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Cytokine mRNA profiles in Epstein–Barr virus-associated post-transplant lymphoproliferative disorders

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Abstract: Cytokine mRNA patterns were analyzed in 11 post-transplant lymphoproliferative disorder (PTLD) specimens using qualitative reverse-transcriptase polymerase chain reaction (RT-PCR). In each case, a pattern of IL2$^-$, IFNγ$^-$, IL4$^+$, IL10$^+$ was seen. A similar pattern was observed in a spleen sample from 1 patient with contemporaneous PTLD elsewhere.
Semiquantitative RT-PCR for cytokine message was performed using RNA from bronchoalveolar lavage (BAL) specimens obtained from 2 patients with pulmonary PTLD. In both cases, IL4 message predominated. Reduction of message coincided with resolution of the tumors. The pattern differed from that seen in 1 patient with acute pulmonary rejection, in which RT-PCR of BAL cells showed predominance of IL6 and IFNγ.
We conclude that at least some PTLDs exist within a T-helper cell type 2 (Th2)-like cytokine microenvironment. The presence of a similar mRNA pattern in an extratumoral specimen at the time of PTLD suggests that it may reflect a systemic phenomenon. Disappearance of this pattern following PTLD resolution indicates its dynamic nature and is consistent with the hypothesis that specific cytokines contribute to the development of PTLDs.

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Post-transplant lymphoproliferative disorders (PTLD) comprise a range of lymphoid tumors divisible into several clinicopathological categories (1). Most PTLDs are of B cell origin and contain the Epstein–Barr virus (EBV), which is considered to be a major cofactor for their development (2, 3).

It has been estimated that between 31 and 50% of all PTLDs may regress following reduction of iatrogenic immunosuppression (4, 5), and this is consistent with the concept that host immunity plays a role in controlling the growth of such lesions (6). Examination of the microenvironment of PTLDs may, therefore, help to define a) the milieu within which tumor growth occurs; and b) intratumoral changes that occur during the process of tumor regression.

The present study analyzes PTLDs for expression of mRNAs encoding several cytokines that may directly or indirectly influence B lymphocyte growth. In addition, semiquantitative and longitudinal evaluations of cytokine mRNAs from bronchoalveolar lavage (BAL) specimens in patients with lung-based PTLDs were performed to detect changes following resolution of these tumors.

Materials and methods

Specimens

Twelve tissue specimens from 7 patients with PTLD were obtained as byproducts of diagnostic or therapeutic procedures performed at the University of Pittsburgh Medical Center, Pittsburgh,

PA from May 1990 to February 1992. Tissues were obtained with approval of the Institutional Review Board. Eleven of these specimens represented PTLD tumors and the twelfth specimen derived from a splenectomy performed on a patient with PTLD at a separate site.

Two spleen specimens from transplant patients without evidence of PTLD were used as control tissues. Six reactive lymph nodes and one splenic specimen from nontransplant patients were also used as technical controls.

Tissues were snap-frozen in liquid nitrogen-cooled isopentane and stored at −80°C until use.

Fresh cells were obtained as byproducts of BAL in two additional patients with pulmonary PTLD, both at the times of tumor diagnosis and following tumor resolution. The clinical course of one of these patients has been reported previously (7). BALs were performed in a third lung transplant recipient without evidence of PTLD both at the time of allograft rejection and following resolution of rejection.

Cytokine mRNA polymerase chain reaction

Total RNA was extracted from frozen tissues or cells and cDNA synthesis was carried out as previously described (7). Polymerase chain reaction (PCR) primers for IL2, IL4, IL6, IL10 and IFNγ were synthesized at the University of Pittsburgh DNA facility. Commercial primers for EBV (Research Genetics, Huntsville, AL) were also utilized, and primers detecting actin were used as positive controls for each specimen.

EBV-negative HUT78 cells were obtained from the American Type Culture Collection (Rockville, MD) and were used as positive controls for IFNγ, IL2 and IL10. Phytohemagglutinin-stimulated peripheral blood mononuclear cells served as positive controls for IL6 mRNA detection. A plasmid containing the IL4 gene was used as a positive control for IL4 primers. EBV-transformed cells were used to test the EBV primers.

Qualitative PCR using cDNA from solid tissue samples was carried out for 30 cycles and the products visualized with ethidium bromide.

BAL cell cDNA samples were analyzed for the expression of cytokine mRNA using a semi-quantitative reverse transcriptase-PCR (RT-PCR) with ^{32}P-deoxycytidine triphosphate-labelled oligoprimers, quantitated on a Betagen radioanalytic scanner and the results were expressed as counts per min. β-actin mRNA was measured as an internal control for each BAL specimen. The results are reported as the ratio of cytokine per actin mRNA.

