

Characteristics of T-Lymphocytes Infiltrating Human B-Cell Lymphomas

by Peter Marquardt* and Hans Konrad Müller-Hermelink*

Using a recently described technique for direct expansion of human T-lymphocytes isolated from small intestine biopsies, we have investigated the local cellular immune response in six patients with B-cell lymphomas of various subtypes. T-cell lines (TCL) were established by seeding tumor-infiltrating T-lymphocytes (TITL) at limiting dilution in the presence of irradiated feeder cells in culture medium containing rIL-2 and phytohemagglutinin (PHA). About 1/50 T-cells gave rise to a TCL; they all were CD3⁺. The CD4/CD8 ratio was 3.8:1 before and after cloning. Of 45 TCLs analyzed so far from one patient with B-cell lymphoma of the lung, 4 were cytotoxic as shown by their ability to exert lectin-dependent cytotoxicity against allogeneic target cells. Of these, 3 demonstrated specificity for the autologous malignant B-cells. Five TCLs lysed the NK-sensitive K562 cell line in a HLA-unrestricted manner. When tested for antigen-specific proliferative activity, 4 TCLs only responded to the autologous lymphoma cells, but 5 TCLs reacted to the autoantigenic ganglioside G_{M1}. Southern Blot analyses did not show a clonal pattern of T-cell receptor gene rearrangement within all TITL populations. The peripheral T-lymphocytes of the lymphoma patients showed a drastically reduced response to the mitogens PHA, Concanavalin A, and pokeweed mitogen. The present report demonstrates that it is possible to analyze TITL at clonal level. This technique may be the only means of investigating the specificity of the TITL and may help us to identify the relevant tumor-associated autoantigens if tumor-induced autoimmunization is indeed one of the mechanisms that control the growth of tumors and metastases.

Introduction

Tumor-infiltrating leukocytes, which are usually found in neoplastic tissue, have attracted the interest of many immunologists (1). Among the components of the lymphoreticular infiltrate of malignancies, macrophages have been studied most extensively. Other researchers are looking for tumor-specific monoclonal antibodies in order to trace tumor-associated antigens and for therapeutical reasons. However, T-lymphocytes represent the major component of these infiltrates. The greatly increased incidence of lymphomas in circumstances of immunosuppression (2-4) has prompted us to study the role of T-lymphocytes infiltrating human B-cell lymphomas. In view of the compartmentalization of T-cell immunity, it is possible that the relevant T-cells may be sequestered into the malignant lesion (5). Therefore, it is essential for us to study the local immune response by using tumor-infiltrating T-lymphocytes (TITL) and not just simply the easily accessible peripheral blood lymphocytes (PBL) of the patients (5,6). Our previous reports of a high enrichment of virus-specific HLA-restricted T-cells at the site of inflammation during the acute phase of viral infections was

an important observation, suggesting that the information obtained from cloned local T-cell populations (Table 1) is in fact reliable (7).

Experimental Protocol

In vivo activated TITL were always directly cloned and expanded without any prior *in vitro* restimulation with lymphoma cells but in the presence of phytohemagglutinin (PHA); we therefore may assume that no selection for certain subpopulations or certain specificities occurred before the cloning procedure, and random growth of TITL could be expected (5,6). As not all ex-

Table 1. Local cellular immune reactivity patterns in different inflammatory conditions.

Parameter	Viral infections		Multiple sclerosis	B-cell lymphomas
	Acute	Chronic		
Proliferating frequency	0.025	0.026	0.04-0.06	0.01-0.03
Phenotype				
CD4 ⁺	20%	66%	75-100%	73.7%
CD8 ⁺	80%	34%	0-25%	26.3%
Antigen-specific	88%	7.0%	—	11.6%
Proliferative	6%	2.7%	—	6.6%
Cytotoxic	82%	4.3%	—	5.0%
Autoreactive				
Proliferative	—	8.7%	~ 25%	8.3%
LDCC	NT ^a	45%	~ 50%	6.6%

^a NT, not tested.

