A cytotoxic phenotype does not predict clinical outcome in anaplastic large cell lymphomas

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Abstract

Aim—To investigate whether anaplastic large cell lymphomas (ALCL) expressing cytotoxic proteins have a relatively worse clinical outcome compared with ALCL lacking a cytotoxic phenotype.

Methods—59 primary cases of ALCL originating from different sites were investigated by immunohistochemistry for the presence of the cytotoxic proteins T cell intracytoplasmic antigen (TIA-1) and granzyme B in the neoplastic cells. Since site of origin and expression of anaplastic lymphoma kinase (ALK) strongly influence prognosis, the presence of a cytotoxic phenotype was also investigated in relation to the primary site of origin (lymph node, gut, or skin) and ALK expression. The prognostic value was investigated by analysis of overall and relapse-free survival time, including Cox regression analysis.

Results—39 of 59 ALCL (66%) appeared to have a cytotoxic phenotype as shown by expression of TIA-1 or granzyme B or both in the neoplastic cells. The presence of a cytotoxic phenotype did not have any influence on prognosis. Even when the survival data were corrected for site of origin and stage at presentation or were analysed separately for ALK positive and negative cases, no prognostic influence of a cytotoxic phenotype was observed.

Conclusions—In primary biopsies of patients with ALCL, the presence of a cytotoxic phenotype is not related to clinical outcome of the disease.
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Keywords: granzyme B; anaplastic large cell lymphoma; prognosis

Anaplastic large cell lymphomas (ALCL) are considered to be a subgroup of non-Hodgkin's lymphomas (NHL), characterised by the presence of cohesive sheets of large CD30 positive anaplastic cells with prominent nucleoli.[1] At present only T cell or null cell proliferations are identified as ALCL in the International Lymphoma Study Group classification proposal.[2] A consistent finding in a significant proportion of ALCL cases is the t(2;5) translocation fusing the nucleophosmin gene (NPM) with a tyrosine kinase gene named anaplastic lymphoma kinase (ALK), resulting in NPM/ALK expression. Although the function of this protein remains unclear,[3–6] the presence of NPM/ALK fusion protein in neoplastic cells of ALCL cases has been found to be correlated with a better prognosis.[3] [6–8]

Recent studies indicate that ALCL have phenotypic features of activated cytotoxic T cells.[9–12] Using antibodies directed against granzyme B, perforin, and T cell restricted intracytoplasmic antigen (TIA-1), it is possible to detect a population of cells with potential cytotoxic activity.[13–15] It has been shown that granzyme B and perforin expression is restricted to activated lymphocytes and natural killer cells,[16] [17] whereas TIA-1 is found in both resting and activated cytotoxic T cells and natural killer cells. In comparison with granzyme B, TIA-1 is more abundantly expressed, in up to 60% of CD8 positive lymphocytes and in a small minority (6%) of CD4 positive T lymphocytes.[18]

It could be hypothesised that lymphomas originating from cytotoxic T cells (or natural killer cells) may be particularly resistant to apoptosis since cytotoxic T cells themselves are resistant to perforin/granzyme B mediated lysis.[19] Several mechanisms are likely to be involved in producing resistance against apoptosis of cytotoxic T cells. There is evidence that an as yet unidentified membrane protein on the surface of cytotoxic T cells and natural killer cells protects these cells against perforin mediated lysis.[19] Furthermore, in vitro experiments have shown that (tumour) cells can escape granzyme B mediated apoptosis by expressing certain intracellular serine protease inhibitors (serpins). A recently cloned serpin called PI-9 was shown to be a potent inhibitor of granzyme B mediated apoptosis.[20] These observations indicate that evasion of granzyme B/perforin mediated apoptosis might be important in tumour cell resistance against cytotoxic T cell mediated cytotoxicity.[11] [21]

Although tumour cells showing resistance to apoptosis induced by cytotoxic T cells are not inherently also resistant to apoptosis induced by either radiotherapy or chemotherapy, several lines of research indicate that the different apoptosis inducing signals share one or more common final lethal pathways.[22–24] Thus resistance to cytotoxic T cell induced apoptosis may also lead to resistance to chemotherapy induced apoptosis, resulting in a poor clinical outcome in ALCL patients in whom the tumour cells show a cytotoxic phenotype.

Since the site of origin and ALK expression strongly influence prognosis,[3] [25] [26] we investigated 59 cases of ALCL (44 nodal, three gastrointestinal, and 12 cutaneous) and determined whether a cytotoxic phenotype, alone or in relation to site or ALK expression,

Table 1 Patient and tumour characteristics at the time of first presentation in all cases used for survival analysis (n = 59)

Characteristic	Nodal ALCL		Gut associated ALCL		Cutaneous ALCL	
	GrB/TIA-1 pos (n=29)	GrB/TIA-1 neg (n=15)	GrB/TIA-1 pos (n=2)	GrB/TIA-1 neg (n=1)	GrB/TIA-1 pos (n=8)	GrB/TIA-1 neg (n=4)
Median age (y)	39	44	45	74	65	50
Sex (M/F)	19/10	10/5	1/1	0/1	5/3	4/0
Disease stage						
I	5	4	0	0	8	4
II	9	4	0	1	0	0
III	7	3	2	0	0	0
IV	8	4	0	0	0	0
B symptoms	14	10	0	1	0	0
CR	24	11	1	1	8	4
Relapse	11	7	2	0	1	2
Cause of death						
ALCL	9	6	2	0	0	0
Other	1	0	0	0	2	0

ALCL, anaplastic large cell lymphoma; CR, complete remission; GrB, granzyme B; TIA-1, T cell intracytoplasmic antigen.

influences the clinical behaviour of these lymphomas in terms of overall survival and relapse-free survival time.