Results

Clinicopathologic features of post-transplant lymphoproliferative disordersg

Clinicopathologic features of the study specimens are shown in Table 1. Solid tissue samples were obtained from patients 1–7, and patients 8–10 were the sources of BAL specimens. Two or more PTLDs were sampled in 3 patients. In one case (patient 1), a pharyngeal lesion arose 5 months following resection of a PTLD that was confined to the allograft liver. Both tumors had the appear-

Table 1. Pathologic features of study patients

Patient no.	Transplant	Interval to tumor (months)	Specimen	Site	PTLD	Histology	Clonality
1	Liver	6	1A	Allograft	+	Monomorphic LCL	Monoclonal
		11	1B	Pharynx	+	Monomorphic LCL	Monoclonal
2	Heart	4	2A	Pulm node	+	Monomorphic NOS	Monoclonal
		4	2B	Gastr node	+	Monomorphic NOS	Monoclonal
		4	2C	Mes node	+	Monomorphic NOS	Monoclonal
3	Liver	1.7	3A	Cerv node	+	Monomorphic IB	ND
		2	3B	Liver	+	Monomorphic IB	ND
		2	3C	Spleen	+	Monomorphic IB	ND
4	Heart	18	4	Liver	+	Monomorphic LCL	ND
5	Liver	4	5	Cerv node	+	Monomorphic LCL	Oligoclonal
6	Liver	88	6	Spleen	−	No PTLD	
7	Lung	54	7	Spleen	+	HD-like, B cell	
8	Lung	11.0	8A	BAL	+	PTLD NOS	ND
	Lung	12.6	8B	BAL	−	Resolved PTLD	
9	Lung	1.6	9A	BAL	+	PTLD NOS	ND
	Lung	5.2	9B	BAL	−	Resolved PTLD	
10		20.3	10A	BAL	−	Acute rejection	
		22.4	10B	BAL	−	No rejection	

Pulm, pulmonary; gastr, gastric; mes, mesenteric; cerv, cervical; LCL, large cell lymphoma; NOS, not otherwise specified; IB, immunoblastic lymphoma; HD, Hodgkin's disease; ND, not done.

Table 2. Qualitative cytokine mRNA evaluation in solid tissue samples from PTLD patients

Specimen	PTLD	IL2	IFNγ	IL4	IL10
1A	+	+		+	+
1B	+	+	+	+	+
2A	+	+	+	+	+
2B	+	+	+	+	+
2C	+	+	+	+	+
3A	+	+	+	+	+
3B	+	+	+	+	+
3C	+	+	+	+	+
4	+	+	+	+	+
5	+	+	+	+	+
6	+	+	+	+	+
7	+	+	+	+	+

IL2, interleukin 2; IFNγ, interferon γ; IL4, interleukin 4; IL10, interleukin 10.

ance of a large cell non-Hodgkin's lymphoma (monomorphic PTLD) and both demonstrated identical rearrangements of the immunoglobulin heavy chain gene. In patients 2 and 3, synchronous and noncontiguous tumors were sampled. In one case (patient 2), the presence of two separate clonal proliferations was documented.

PTLD was not further categorized in patients 8 and 9, both of whom had pulmonary lesions.

Treatment included reduction of immunosuppression and acyclovir in all cases. Patient 3 required allograft resection and patient 7 received subsequent chemotherapy. Patient 9 received additional therapy with α-interferon.

PTLD resolved in 5 patients (Nos. 1, 4, 7–9) and the remaining 4 tumor patients (Nos. 2, 3, 5, 6) expired with PTLD. Patient 10, included as a control, did not have PTLD at any time during the post-transplant course.

EBV status of PTLDs

All 11 solid tissue PTLD specimens were positive for EBV by PCR analysis (Table 2). Specimen 6, taken from uninvolved spleen in a PTLD patient, was negative for this target. A pulmonary biopsy from patient 9 was positive for EBV by *in situ* hybridization for Epstein–Barr early RNA (EBER). Insufficient tissue was available from patient 8 to perform this assay.

Cytokine mRNA profiles of PTLDs

All 12 specimens from PTLD patients showed a similar qualitative cytokine mRNA profile of $IL2^-$, $IFN\gamma^-$, $IL4^+$, $IL10^+$ (Table 2). Representative patterns of two separate tumors resected 5 months apart from patient 1 are shown in Fig. 1. In this example, the first tumor represented a lymphomatous PTLD, which was localized to the allograft. The second tumor arose in the pharynx after a 5-month tumor-free interval. Pathologic evaluation of this case was previously reported and it was concluded that the second lesion represented a true recurrence of the primary tumor (8).

