Cutaneous T Cell Lymphoma Reactive CD4+ Cytotoxic T Lymphocyte Clones Display a Th1 Cytokine Profile and Use a Fas-Independent Pathway for Specific Tumor Cell Lysis

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We have previously described two cytotoxic T lymphocyte clones isolated from lymphocytes infiltrating a human major histocompatibility complex class II−/class I+, CD4+ cutaneous T cell lymphoma. These clones displayed a CD4+CD8dim+ (TC5) and CD4+ CD8− (TC7) phenotype and mediated a specific major histocompatibility complex class I-restricted cytotoxic activity toward Cou-LB autologous tumor cell line. Our studies were performed to elucidate the mechanism involved in T-cell-clone-mediated cytotoxicity and to determine the cytokine profile of both the lymphoma cell line and specific cytotoxic T lymphocyte clones. The results indicate that, despite surface expression of Fas receptor on Cou-LB and Fas ligand induction on TC5 and TC7 cell membranes, the CD4+ cytotoxic T lymphocyte clones do not use this cytotoxic mechanism to lyse their specific target. The TC7 clone uses instead a granzyme–perforin-dependent pathway. Furthermore, quantitative analysis of Th1 and Th2 cytokine mRNA expression in the cutaneous T cell lymphoma cell line as well as in TC5 and TC7 clones indicated that, whereas the tumor cells display a Th2-type profile (interleukin-4, interleukin-6, and interleukin-10), the cytotoxic T lymphocyte clones express Th1-type cytokines (interferon-γ, granulocyte macrophage colony stimulating factor, and interleukin-2). In addition, preincubation of the tumor-infiltrating lymphocyte clones with autologous tumor cells induced their activation and subsequent amplification of the Th1-type response. These results indicate a direct contribution of the malignant cells in the Th1/Th2 imbalance observed frequently in cutaneous T cell lymphoma patients and suggest their potential role in depressed cell-mediated immunity. Identification of CD4+ Th1-type cytotoxic T lymphocyte clones, the tumor antigen they recognize, and optimization of their cytokine expression profile should be useful for the design of new immunotherapy protocols in cutaneous T cell lymphoma. ***Key words: CTCL/cytokine profiles/cytotoxic pathways/TIL. J Invest Dermatol 115:74–80, 2000***

Cutaneous T cell lymphomas (CTCLs) are a heterogeneous group of lymphoproliferative diseases with skin-homing properties (Willemze *et al*, 1997). Mycosis fungoides is characterized by skin invasion of clonally derived malignant CD4+ T lymphocytes that resemble phenotypically mature T helper cells. Sezary syndrome is a leukemic form of CTCL characterized by erythroderma and invasion of patient peripheral blood by the malignant T cell population. It has been reported that skin lesions in CTCL contain heterogeneous cell infiltrates, including tumor cells and reactive non-neoplastic T cells with CD4+ and CD8+ lymphocytes (Wood *et al*, 1994). Therefore, CTCL represents a unique tumor model where both the neoplastic and the reactive cells are T lymphocytes.

Previous studies have demonstrated that skin biopsies of patients with different stages of CTCL express variable levels of Th1- and Th2-type cytokines (Saed *et al*, 1994; Vowels *et al*, 1994). Sezary skin and blood specimens were found to express a Th2-type cytokine mRNA pattern, with increased production of interleukin-4 (IL-4), IL-5, and IL-10, and deficient production of IL-2, IL-12, and interferon-γ (IFN-γ) (Vowels *et al*, 1992; Saed *et al*, 1994; Rook *et al*, 1997). Furthermore, clonal T lymphocytes isolated from the blood of patients with Sezary syndrome were described to display a Th2-type cytokine production pattern (Dummer *et al*, 1996). Sezary syndrome with a Th1 cytokine profile, however, has also been reported (Yagi *et al*, 1996). It should be noted that most of these studies were performed on a mixture of reactive and neoplastic T cells, particularly when studies were performed on skin lesions. Thus, the contributions of the reactive and the malignant T cells in the observed Th2 cytokine pattern are not clearly determined. Furthermore, the interactions of tumor-infiltrating lymphocyte (TIL) effector cells and tumor T cells as well as the mechanisms underlying the cytotoxic T lymphocyte (CTL) lysis of the