Methods

PATIENT SELECTION
Paraffin wax embedded tissue specimens of 59 primary anaplastic large cell lymphomas were selected from the archives of the Comprehen- sive Cancer Centre, Amsterdam (n = 56) and the St Elizabeth Hospital, Tilburg (n = 3). ALCL from different sites of origin—that is, lymph nodes (n = 44), gastrointestinal tract (n = 3), and skin (n = 12)—were included.

The diagnosis of ALCL was made according to the criteria of the updated Kiel classification, which is also incorporated in the REAL classification.[2 27]

Table 2 Patient and tumour characteristics in primary nodal anaplastic large cell lymphomas (ALCL)

Case	GrB*	TIA-1*	ALK	CD3	CD4	CD8	Stage	Rx	CR	Rel	Surv†	Status
Nodal ALCL with a cytotoxic phenotype												
1	86	18	+	+	−	−	II	PC	+	−	15	AAW
2	60	86	+	+	−	−	II	PC+R	+	−	46	AAW
3	47	92	+	−	nd	nd	III	PC	+	−	71	AAW
4	30	30	+	+	+	−	II	PC+R	+	−	51	AAW
5	30	30	+	−	+/−	−	IV	PC	+	−	36	AAW
6	30	30	+	−	+	−	I	R	+	+	34	AWD
7	30	30	+	−	+	−	III	PC	+	−	27	AAW
8	30	30	+	−	−	−	I	PC	+	−	21	AAW
9	10	30	+	−	−	−	I	PC+R	+	−	9	AAW
10	7	27	+	+	+	−	II	PC+R	+	+	126	AWD
11	1	57	+	+	−	−	II	PC	+	−	106	AAW
12	0	72	+	−	nd	−	II	PC	+	−	33	AAW
13	92	90	−	+	−	+	III	PC	+	+	12	DOD
14	69	38	−	+	−	−	III	PC	+	−	64	AAW
15	31	32	−	−	nd	−	IV	PC	+	−	207	AAW
16	30	20	−	+	−	−	IV	PC	+	−	32	AAW
17	21	44	−	+	+	−	IV	PC	−	+	1	DOD
18	20	63	−	−	+	−	II	PC+R	+	+	26	DOD
19	20	17	−	−	nd	−	III	PC+R	+	+	8	DOD
20	10	30	−	−	−	−	IV	PC	−	+	4	DOD
21	10	30	−	−	+	−	III	PC	+	+	20	DOD
22	10	10	−	−	−	−	I	PC	+	−	54	AAW
23	10	30	−	+	+	−	IV	PC	−	+	1	DOD
24	10	25	−	−	+	−	IV	PC	+	−	7	DOC
25	10	10	−	+	+/−	−	IV	PC	+	−	29	AAW
26	10	10	−	−	+	−	II	PC	−	+	2	DOD
27	1	19	−	−	−	−	I	PC	+	−	66	AAW
28	0	10	−	−	+	−	III	PC	−	+	12	DOD
29	5	74	nd	−	−	−	II	PC	+	−	32	AAW
Nodal ALCL without a cytotoxic phenotype												
30	0	0	+	−	+	−	I	PC+R	+	−	75	AAW
31	0	0	−	−	+	−	I	PC+R	+	−	100	AAW
32	0	0	−	+	−	−	I	R	+	+	13	DOD
33	0	0	−	+	nd	nd	III	PC	+	+	74	AAW
34	0	0	−	+	+	−	II	PC+R	+	−	49	AAW
35	0	0	−	+	−	−	IV	PC	+	−	44	AAW
36	0	0	−	−	+	−	I	R	+	+	25	DOD
37	0	0	−	−	+	−	III	PC	+	−	22	AAW
38	0	0	−	−	−	−	III	PC	−	+	2	DOD
39	0	0	−	−	−	−	IV	none	−	+	0	DOD
40	0	0	−	−	−	−	IV	none	−	+	1	DOD
41	0	0	−	−	+	−	II	PC	+	−	5	AAW
42	0	0	−	−	+	−	II	PC	+	−	12	AAW
43	0	0	−	+	+	nd	IV	PC	−	+	3	DOD
44	0	0	nd	nd	nd	nd	II	PC	+	−	10	AAW