*Institute of Pathology, University of Würzburg, D-8700 Würzburg, Federal Republic of Germany.

Address reprint requests to P. Marquardt, Institute of Pathology, University of Würzburg, D-8700 Würzburg, Federal Republic of Germany

panded T-cell populations were monoclonal in the strict sense of the word, the term TCL will be used. The growing TCLs were analyzed for proliferating frequency by using Lefkovits calculation and were analyzed for phenotype by using monoclonal antibodies specific for T-cell subpopulations (CD3, CD4, CD8) and HLA-DR. TCLs were analyzed for function by using conventional cytotoxicity assays and analyzed for specific reactivity by using proliferation assays against the malignant B-cells and the autoantigens actin, cardiolipin (phospholipid), and various tumor-associated glycolipids (G_{M1} , G_{D1a} , G_{T1} , L_{1-A}). Southern Blot analyses were carried out using conventional techniques (8).

Results

The analysis of B-cell lymphoma-infiltrating T-cells revealed patterns of reactivity as far as proliferating frequency, phenotype, functions, antigen-specificities, and restriction elements were concerned that were almost identical to our previous observations in patients with chronic viral infections, autoimmune diseases (Table 1), and gastric carcinomas (7,9,10). In 6 patients with B-cell lymphomas of various subtypes, about 1 in 50 T-cells gave rise to a TCL (proliferating frequency ~ 0.02) (Fig. 1); they all were $CD3^+$. As compared to normal PBL, all of which grow under these culture conditions (11), this poor response to PHA indicates a reduced proliferative capacity of the TITL. A substantial HLA-DR⁺ fraction of TITL had obviously been activated *in vivo*. There is a predominance of $CD4^+$ T-cells in the infiltrate. The $CD4/CD8$ ratio was 3.8:1 before and after cloning, suggesting that there is no selection for certain subpopulations due to our cloning procedure. Out of 45 TCLs analyzed so far from one patient with B-cell lymphoma of the lung, 4 were cytotoxic as shown by their ability to exert lectin-dependent cytotoxicity (LDCC) against allogeneic target cells. Of these, 3 demonstrated cytotoxicity specific for the autologous malignant B-cells (2 of which were $CD4^+$). Five TCLs lysed

the NK-sensitive K562 cell line in a HLA-unrestricted manner.

All TCLs were also tested for proliferative reactivity to the autologous lymphoma cells, but only four TCLs ($CD4^+$) responded with a 3.3-, 3.5-, 6.0-, and 66-fold 3H -TdR incorporation (Table 2). When tested against tumor-associated glycolipids, five TCLs ($CD4^+$) reacted to the autoantigenic ganglioside G_{M1} with stimulation indices of $3.3 \times$, $4.5 \times$, $5.0 \times$, $5.2 \times$, and $8.8 \times$ (Table 2). Though not identified sufficiently in this study, the restriction elements are most likely class II HLA molecules.

Using cells directly after isolation from the lymphoma tissue ($\sim 35\%$ TITL), Southern Blot analyses did not reveal a clonal TcR gene rearrangement within all TITL populations (sensitivity $\geq 3\%$). The PBL of all lymphoma patients showed a drastically reduced response to mitogens (PHA, Concanavalin A, pokeweed mitogen) (Table 3).

Discussion

The present report describes for the first time the establishment of TCLs directly from surgically resected

Table 2. Proliferative response of $CD4^+$ TCLs derived from cloned TITL from a patient with B-cell lymphoma.

Antigen specificity	Medium	PHA
Autologous lymphoma cells		
15410 ^a	234	28428
3440	572 ^a	3330
697	198	5439
725	222	6794
Ganglioside G_{M1}		
4890	1083	8112
627	188	6222
1911	218	4549
883	274	11015
856	171	3924

^a All numbers represent cpm 3H -TdR uptake.