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Abbreviations: CTL, cytotoxic T lymphocyte; FasL, Fas ligand; TGF-β1, tumor growth factor 1; TIL, tumor infiltrating lymphocyte

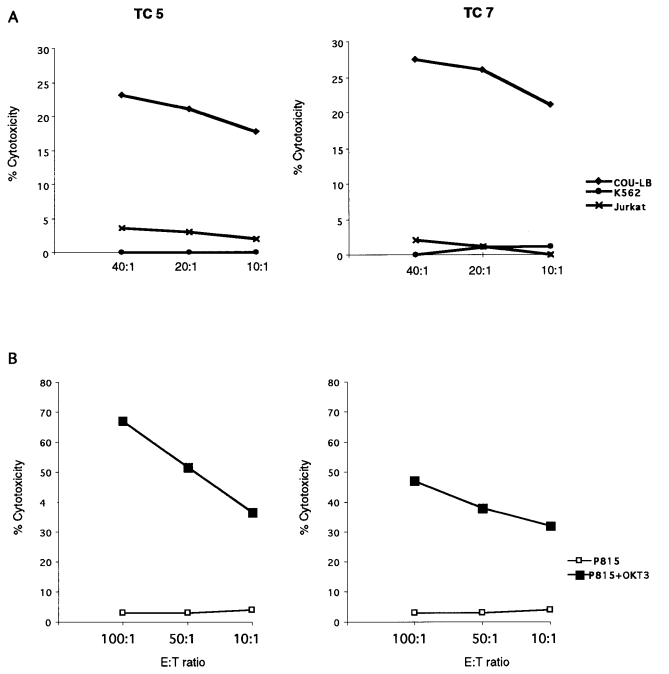

Figure 1. (*A*) Cytotoxic activity of TC5 (*left panel*) and TC7 (*right panel*) T cell clones against the autologous tumor cell line Cou-LB, K562, and Jurkat cells. (*B*) CD3-redirected lysis of TC5 (*left panel*) and TC7 (*right panel*) CTL clones toward P815 precoated with OKT3 MoAb. Assays were performed in triplicate at the indicated effector to target (E:T) ratios.

CTCL tumor remain to be determined. These issues are of great interest for the development of specific cellular immune therapy for CTCL.

We have previously isolated, from lymphocytes infiltrating a CTCL, two $CD4^+$ CTL clones (TC5 and TC7) recognizing the autologous tumor cells in a major histocompatibility complex (MHC) class I restricted manner (Bagot *et al*, 1998). To further study the interactions of these peculiar effector T lymphocytes and the specific tumor cells, we have investigated the mechanisms underlying specific CTL clone lysis and the cytokine profile of both malignant and reactive T lymphocyte populations. Our results indicate that the cytotoxic activity of TC5 and TC7 does not involve tumor necrosis factor (TNF)/TNF receptor (TNF-R) and Fas (CD95, Apo-1)/Fas ligand (FasL) pathways and that TC7 instead uses a granzyme–perforin-dependent pathway. In addition, while the tumor cells produced a Th2-type cytokine profile (IL-4, IL-6, and IL-10), both CTL clones expressed a Th1 cytokine profile [IL-2, granulocyte macrophage colony stimulating factor (GM-CSF) and IFN-γ] as well as tumor growth factor 1 (TGF-β1).

MATERIALS AND METHODS

Patient Cou was an 82-y-old man with a mycosis fungoides initially presenting as disseminated infiltrated patches and plaques with no extracutaneous involvement (T2N0M0, Ib). At a 5 y follow-up, this mycosis fungoides had evolved into a pleomorphic large T cell lymphoma presenting as disseminated cutaneous tumors and 30% atypical lymphocytes in the peripheral blood (T3N3M1, IVb; Lamberg *et al*, 1984).