AAW, alive and well; ALK, anaplastic lymphoma kinase; AWD, alive with disease; CR, complete remission; DOC, died of other cause; DOD, died of disease; GrB, granzyme B; nd, not determined; PC, polychemotherapy; Rel, relapse; Rx, treatment; Surv, survival; TIA-1, T cell intracytoplasmic antigen.
*Percentage of positive neoplastic cells determined using the Q-Prodit system.
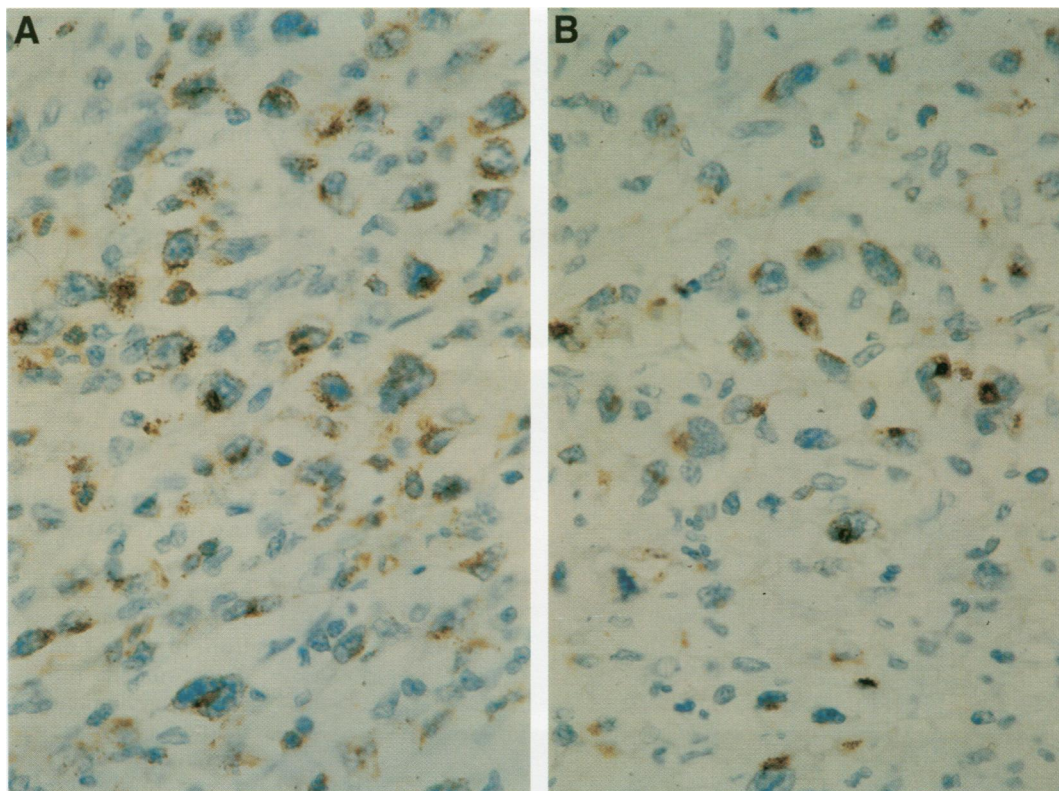†Survival in months.

Figure 1 Anaplastic large cell lymphoma. (A) A strong granular TIA-1 specific signal (brown) is observed in the cytoplasm of the large majority of neoplastic cells. (B) A granular dot-like staining of granzyme B (brown) in the neoplastic cells of the same anaplastic large cell lymphoma. (Haematoxylin counterstaining.)

Patient characteristics are summarised in table 1. The stage of the disease at first presentation was determined by standard staging procedures including full blood count, x rays of the chest, computed tomography of the abdomen, bone marrow aspiration, and biopsy.

Except in three cases, all patients with nodal and gut associated tumours received polychemotherapy consisting of CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) or CHOP-like regimens, some receiving involved field radiation alone (n = 3) or in addition to chemotherapy (n= 9, table 2). One patient (No 39, table 2) died before treatment could be given, while another patient (No 40, table 2) declined treatment on personal grounds. In 49 of the 59 cases, complete remission was achieved but 23 patients had a relapse.

IMMUNOHISTOCHEMISTRY

In all cases formalin fixed paraffin embedded tissue sections were used for immunohistochemical detection of granzyme B and TIA-1. The antibody raised against TIA-1 (Coulter Corporation) recognises a 15 kDa protein in the cytoplasmic granules of potentially cytotoxic T cells and belongs to the IgG1 subclass.[13][28] The production and characterisation of a granzyme B directed monoclonal antibody (GB7) has been described previously.[15] Briefly, the IgG2a subclass monoclonal antibody was raised against recombinant human granzyme B and reacted with granzyme B isolated from activated human cytotoxic lymphocytes and natural killer cells. Both

TIA-1 and GB7 can be applied on routine formalin fixed, paraffin embedded tissue specimens.

