magnification, ×500.* D: *The large majority of lymphoblasts in involved bone marrow show immunoreactivity with ACT-1 antibody. Original magnification, ×625.*

pathological lymph nodes showed a concomitant immunoreactivity with the ACT-1 antibody in nearly all blasts infiltrating the bone marrow (Figure 5D). Two metachronous localizations of the disease from 2 $\alpha4\beta7$-negative cases were also investigated: 1 testicular recurrence from patient 8 after 23 months from the diagnosis and an additional neoplastic lymphadenopathy from patient 11 after 51 months from the onset of the disease. No evidence of $\alpha4\beta7$ integrin expression was obtained in either of these samples (data not shown), suggesting that $\alpha4\beta7$-negative cases remain as such over time. Of note, neither of these two cases showed bone marrow involvement during the course of the disease, apart from the preterminal phase when a fatal, generalized dissemination of T lymphoblasts occurred. When compared with negative cases, $\alpha4\beta7$-expressing T-LBLs were significantly more prevalent in younger patients (<11 years of age; $P = 0.02$; Table 2). No significant correlation was found between $\alpha4\beta7$ integrin expression and sex, proliferative rate (as indicated by the percentage of Mib-1-positive cells), and immunophenotypic features of T-LBLs (Table 2).

Discussion

The present study aimed at elucidating whether $\alpha4\beta7$ integrin expression correlated with the leukemic behavior of murine and human T-LBLs. The results obtained in the AKR mouse system extend our previous observations[13] and confirm that the *in vivo* up-regulation of the $\alpha4\beta7$ heterodimeric complex is associated with widespread dissemination of T-LBL cells and a leukemic pattern of tissue infiltration. This correlation was demonstrated not only in a series of AKR T-LBL lines with differential leukemic properties but also by analyzing comparatively the expression of $\alpha4\beta7$ in the highly leukemic cell line NQ22 and the relative variant NQ22V characterized by a reduced leukemic potential. In fact, when compared with the parental cell line, NQ22V induced larger s.c. tumors containing significantly fewer $\alpha4\beta7$-positive cells. Nevertheless, as for NQ22, high expression levels of $\alpha4\beta7$ were detected in the large majority of NQ22V cells present in the peripheral blood or infiltrating the spleen and lymph nodes of leukemic animals. These results indicate that the decreased spreading ability

of NQ22V is associated with a delayed *in vivo* up-regulation of α4β7 and provide indirect evidence supporting a possible role for α4β7 in mediating the hematogenous dissemination of T-LBL cells. Interestingly, the correlation between α4β7 and leukemic behavior of T-LBL was not restricted to the AKR model as the highly leukemic cell line SJ-1, derived from a spontaneous T-LBL of the SJL strain, also displayed the same high levels of α4β7 expression observed in NQ22 cells and a similar pattern of tissue distribution. Of note, immunohistochemical analyses revealed that, in s.c. tumors induced by NQ22, NQ22V, and SJ-1 cell lines, the α4β7-positive cells had a prevalent perivascular location. In particular, the detection of high levels of VCAM-1 expression on peri-endothelial branching cells is consistent with the possibility that the interaction of α4β7-positive blasts with these cells through VCAM-1 may help direct T-LBL cells into vessels. These findings, together with the observation that integrins can transduce signals promoting cell migration and basement membrane invasion,[30,31] suggest that α4β7 may contribute to leukemic cell dissemination from the earlier phases of this process, perhaps favoring intravasation. These observations open an interesting area for future investigation and emphasize the usefulness of experimental models of spontaneous cell dissemination.

Available experimental data indicate that, like the closely related α4β1 integrin,[14,32] the α4β7 heterodimer also mediates interactions between lymphocytes and endothelial cells favoring primary contact and supporting subsequent rolling as well as rapid sticking and arrest, which may ultimately result in cell extravasation.[33] Besides playing an important role in physiological processes, these α4β7-mediated interactions, and in particular the binding to VCAM-1 and MAdCAM-1, may also contribute to leukemic evolution of murine T-cell lymphomas. In particular, our results support the hypothesis that the widespread pattern of tissue infiltration displayed by the NQ22 cell line may be largely determined by the binding to VCAM-1. In fact, in most of the tissues involved by NQ22 dissemination, and particularly in liver, kidney, and lung, leukemic cell infiltrates surrounded strongly VCAM-1-positive vascular spaces. Studies aimed at characterizing the functional properties of α4β7-positive murine lymphoma cells are in progress (manuscript in preparation).