Table 3. Proliferative response of autologous PBL to mitogens in whole blood cultures.^a

PBL source	Maximal cpm 3H -TdR uptake/PBL in response to		
	PHA	Con A	PWM
Normals, $n = 20$	11.13 (0.57) ^b	2.74 (0.26)	3.86 (0.16)
Chronic viral infection (RB)	5.48	1.78	2.76
Morbus Hodgkin type I (KG)	3.02	1.81	1.26
T-cell lymphoma, low grade (JK)	0.97	1.20	0.35
B-cell lymphomas			
High grade (PR)	6.29	1.83	2.59
Low grade (BR)	1.48	0.76	1.46
Low grade (FL)	0.05	0.94	0.19
Low grade (IS)	5.66	1.58	1.88

^a Mitogens are PHA, Concanavalin A (Con A), and pokeweed mitogen (PWM). Letters in parentheses are patients' initials.

^b Numbers in parentheses are SEM.

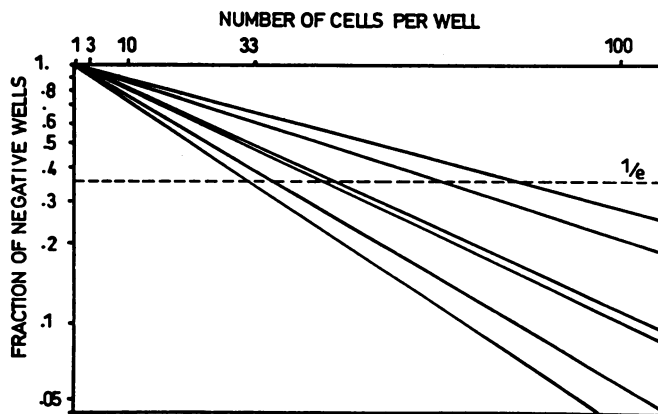


FIGURE 1. Limiting dilution of T-lymphocytes infiltrating human B-cell lymphomas. Wells were screened for colonies on days 6, 10, 14, and 21 after seeding. A well was scored positive if one-fourth of its surface was covered with growing cells. Each value is based on the evaluation of at least 200 wells.

human B-cell lymphoma tissue. In contrast to 100% growth of normal PBL (11), identical proliferating frequencies of infiltrating T-cells in various chronic diseases [viral infections (9), autoimmune diseases (7), gastric cancers (10), and B-cell lymphomas] argue against a tumor-induced unresponsiveness in the tumor environment. However, reduced responses, also of the patients' PBL to mitogens (Table 3), suggest a nonspecific immunodeficiency state (12). Our previous results as to T-cell reactivity in chronic inflammatory conditions where CD4⁺ T-cells represent the major component of the infiltrate (7) indicate that the incidence of B-lymphoma-reactive T-cells in such a similarly protracted disease of long duration may be extremely low. This conforms to observations of other groups (13). Thus, and in accordance with a polyclonal TcR gene rearrangement within the TITL populations, it is questionable whether one should test hundreds of clones for tumor reactivity. We favor the view that HLA class I-restricted cytotoxic T-cells of the CD8⁺ phenotype, specific for the etiological agent, are limited to the initial phase of an acute inflammation (Table 1), whereas CD4⁺ cells are a hallmark of a chronic inflammatory process (9). The function of the CD4⁺ TITL has yet to be defined. Whether they are cytotoxic or noncytotoxic, they obviously do not recognize an antigen on the tumor cell surface by which the malignant cells are eliminated from the host. According to the literature, they might even have suppressive activity (14–16). Therefore, cloning TITL may be the only means of investigating the specificity of these CD4⁺ T-cell populations and of finding out whether they respond to tumor-associated autoantigens (e.g., glycolipids, glycoproteins). In order to maintain self-tolerance, such ubiquitous autoantigens, known to be highly expressed on certain tumor cell surfaces, have to be protected by the immune system. Consequently, they would have to induce T-suppressor cells (probably the CD4⁺ TITL population) that would facilitate oncogenesis by suppressing antitumor (anti-self) reactivity (14–16). Allowing malignant cells to escape from the immune system would be an important way by which immune effectors could produce tumor progression.