For granzyme B and TIA-1 detection, sections were boiled in citrate buffer (0.01 M pH 6.0) using a microwave oven (2 × 5 minutes at 100°C; maximum power 700 W). They were then stained with monoclonal antibodies against granzyme B and TIA-1 (diluted 1:500 and 1:250, respectively) and incubated for one hour at room temperature, employing a three step method using a biotinylated rabbit anti-mouse monoclonal antibody and streptavidin–biotin–horseradish peroxidase (HRP) complex (Dako). Cases were further characterised using antibodies directed against CD3, CD4, CD8, and CD30 as well as B cell markers (CD20 and MB2 or CD79a).

ALK expression was detected using the monoclonal antibody ALK1 as described previously,[29] with slight modifications. The ALK antibody was diluted 1:75 and slides were incubated overnight at 4°C and staining was enhanced using the CARD method.[30]

When double staining procedures were performed (n = 4), granzyme B and TIA-1 were applied simultaneously for 60 minutes at room temperature. Subsequently TIA-1 was detected using biotinylated goat anti-IgG1 and GB7 with goat anti-IgG2a conjugated with HRP and applied simultaneously for 30 minutes. Biotinylated goat anti-IgG1 (Southern Biotechnology Association) was detected using streptavidin Cy3 (Jackson Immunoresearch Laboratories) for 90 minutes, while goat anti-IgG2a HRP was detected by the deposition of fluorescein isothiocyanate
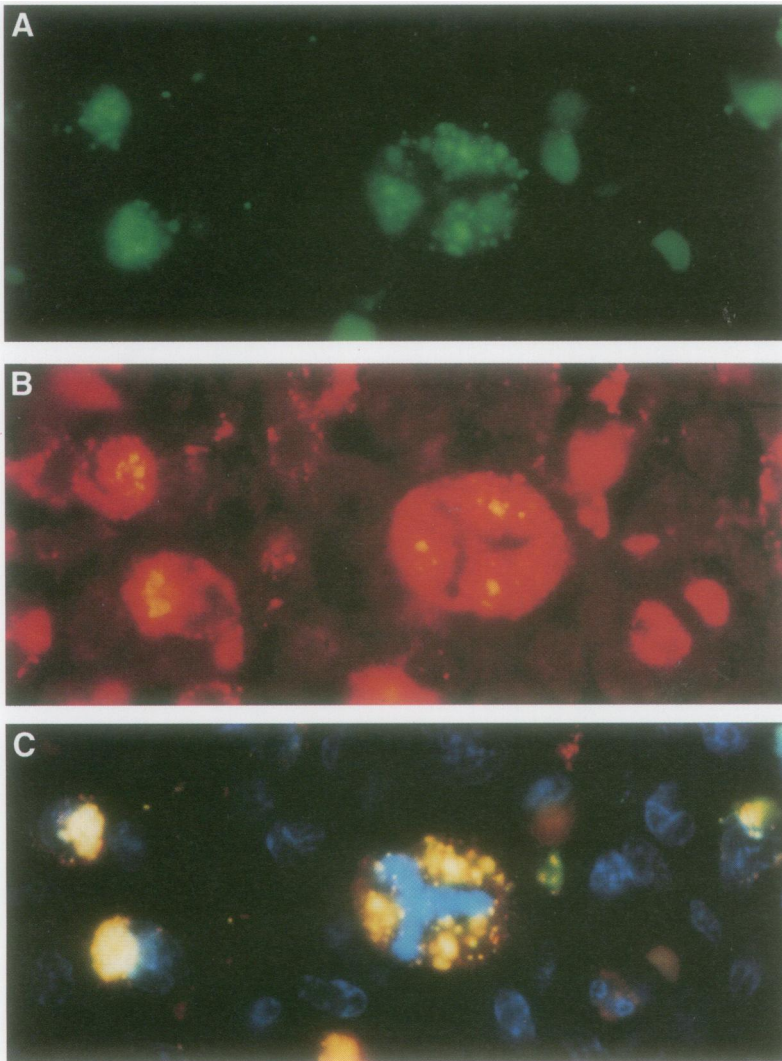
Figure 2   Double staining of granzyme B and TIA-1 in a nodal anaplastic large cell lymphoma. (A) green staining (FITC conjugated tyramine) indicates granzyme B expression in neoplastic cells. (B) TIA-1 (red), visualised by cy3 labelled streptavidin. Tumour cells showing a granular expression of TIA-1 in the cytoplasm. (C) Granzyme B/TIA-1 double staining as detected using a triple filter, simultaneously visualising coexpression of TIA-1 and granzyme B (yellow) and DAPI counterstained nuclei (blue).

(FITC) conjugated tyramine (15 minutes) as described previously.[31] The sections were counterstained with DAPI and evaluated with a Leica DMRB fluorescence microscope.

Surrounding reactive lymphocytes were used as a positive internal control. Negative controls consisted of simultaneously processed slides with isotype specific antibodies instead of GB7 and TIA-1, respectively.

