Published in final edited form as:

Clin Transplant. 1999 February ; 13(1 Pt 1): 39–44.

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

Michael A Nalesnik^{a,c,d}, Adriana Zeevi^{a,c}, Parmjeet S Randhawa^{a,c}, Albert Faro^e, Kathy J Spichty^{a,c}, Anthony J Demetris^{a,c}, John J Fung^{b,c}, Theresa L Whiteside^{a,d}, and Thomas E Starzl^{b,d}

^a Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, PA

^b Department of Surgery, University of Pittsburgh Medical Center, Pittsburgh, PA

^c Thomas E Starzl Transplantation Institute, University of Pittsburgh Medical Center, Pittsburgh, PA

^d University of Pittsburgh Cancer Institute, University of Pittsburgh Medical Center, Pittsburgh, PA

^e Mailman Center for Child Development, Miami, FL, USA

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⁺, IFN γ ⁺, 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 IFN γ .

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.

Keywords

cytokine; Epstein; Barr virus; interleukin 4; interleukin 10; lymphoma; lymphoproliferative disorder; organ transplantation

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 IFN γ 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 IFN γ , 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 PTLT that was confined to the allograft liver. Both tumors had the appearance of a large cell non-Hodgkin's lymphoma (monomorphic PTLT) 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.

PTLT 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.

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

EBV status of PTLTs

All 11 solid tissue PTLT specimens were positive for EBV by PCR analysis (Table 2). Specimen 6, taken from uninvolved spleen in a PTLT 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 PTLTs

All 12 specimens from PTLT patients showed a similar qualitative cytokine mRNA profile of IL2⁺, IFN γ ⁺, 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 PTLT, 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 PTLT. Although the spleen itself was not involved by tumor, it demonstrated a cytokine mRNA profile similar to that seen in PTLT 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 γ ⁺, IL4⁺, IL10⁺ and in the other it was IL2⁺, IFN γ ⁺, IL4⁺, IL10⁺. A splenic specimen obtained from 1 nontransplant patient was associated with a pattern similar to that found in the PTLTs, 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 of IL2. A fifth example showed expression of all four cytokine mRNAs, and in the sixth case the pattern was IL2⁺, IFN γ ⁺, IL4⁻, IL10⁺.

BAL cells from the 2 lung transplant recipients with biopsy-proven pulmonary PTLT 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 IFN γ 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 PTLT resolution.

In contrast to this pattern, IL6 and IFN γ 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 PTLT 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 PTLTs exist within a T-helper cell type 2 (Th2)-like (9) cytokine environment. In all PTLT specimens analyzed by qualitative RT-PCR in this series, IL4 and IL10 mRNA were present and neither IL2 nor IFN γ were found. This pattern was not seen in either of two splenic specimens obtained from transplant patients without PTLT, 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 IFN γ 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 PTLTs.

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 PTLT. Immunosuppressed transplant recipients without PTLT also had elevated IL4 levels relative to a nontransplant population, but did not demonstrate a depression of α -interferon as did the PTLT patients. Similarly, Burke et al. (13) demonstrated decreased serum IL2 in conjunction with a rise in serum IL4 in a patient with disseminated PTLT. The presence of a systemic Th2-like cytokine profile during PTLT is also suggested by the specimen from patient 6 in the current study. In this case, the patient had a PTLT 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 PTLT as suggested by others (12,13).

The role of individual cytokines within PTLT 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 IFN γ 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.

Acknowledgments

The assistance of Carole Frye, BS, in performing the qualitative RT-PCR is gratefully appreciated. This study was supported in part by NIH grant PO1-CA 47445 and by funds from the Pathology Education and Research Foundation, Pittsburgh PA and the Thomas E Starzl Transplantation Institute, Pittsburgh, PA, USA.

References

1. Harris NL, Ferry JA, Swerdlow SH. Post-transplant lymphoproliferative disorders (PTLD): summary of Society for Hematopathology Workshop. *Semin Diag Pathol* 1997;14:8.
2. Hanto DW, Gajl-Peczalska KJ, Frizzera G, Arthur DC, Balfour HH Jr, McClain K, Simmons RL, Najarian JS. Epstein-Barr (EBV) induced polyclonal and monoclonal B-cell lymphoproliferative diseases occurring after renal transplantation. *Ann Surg* 1983;198:356. [PubMed: 6311121]
3. Klein G. Epstein-Barr virus strategy in normal and neoplastic B cells. *Cell* 1994;77:791. [PubMed: 8004668]
4. Penn I. Immunosuppression – a contributing factor in lymphoma formation. *Clin Transplant* 1992;6:214.
5. Heslop HE, Brenner MK, Rooney C, Krance RA, Roberts WM, Rochester R, Smith CA, Turner V, Sixbey J, Moen R, Boyett JM. Clinical Protocol. Administration of neomycin resistance gene marked EBV specific cytotoxic T lymphocytes to recipients of mismatched-related or phenotypically similar unrelated donor marrow grafts. *Hum Gene Ther* 1994;5:381. [PubMed: 8018749]
6. Starzl TE, Nalesnik MA, Porter KA, Ho M, Iwatsuki S, Griffith BP, Rosenthal JT, Hakala TR, Shaw BW Jr, Hardesty RL, Atchison RW, Jaffe R, Bahnson HT. Reversibility of lymphomas and