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REFERENCES

1. Mantovani, A., and Evans, R. Tumor-infiltrating leukocytes. *Immunol. Today* 6: 143–145 (1985).
2. Schneck, S. A., and Penn, I. De-novo brain tumours in renal-transplant recipients. *Lancet* i: 983–986 (1971).
3. Penn, I. Depressed immunity and the development of cancer. *Clin. Exp. Immunol.* 46: 459–474 (1981).
4. Schulof, R. S., Lacher, M. J., and Gupta, S. Abnormal phytohemagglutinin induced T-cell proliferative responses in Hodgkin's disease. *Blood* 57: 607–613 (1981).
5. Marquardt, P., Martin, R., and Kreth, H. W. Local cell-mediated immunity: gliadin-specific T-cells sequestered into coeliac mucosal lesions. *J. Immunobiol.* 168: 109 (1985).
6. Fleischer, B., Marquardt, P., Poser, S., and Kreth, H. W. Phenotypic markers and functional characteristics of T-lymphocyte clones from cerebrospinal fluid in multiple sclerosis. *J. Neuroimmunol.* 7: 151–162 (1984).
7. Kreth, H. W., Marquardt, P., and Martin, R. Specificity of clones and T-cell lines from cerebrospinal fluid in viral meningoencephalitis and multiple sclerosis. In: *Cellular and Humoral Immunological Components of Cerebrospinal Fluid in Multiple Sclerosis* (A. Lowenthal and J. Raus, Eds.), Plenum Publishing Corp., New York, 1987, pp. 283–290.
8. Southern, E. M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98: 503–517 (1975).
9. Martin, R., Marquardt, P., O'Shea, S., Borkenstein, M., and Kreth, H. W. Virus-specific and autoreactive T cell lines isolated from cerebrospinal fluid of a patient with chronic rubella panencephalitis. *J. Neuroimmunol.* 23: 1–10 (1989).
10. Marquardt, P., Stulle, K., and Müller-Hermelink, H. K. Direct expansion, phenotypic markers, and functional characteristics of human T-lymphocyte clones isolated from gastric tumor tissue. *Clin. Exp. Metast.* 6: 77 (1988).
11. Moretta, A., Pantaleo, G., Moretta, L., Cerottini, J. -C., and Mingari, M. C. Direct demonstration of the clonogenic potential of every human peripheral blood T cell. *J. Exp. Med.* 157: 743–754 (1983).
12. Fletcher, M. A., Baron, G. C., Ashman, M. R., Fischl, M. A., and Klimas, N. G. Use of whole blood methods in assessment of immune parameters in immunodeficiency states. *Diag. Immunol.* 5: 69–81 (1987).
13. Anchini, A., Fossati, G., and Parmiani, G. Clonal analysis of the cytolytic T-cell response to human tumors. *Immunol. Today* 8: 385–389 (1987).
14. Vose, B. M., and Moore, M. Suppressor cell activity of lymphocytes infiltrating human lung and breast tumours. *Int. J. Cancer* 24: 579–585 (1979).
15. Takahashi, K., Ono, K., Hirabayashi, Y., and Taniguchi, M. Escape mechanisms of melanoma from immune system by soluble melanoma antigen. *J. Immunol.* 140: 3244–3248 (1988).
16. North, R. J., Awwad, M., and Dunn, P. L. Regulatory aspects of the antitumor immune response. In: *Cancer Metastasis* (V. Schirrmacher and R. Schwartz-Albiez, Eds.), Springer-Verlag, Berlin, 1989, pp. 150–154.