In patient 6, splenectomy was performed at the time of active PTLD. Although the spleen itself was not involved by tumor, it demonstrated a cytokine mRNA profile similar to that seen in PTLD specimens, except for the absence of EBV.

Two splenic specimens were obtained from transplant recipients at the time of acute rejection. Both specimens were negative for EBV. In one case the cytokine mRNA pattern was $IL2^-$, $IFN\gamma^+$, $IL4^+$, $IL10^-$ and in the other it was $IL2^+$, $IFN\gamma^-$, $IL4^-$, $IL10^-$. A splenic specimen obtained from 1 nontransplant patient was associated with a pattern similar to that found in the PTLDs, except for the absence of EBV. Six lymph nodes from 5 nontransplant patients were also analyzed. All were EBV negative. In one case no cytokine mRNA was detected, in two cases only weak expression of IL10 mRNA was seen, and in one case IL10 positivity was coupled with weak expression

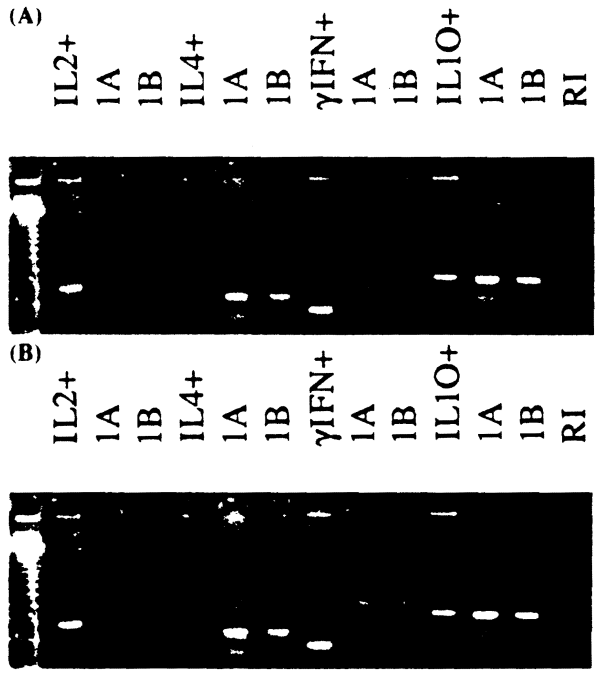

Fig. 1. Ethidium bromide-stained gel electrophoresis of RT-PCR reaction products for cytokines. 1A and 1B refer to two separate specimens from patient 1. These noncontiguous clonal PTLDs occurred 5 months apart. Lane 1: DNA size ladder; Lanes 2–4: positive IL2 control and patient samples; Lanes 5–7: positive IL4 control and patient samples; Lanes 8–10: positive IFNγ control and patient samples; Lanes 11–13: IL10 positive control and patient samples; Lanes 14: RI, negative plasmid DNA control.

Table 3. Semiquantitative cytokine RT-PCR performed on BAL specimens. The results are expressed as the ratio of cytokine mRNA to β-actin mRNA

Specimen	PTLD	IL2	IFNγ	IL4	IL6	IL10
8A	+	0.05	0.01	0.15	0.02	0.02
8B	-	0.04	<0.01	0	<0.01	<0.01
9A	+	0.25	0.06	0.74	0.02	14.5
9B	-	0.01	0	0.09	0	2.6
10A	-	<0.01	1.7	0	1.6	0.08
10B	-	<0.01	0.3	0	<0.01	<0.01

IL2, interleukin 2; IFNγ, interferon γ; IL4, interleukin 4; IL10, interleukin 10.

of IL2. A fifth example showed expression of all four cytokine mRNAs, and in the sixth case the pattern was IL2$^+$, IFNγ$^+$, IL4$^-$, IL10$^+$.

BAL cells from the 2 lung transplant recipients with biopsy-proven pulmonary PTLD revealed strong expression of IL4 mRNA when analyzed by semiquantitative RT-PCR (Table 3). Prominent IL10 mRNA expression was also seen in one case (specimen 9A, Table 3) and was expressed to a lesser extent in the second case. In contrast to the results of qualitative analysis, some expression of IL2 and IFNγ mRNA was observed in both of these patients. IL6 mRNA was also detected in both specimens.