RNA IN SITU HYBRIDISATION

To demonstrate that granzyme B positive staining of tumour cells represented intracellular production rather than passive uptake from surrounding non-neoplastic reactive lymphocytes, RNA in situ hybridisation (RISH) was performed to detect granzyme B at the mRNA level (n = 4). The RISH procedure used has been described previously.[32]

INTERPRETATION OF IMMUNOHISTOCHEMICAL RESULTS

A case was considered to possess a cytotoxic phenotype when unequivocal staining of neoplastic cells (irrespective of the number of positive cells) for either TIA-1 or granzyme B

was detected according to criteria described by Foss *et al.*[10] The percentage of tumour cells staining positive for granzyme B and TIA-1 was quantified using a commercially available interactive video-overlay based measuring system (Q-Prodit, Leica), as described previously.[21] The microscopic image was recorded by a charged coupled device camera and displayed on a monitor. A 40× objective was used, giving a final magnification of 1200×, which appeared the most suitable for this purpose. One hundred fields of vision were systematically randomly selected using an automatic scanning stage controlled by Q-Prodit. In these fields at least 100 tumour cells, as determined by morphology, were sampled using a Weibel six point test grid (point distance 50 μm) and scored positive and negative for granzyme B or TIA-1.[33]

ANALYSIS OF CLINICAL DATA

For statistical analysis we used 59 biopsies from primary tumours from which the clinical data could be retrieved. For each patient the following characteristics were noted from the medical records: age, sex, clinical stage at first presentation, presence of B symptoms, response to treatment, occurrence of relapses, and cause of death. The median follow up time was 25 months (range 0 to 207 months). Survival time was measured from the time of initial diagnosis until death from the disease or until the date of last follow up. Patients who died of causes unrelated to the disease were censored at the time of death. Relapse-free survival time was measured from time of initial diagnosis until time of disease relapse. Patients who did not enter complete remission were assigned a relapse-free survival time of zero in the analysis.

STATISTICAL ANALYSIS

Survival curves were constructed with the Kaplan–Meier method. Differences between the curves were analysed using the log-rank test. Cox regression analysis[34] (enter and remove limits 0.1) was used to investigate whether the percentage of granzyme B/TIA-1 positive tumour cells (when entered as a continuous variable) was related to prognosis. Comparisons of means were performed using the Student t test or the Mann Whitney U test, where appropriate. Qualitative variables were analysed by Pearson χ^2 test or by the Fisher exact test, where appropriate. All values were based on two tail statistical analysis. Probability (p) values below 0.05 were considered significant. All analyses were performed using the SPSS statistical software package.

Results

EXPRESSION OF CYTOTOXIC PROTEINS IN REACTIVE CELLS IN ALCL

In all cases tested, both granzyme B and TIA-1 positive non-neoplastic small reactive lymphocytes were found, thus serving as positive controls. In these cells a granular staining pattern was observed for either GB7 or TIA-1. The percentage of reactive lymphocytes

Table 3   Patient and tumour characteristics of primary gut associated and cutaneous anaplastic large cell lymphomas (ALCL)

Case	GrB*	TIA-1*	ALK	CD3	CD4	CD8	Stage	Rx	CR	Rel	Surv†	Status
Gut associated ALCL with a cytotoxic phenotype												
45	90	92	−	+	−	−	III	PC	−	+	1	DOD
46	79	88	−	+	+	−	III	PC	+	+	13	DOD
Gut associated ALCL without a cytotoxic phenotype												
47	0	0	−	+	+	−	II	PC+R	+	−	80	AAW
Cutaneous ALCL with a cytotoxic phenotype												
48	57	44	−	+	+	−	I	R	+	−	16	DOC
49	20	60	−	+	+	−	I	R	+	−	13	DOC
50	18	20	−	+	+	−	I	None	+	−	68	AAW
51	14	23	−	+	+	−	I	PC	+	−	57	AAW
52	11	15	−	+	+	−	I	PC	+	−	61	AWD
53	9	15	−	+/−	+	−	I	PC	+	+	85	AWD
54	7	17	−	+	+	−	I	R	+	−	246	AAW
55	5	11	−	+	+	−	I	None	+	−	32	AAW
Cutaneous ALCL without a cytotoxic phenotype												
56	0	0	−	+/−	+	−	I	PC	+	−	123	AAW
57	0	0	−	+	−	−	I	PC	+	+	47	AAW
58	0	0	−	+	+	−	I	None	+	+	24	AWD
59	0	0	−	−	+	−	I	None	+	−	36	AAW

AAW, alive and well; ALK, anaplastic lymphoma kinase; AWD, alive with disease; CR, complete remission; DOC, died of other cause; DOD, died of disease; GrB, granzyme B; nd, not determined; PC, polychemotherapy; Rel, relapse; Rx, treatment; Surv, survival; TIA-1, T cell intracytoplasmic antigen.
*Percentage of positive neoplastic cells determined using the Q-Prodit system.
†Survival in months.

expressing granzyme B ranged from 1% to 61%, whereas TIA-1 expression ranged from 9% to 75% of reactive lymphocytes.