- lymphoproliferative lesions developing under cyclosporine–steroid therapy. *Lancet* 1984;i:583. [PubMed: 6142304]
7. Faro A, Kurland G, Michaels MG, Dickman PS, Greally PG, Spichty KJ, Noyes BB, Boas SR, Fricker FJ, Armitage JM, Zeevi A. Interferon-alpha affects the immune response in post-transplant lymphoproliferative disorder. *Am J Respir Crit Care Med* 1996;153:1442. [PubMed: 8616579]
  8. Wu TT, Swerdlow SH, Locker J, Bahler D, Randhawa P, Yunis EJ, Dickman PS, Nalesnik MA. Recurrent Epstein–Barr virus-associated lesions in organ transplant recipients. *Hum Pathol* 1996;27:157. [PubMed: 8617457]
  9. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Ann Rev Immunol* 1989;7:145. [PubMed: 2523712]
  10. Iacono A, Dauber J, Keenan R, Spichty K, Cai J, Grgurich W, Burkart G, Smaldonef G, Pham S, Ohori NP, Yousem S, Williams P, Griffith B, Zeevi A. Interleukin-6 and gamma interferon gene expression in lung transplant recipients with refractory acute cellular rejection: implications for monitoring and inhibition by treatment with aerosolized cyclosporin A. *Transplantation* 1997;64:263. [PubMed: 9256185]
  11. Mitruka SN, Pham SM, Zeevi A, Li S, Cai J, Burckart GJ, Yousem SA, Keenan RJ, Griffith BP. Aerosol cyclosporine prevents acute allograft rejection in experimental lung transplantation. *J Thorac Cardiovasc Surg* 1998;115:28. [PubMed: 9451042]
  12. Mathur A, Kamat DM, Filipovich AH, Steinbuch M, Shapiro RS. Immunoregulatory abnormalities in patients with Epstein–Barr virus-associated B cell lymphoproliferative disorders. *Transplantation* 1994;57:1042. [PubMed: 8165700]
  13. Burke GW, Cirocco R, Hensley G, Reddy R, Jeffers L, Schiff E, Miller J. The rapid development of a fatal, disseminated B cell lymphoma following liver transplantation – serial changes in levels of soluble serum interleukin 2 and interleukin 4 (B cell growth factor). *Transplantation* 1992;53:1148. [PubMed: 1585481]
  14. Howard, MC.; Miyajima, A.; Coffman, R. T-cell-derived cytokines and their receptors. In: Paul, WE., editor. *Fundamental Immunology*. 3. New York: Raven Press; 1993.
  15. Azzawi M, Hasleton PS, Grant SCD, Stewart JP, Hutchinson IV. Interleukin-10 in human heart transplantation: an in situ hybridization study. *J Heart Lung Transplant* 1994;14:519. [PubMed: 7654735]
  16. Xu GP, Sharma VK, Li B, Bologa R, Li Y, Mouradian J, Wang J, Serur D, Rao V, Stenzel KH, Suthanthiran M. Intragraft expression of IL-10 messenger RNA: a novel correlate of renal allograft rejection. *Kid Internat* 1995;48:1504.
  17. Beatty PR, Krams SM, Martinez OM. Involvement of IL-10 in the autonomous growth of EBV-transformed B cell lines. *J Immunol* 1997;158:4045. [PubMed: 9126962]
  18. Randhawa P, Whiteside T, Zeevi A, Nalesnik M, Alvares C, Gollin SM, Demetris J, Locker J. In vitro culture of B-lymphocytes derived from Epstein–Barr virus-associated post-transplant lymphoproliferative disease: cytokine production and effect of interferon alpha. *In Vitro Cell Dev Biol* 1997;33:803.
  19. Kanno H, Naka N, Yasunaga Y, Iuchi K, Yamauchi S, Hashimoto M, Aozasa K. Production of the immunosuppressive cytokine interleukin-10 by Epstein–Barr-virus-expressing pyothorax-associated lymphoma. Possible role in the development of overt lymphoma in immunocompetent hosts. *Am J Pathol* 1997;150:349. [PubMed: 9006350]
  20. Randhawa PS, Demetris AJ, Nalesnik MA. The potential role of cytokines in the pathogenesis of Epstein–Barr virus associated post-transplant lymphoproliferative disease. *Leuk Lymphoma* 1994;15:383. [PubMed: 7873995]
  21. Papadopoulos EB, Ladanyi M, Emanuel D, Mackinnon S, Boulad F, Carabasi MH, Castro-Malaspina H, Childs BH, Gillio AP, Small TN, Young JW, Kernan NA, O'Reilly RJ. Infusions of donor leukocytes to treat Epstein–Barr virus-associated lymphoproliferative disorders after allogeneic bone marrow transplantation. *N Engl J Med* 1994;330:1185. [PubMed: 8093146]
  22. Rooney CM, Smith CA, Ng CYC, Loftin S, Li C, Krance RA, Brenner MK, Heslop HE. Use of gene-modified virus-specific T lymphocytes to control Epstein–Barr-virus-related lymphoproliferation. *Lancet* 1995;345:9. [PubMed: 7799740]

23. Nalesnik MA, Rao AS, Furukawa H, Pham S, Zeevi A, Fung JJ, Klein G, Gritsch HA, Elder E, Whiteside TL, Starzl TE. Autologous lymphokine-activated killer cell therapy of Epstein–Barr virus-positive and -negative lymphoproliferative disorders arising in organ transplant recipients. *Transplantation* 1997;63:1200. [PubMed: 9158009]

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 IFN γ control and patient samples; Lanes 11–13: IL10 positive control and patient samples; Lanes 14: RI, negative plasmid DNA control.

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	IFN γ	IL4	IL10
1A	+	-	-	+	+
1B	+	-	-	+	+
2A	+	-	-	+	+
2B	+	-	-	+	+
2C	+	-	-	+	+
3A	+	-	-	+	+
3B	+	-	-	+	+
3C	+	-	-	+	+
4	+	-	-	+	+
5	+	-	-	+	+
6	-	-	-	+	+
7	+	-	-	+	+

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

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	IFN γ	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; IFN γ , interferon γ ; IL4, interleukin 4; IL10, interleukin 10.