Perhaps the most relevant finding of the present study is the close association between α4β7 expression and human T-LBLs displaying bone marrow involvement at diagnosis. In fact, pathological lymph nodes from all seven cases with bone marrow infiltration carried ACT-1-staining cells, whereas all four aleukemic T-LBLs were repeatedly α4β7 negative, also in metachronous lesions. Interestingly, the pattern of α4β7 positivity in involved lymph nodes was mainly focal, whereas nearly all neoplastic cells infiltrating bone marrow expressed the α4β7 integrin. These characteristics are strikingly similar to those displayed by murine T-cell lines with high leukemic potential and support the hypothesis that the α4β7 heterodimer may contribute to the leukemic dissemination of T-LBLs. It is worth considering in this respect that human leukemia/lymphoma cells bind to marrow stromal cells mainly through interactions between VCAM-1 and α4 integrins.[34–36] Of note, anti-α4 but not anti-β1 antibodies were shown to significantly inhibit adhesion of T-LBL cells to unstimulated marrow stromal cells.[36] These findings suggest that T-LBLs may preferentially use the α4β7 heterodimer to interact with VCAM-1-positive marrow stromal cells, a hypothesis that, however, needs to be confirmed.

The clinicopathological distinction between T-LBL and T-ALL has long been a matter of debate,[1–6] and some authors have proposed the common designation of lymphoblastic lymphoma/leukemia, indicating that these manifestations may represent parts of a single disease spectrum, as suggested by Lennert and Feller[37] and, more recently, by the revised European-American classification of lymphoid neoplasms.[38] Initial studies aimed at defining the differentiation profile of T blasts have shown a marked degree of immunophenotypic heterogeneity, with a substantial overlap between the two disorders, although T-ALL often displayed a more immature thymic phenotype.[3–5] More recently, the observation that the T-cell receptor-γδ is more frequently used in T-ALL, whereas the αβ-heterodimer is expressed in most T-LBLs suggested that these diseases may have a different origin.[6] Our results provide further support to this hypothesis, as expression of α4β7 integrin appears to discriminate between two different groups of T-LBLs otherwise indistinguishable on the basis of conventional histopathological and immunocytochemical findings: α4β7-negative cases, which remain aleukemic throughout the course of the disease, and α4β7-positive cases displaying limited but detectable bone marrow involvement at diagnosis and frequent evolution to an overtly leukemic picture. Moreover, in our T-LBL series, α4β7 expression was significantly more prevalent in younger patients (<11 years; $P = 0.02$), an observation that provides added support to the possible existence of two different clinicopathological entities. Such a hypothesis has also been substantiated by recent cy-

togenetic findings showing that approximately 40 to 70% of T-ALLs/T-LBLs with mediastinal masses shared similar chromosomal translocations, particularly those involving 14q11–13 or 7q35,[39] whereas a t(9;17) translocation was detected only in a subset of T-LBLs without bone marrow involvement, either at presentation or during the natural history of the disease.[39,40]

In conclusion, the results of the present study demonstrate that the expression of $\alpha4\beta7$ integrin is closely correlated with the leukemic behavior of both murine and human T-LBLs. Moreover, $\alpha4\beta7$ appears to constitute a useful marker to distinguish between human T-LBLs with bone marrow involvement and cases that remain aleukemic throughout the course of the disease. It is well known that lymphoblastic lymphomas and leukemias of T-cell lineage share a highly malignant behavior, and clinical experience accumulated so far has indicated that these disorders should be treated with similar aggressive therapeutic regimens. Although the recent therapeutic advances have markedly improved the overall prognosis of T-LBL, many patients still fail to achieve complete remission and cannot be ultimately cured. The possibility of using an immunophenotypic marker such as $\alpha4\beta7$ may potentially allow discrimination between aleukemic T-LBLs and cases with bone marrow involvement at presentation. This may allow verification of the effectiveness of current regimens on more homogeneous groups of patients, and of the design of new strategies of intervention for a better clinical control of these disorders.

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