EXPRESSION OF CYTOTOXIC PROTEINS IN NEOPLASTIC CELLS OF ALCL

In 37 of the 59 cases studied (63%), the neoplastic cells showed expression of TIA-1 and granzyme B in the tumour cells. In two additional cases only expression of TIA-1 was detected. For both TIA-1 and granzyme B, the characteristic granular or dot-like cytoplasmic staining in the neoplastic cells was observed (fig 1). The number of tumour cells staining positive for either granzyme B or TIA-1 ranged from a few positive tumour cells to over 90% of all tumour cells. All cases positive for granzyme B were also positive for TIA-1. In all but two cases, TIA-1 positive tumour cells outnumbered the granzyme B positive tumour cells (table 2). Coexpression of TIA-1 and granzyme B in the tumour cells was confirmed using double staining procedures, showing colocalisation of granzyme B and TIA-1 in the cytoplasmic granules in all four cases tested (fig 2).

CYTOTOXIC PHENOTYPE OF ALCL IN RELATION TO SITE OF ORIGIN

In nodal ALCL, both TIA-1 and granzyme B expression was found in tumour cells in 27 of 44 cases (61%), with an additional two cases showing only TIA-1 expression. In cutaneous ALCL, expression of both TIA-1 and granzyme B in tumour cells was found in eight of 12 cases. In gut associated ALCL, two of three cases showed a cytotoxic phenotype (table 3). Thus the cytotoxic phenotype of ALCL was not associated with a certain site of origin.

Of the ALCL with a cytotoxic phenotype, the gut associated ALCL had the highest mean percentage of neoplastic cells expressing both TIA-1 and granzyme B, whereas in cutaneous

lymphomas only a minority of the neoplastic cells showed expression of TIA-1 or granzyme B or both. The nodal ALCL cases showed a highly variable percentage of tumour cells with a cytotoxic phenotype, ranging from a few to more than 90% of tumour cells (table 2).

CYTOTOXIC PHENOTYPE OF ALCL IN RELATION TO ALK EXPRESSION

In nodal ALCL with a cytotoxic phenotype, 12 of 28 cases were ALK positive, compared with only one ALK positive case of 14 nodal ALCL without a cytotoxic phenotype (p = 0.02). No ALK expression was observed in gut associated and cutaneous cases (table 3).

CYTOTOXIC PHENOTYPE OF ALCL IN RELATION TO IMMUNOPHENOTYPE

It appeared that with two exceptions, all primary skin and gut associated ALCL were CD3+ and CD4+, irrespective of granzyme B/TIA-1 expression, whereas expression of T cell markers in nodal ALCL was found to be more heterogeneous. In nodal granzyme B/TIA-1 positive cases, five CD3+/CD4+/CD8− cases were found, six cases positive for CD3 only, eight positive for CD4 only, one CD3−/CD4−/CD8+ case, and six CD3−/CD4−/CD8− cases. These marker profiles did not differ significantly from nodal granzyme B/TIA-1 negative cases: three CD3+/CD4+/CD8− cases, one case positive for CD3 only, five positive for CD4+ only, and three CD3−/CD4−/CD8− cases (table 4).

RNA IN SITU HYBRIDISATION

Two cases (one nodal case and one gut associated ALCL) which were strongly positive for granzyme B by immunohistochemistry, and two cases (nodal ALCL) which were negative for granzyme B, were used for RISH. In these cases good RNA quality was shown by clear RISH signals for β_2 microglobulin. In both

Table 4   Anaplastic lymphoma kinase (ALK) and T cell marker expression on neoplastic cells of primary nodal anaplastic large cell lymphomas (ALCL) with and without a cytotoxic phenotype

	Cytotoxic phenotype of ALCL†		
	Positive	Negative	p Value*
ALK	(n = 28)	(n = 14)	
Positive	12	1	
Negative	16	13	0.02
CD3	(n = 29)	(n = 14)	
Positive	11	6	
Negative	18	8	NS
CD4	(n = 25)	(n = 13)	
Positive	13	8	
Negative	12	5	NS
CD8	(n = 28)	(n = 12)	
Positive	1	0	
Negative	27	12	NS

†A case was considered to possess a cytotoxic phenotype when unequivocal staining of neoplastic cells was observed irrespective of the number of cells staining positive.
*As determined by Pearson χ^2 test or Fisher exact test.

granzyme B positive cases, granzyme B could also be demonstrated at the mRNA level in the neoplastic cells in addition to some reactive infiltrating lymphocytes (fig 3). In cases with granzyme B negative tumour cells, no expression of granzyme B mRNA was detected in the neoplastic cells, whereas some infiltrating lymphocytes did express granzyme B specific mRNA. These data show that granzyme B positive staining of tumour cells is not the result of passive uptake from surrounding non-neoplastic reactive lymphocytes but represents intracellular production.

PATIENT CHARACTERISTICS AND PROGNOSTIC VALUE OF NEOPLASTIC CELLS WITH A CYTOTOXIC PHENOTYPE

The clinical features of the 59 patients are summarised in table 1. When all sites were included, no major differences in clinical presentation were observed between granzyme B+/TIA-1+ cases and granzyme B−/TIA-1− cases. In both groups the majority of patients were between 25 and 50 years of age, with a mean of 46 years. Also no differences in stage at presentation were noticed.