Decreased levels of all of the above cytokine mRNAs relative to actin mRNA were seen in BAL specimens taken from both of these patients at time of PTLD resolution.

In contrast to this pattern, IL6 and IFNγ were the predominant cytokine mRNAs found during an acute rejection episode in patient 10 (specimen 10A, Table 3). Relative values for these cytokines also decreased following successful treatment of rejection.

No correlation between the original PTLD cytokine profile and histology, clonality, time interval from transplant to tumor, tumor location or response to treatment could be inferred from this study.

Discussion

The results of this study suggest that many PTLDs exist within a T-helper cell type 2 (Th2)-like (9) cytokine environment. In all PTLD specimens analyzed by qualitative RT-PCR in this series, IL4 and IL10 mRNA were present and neither IL2 nor IFNγ were found. This pattern was not seen in either of two splenic specimens obtained from transplant patients without PTLD, nor in any of six control lymph nodes resected from nontransplant patients. The sole exception occurred in a spleen resected from 1 nontransplant patient. Use of a more sensitive semiquantitative technique again showed predominance of IL4 or IL4 together with IL10 and this pattern differed from that seen in acute rejection, in which IFNγ and IL6 predominated. Although the sensitivity and specificity of this assay are not yet known, this approach has been shown to be useful in the evaluation of pulmonary rejection, both in a rodent model and in clinical specimens (10, 11). In the case of BAL specimens, a variety of cell types, such as epithelial cells, macrophages and granulocytes, may contribute to the final cytokine mRNA profile. Thus the longitudinal changes shown here may or may not reflect the events that occur during regression of extrapulmonary PTLDs.

The results of these intratumoral studies are compatible with those of Shapiro et al. (12) who were the first to describe a decrease in blood levels of α-interferon and an increase in circulating IL4 levels in a cohort of bone marrow transplant patients with PTLD. Immunosuppressed transplant recipients without PTLD also had elevated IL4 levels relative to a nontransplant population, but did not demonstrate a depression of α-interferon as did the PTLD patients. Similarly, Burke et al. (13) demonstrated decreased serum IL2 in conjunction with a rise in serum IL4 in a patient with disseminated PTLD. The presence of a systemic Th2-like cytokine profile during PTLD is also suggested by the specimen from patient 6 in the current study. In this case, the patient had a PTLD that did not involve the spleen. Nevertheless, a Th2-like cytokine mRNA profile was identified in the test sample derived from this organ. This may be coincidental, or it may reflect a systemic Th2-like pattern during PTLD as suggested by others (12, 13).

The role of individual cytokines within PTLD remains speculative. For example, IL4 may selectively drive T cells toward a Th2 phenotype, in addition to having a stimulatory effect on B cells (14). It seems likely that either or both of these activities may contribute to PTLD development, but this has not yet been formally proven.

IL10 is also known to suppress the Th1 cytokine response (14), and the absence of detectable Th1-associated cytokines IL2 or IFNγ in the solid

PTLD specimens is consistent with this role. However, IL10 may also be demonstrated locally in the context of acute cellular rejection, which is not associated with a Th2-predominant pattern (15, 16). Thus, the demonstration of this cytokine alone does not predict the overall cytokine milieu.

IL10 may also serve as an autocrine paracrine growth factor for B-lymphocytes, and one study showed this effect using spontaneous lymphoblastoid B cell lines derived from the blood of a patient with PTLD (17). However, Randhawa et al. (18) failed to show growth stimulation of a PTLD-derived B cell line upon addition of recombinant IL10, despite the fact that the cell line itself produced IL10. Similarly, IL10-stimulated B cell growth could not be demonstrated by Kanno et al. (19) in a separate study of a pyothorax-associated EBV-positive B cell lymphoma from a nonimmunosuppressed patient.

Reversal of the Th2 pattern coincident with PTLD resolution in 2 patients with longitudinal sample analysis lends support to efforts designed to bring about a host antitumor response by use of immunomodulation, like the withdrawal of immunosuppression or administration of Th1-associated cytokines (20), such as α-interferon (7). It is also conceivable that the use of anti-cytokine antibodies such as anti-IL4 and possibly anti-IL-10 antibodies may alter the PTLD microenvironment and thus facilitate a cytotoxic host antitumor response. Such approaches could conceivably be combined with newer methods of adoptive cellular immunotherapy (21–23) in order to generate an enhanced anti-tumor response with a reduced risk of organ rejection. Toward this end, further description of PTLD-associated cytokines and an analysis of the function of these cytokines within these tumors appear warranted.

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