Site of first presentation strongly influenced prognosis (table 5). This ranged from excellent in all cutaneous cases to moderate in nodal cases (five year survival 63%). Two of three patients with a gut associated ALCL died as a result of the disease. As expected, ALK expression in nodal cases was found to be a highly favourable prognostic marker; none of 13 patients with ALK positive ALCL died, whereas 17 of 44 patients with ALK negative ALCL died as result of the disease (p = 0.008).

The presence of a cytotoxic phenotype did not appear to have any impact on prognosis for either overall survival or relapse-free survival time when determined by either Cox regression analysis or by log-rank test. Even when different arbitrary thresholds for a minimum percentage of tumour cells with a cytotoxic phenotype was applied (that is, 5%, 10%, 15%, 20%, 30%, or 50% positive tumour cells), no group with a relatively poor prognosis could be defined (fig 4). Also, when adjusted for either stage at presentation, site, or ALK status, no differences were found in either overall survival or relapse-free survival time.
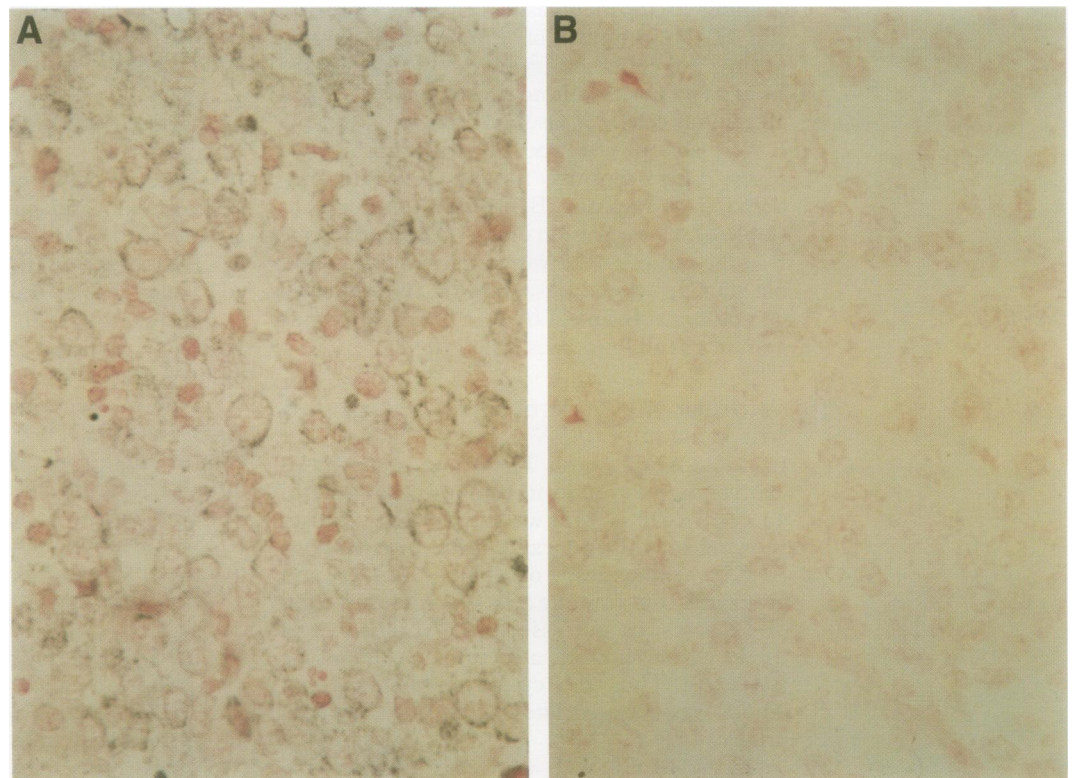
Figure 3   Granzyme B expression at the mRNA level in neoplastic cells using RNA in situ hybridisation. (A) Using an anti-sense probe, strong black staining is observed in the cytoplasm of the neoplastic cells, indicating the presence of granzyme B specific mRNA. (B) Sense control (fast nuclear red counterstaining).

Table 5 Data obtained by univariate survival analysis

Characteristic	n	Deaths due to ALCL	Patients alive after 5 years (%)*	p Value
Cytotoxic phenotype				
Positive	39	11	67	
Negative	15	6	53	NS
ALK				
Positive	13	0	100	
Negative	44	17	59	0.008
Stage				
I	21	2	90	
II	14	2	83	
III	12	7	36	
IV	12	6	50	0.0003
Site				
Nodal	44	15	63	
Gastrointestinal	3	2	33	
Cutaneous	12	0	100	0.03

†As estimated from Kaplan–Meier curves.
*Determined by log-rank test.
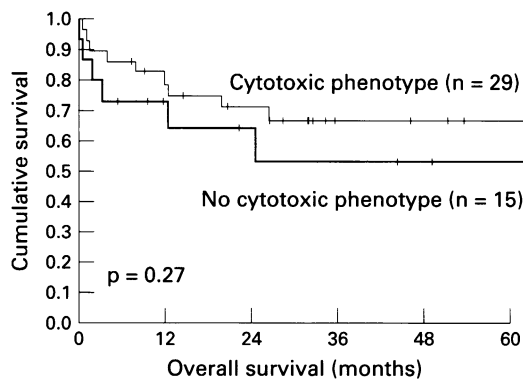
Figure 4 Comparison of overall survival time according to the presence of a cytotoxic phenotype in the neoplastic cells of primary nodal anaplastic large cell lymphomas.

When survival analysis was performed separately for ALK positive and ALK negative cases, a cytotoxic phenotype did not have any impact on clinical outcome.

Discussion

Our study shows that approximately 66% of all ALCL, irrespective of their site of origin, have a cytotoxic phenotype, indicating that—although the number of gut associated ALCL is small—the presence of a cytotoxic phenotype of ALCL is not related to the site of origin. These data are in line with those published by Krenacs et al and Boulland et al.[11] [35] Interestingly, the expression of ALK was nearly always associated with a cytotoxic phenotype and a good prognosis. This confirms previous findings that nodal ALCL showing ALK expression is a separate clinicopathological entity with a good prognosis.[3] Detection of granzyme B at the mRNA level excluded the possibility that detection of granzyme B expression was the result of passive uptake of granzyme B from the surrounding cytotoxic T cells.

Theoretically, the prognosis of ALCL originating from neoplastic equivalents of (activated) cytotoxic T cells should be relatively poor, since these cells are themselves resistant to cytotoxic T cell induced, and possibly also to treatment induced, apoptosis. However, our results clearly show that the presence of a cytotoxic phenotype does not have any prognostic significance on its own. Even when several arbitrary thresholds for a minimal percentage of tumour cells with a cytotoxic phenotype were applied, no group

with a relatively poor prognosis could be defined. Since (almost) equal percentages were observed for granzyme B/TIA-1 positive cases in nodal, cutaneous, and gut associated lymphomas, differences in clinical outcome between the different sites of origin cannot be ascribed to a cytotoxic phenotype of the neoplastic cells. In addition, the fact that nearly all ALK positive cases (with an excellent prognosis) also show a cytotoxic phenotype further emphasises that a cytotoxic phenotype is not necessarily related to a poor clinical outcome.

ALCL with a cytotoxic phenotype were hardly ever CD8 positive. In only one of 38 tested lymphomas possessing a cytotoxic phenotype did the tumour cells express CD8 (a nodal case), whereas 22 of 35 tested granzyme B/TIA-1 positive cases were CD4 positive. However, differences were noted for the different sites: all primary granzyme B/TIA-1 positive cutaneous cases and one of two gut associated cases were CD3+/CD4+/CD8−, whereas granzyme B/TIA-1 positive nodal ALCL cases showed considerable heterogeneity in T cell marker phenotype: CD4+/CD8−, CD4−/CD8+, or CD4− and CD8−. The fact that many granzyme B/TIA-1 positive cases are CD4 rather than CD8 positive is remarkable since in healthy individuals the majority of granzyme B/TIA-1 positive cytotoxic T lymphocytes are CD8 positive. Investigation of tumours without T cell marker expression revealed that these cases were also negative for natural killer cell markers CD56 and CD57 (data not shown). The biological relevance of CD4 positive cytotoxic T lymphocytes in normal immune surveillance is still unclear, but seems limited since these cells are sporadically detected in vivo and can be found only after prolonged cultivation in vitro in the presence of cytokines and antigen.[36–40] Recent data indicate that CD4 positive cytotoxic T cells can acquire an activated cytotoxic phenotype in the presence of γ interferon at high concentrations.[41] However, this was only observed in the absence of activated CD8 positive cytotoxic T cells.

Since cutaneous ALCL are consistently CD3/CD4 positive and CD8 negative, these lymphomas may be the neoplastic counterparts of a unique site specific subset of cytotoxic CD4 positive T cells,[12] whereas nodal cases may originate from different subsets of potentially cytotoxic T cells. Another possibility is that these cases arise from CD4 positive, CD8 negative T cells that acquire a cytotoxic phenotype on long term antigenic stimulation or on activation by the presence of high levels of certain cytokines.[42] One indication that cytokine levels may be involved in the cytotoxic phenotype of tumour cells is the fact that TIA-1 positive tumour cells usually outnumbered the number of granzyme B positive tumour cells. Cytotoxic T cells express granzyme B only after activation with interleukin (IL)-2, IL-9, or IL-12,[43–45] whereas TIA-1 is expressed in all potentially cytotoxic cells. Thus differences in local cytokine concentrations might account for the differences in activation stage and thus for the different expression patterns for TIA-1 and granzyme B.

In conclusion, in the majority of cases, ALCL are a proliferation of CD4 positive T lymphocytes with an activated cytotoxic phenotype. The presence of such a cytotoxic phenotype in primary biopsies of patients with ALCL is not related to site of origin, ALK expression, or clinical outcome of the disease.

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