Tumor cell line and T cell clones Cou-LB [T cell receptor (TCR) Vβ13+] was established *in vitro* from Cou patient peripheral blood as described previously (Bagot *et al*, 1998). TC5 and TC7 were isolated from TILs and cultured in the presence of IL-2 and IL-7 (Bagot *et al*, 1998).

Monoclonal antibodies (MoAbs) and flow cytometry studies Anti-TNF-R MoAbs (htr-9 and utr-1) were generously provided by Dr. M. Brokhaus (Hoffman-La Roche, Basel, Switzerland). CH-11 (anti-Fas apoptosis inducing IgM MoAb), UB2 (anti-Fas, IgG MoAb used for cell surface staining), ZB4 (anti-Fas apoptosis blocking IgG MoAb), and 4H9 (anti-FasL) were provided from Immunotech (Marseille, France).

Indirect immunofluorescence analysis was performed by incubating 3×10^5 cells with each MoAb (at 10 μg per ml) for 30 min at 4°C. Cells were then washed three times with phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA) and incubated with biotinylated affinity-purified goat antimouse IgG for an additional 30 min at 4°C. After three washings, cells were resuspended in 50 μl of streptavidin–phycoerythrin solution for 30 min at 4°C. Cells were then extensively washed before flow cytometric analysis using an EPICS profile II Coulter (Coultronics, Margency, France). FasL expression was performed on CTL clones activated for 2 h in the presence of Cou-LB cells or phorbol 12β-myristate 13α-acetate (PMA; Sigma Biochemicals, St. Quentin, France) and Ca^{2+} ionophore (ionomycin; Calbiochem, La Jolla, CA) as described previously (Mami-Chouaib *et al*, 1996).

Cytotoxicity assays Cytotoxic assays were performed according to a standard ^{51}Cr-release method. Target cells were labeled with 100 μCi sodium chromate (^{51}Cr; Amersham, France) for 1 h at 37°C, washed twice in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and then incubated at 37°C for 2 h to minimize spontaneous release. After a final wash, labeled cells were aliquoted at 2000 cells per well in 96 well U-bottomed microtiter plates in the presence of TC5 and TC7 effector cells. After incubation for 4 h at 37°C in 5% CO_2 incubator, supernatants were assayed for radioactivity using a γ-counter. Percent specific cytotoxicity was calculated conventionally; SD <5%.

The functional effect of the antibodies on target cells (anti-Fas neutralizing ZB4 MoAb, NKTa control MoAb) was tested by preincubating them for 1 h at 37°C before the assay at the predetermined saturating concentration. Ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) (4 mM) and $MgCl_2$ (3 mM) added during the assay were used to inhibit the Ca^{2+}-dependent perforin–granzyme-mediated lysis.

Target cells K562 (derived from a patient with chronic myelogenous leukemia), E418 (an Epstein–Barr-transformed B cell line; Mami-Chouaib *et al*, 1990), Jurkat (TCR-α/β leukemia), CEM (T lymphoblastoid line obtained from a patient with acute lymphoblastoid leukemia), and P815 (mouse DBA/2 mastocytoma) were used as targets in cytotoxicity assays.

Determination of cellular sensitivity to TNF Cell viability to TNF was determined using crystal violet staining as previously described (Zyad *et al*, 1994). Briefly, cells were seeded in flat-bottomed 96 well plates (7500 cells per well) and human recombinant TNF-α was added. After 72 h incubation at 37°C, the medium was replaced with 100 μl of 0.5% crystal violet solution. After 10 min incubation at room temperature, the plates were washed and viable crystal-violet-stained cells were lysed with 1% sodium dodecyl sulfate solution. The absorbance, which is proportional to cell viability, was measured at 540 nm.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis and oligonucleotide primer pairs Total RNA was extracted from the Cou-LB tumor cell line and the TC5 and TC7 T cell clones by an RNA-zol method (bioprobe system, Montreuil sous Bois, France) and reverse transcribed using a cDNA synthesis kit (Boehringer Mannheim, Meylan, France). cDNA was amplified by PCR as follows: 94°C for 90 s (94°C for 15 s, 55°C for 30 s, and 72°C for 50 s) for 25 cycles for β-actin or 30 cycles for FasL, perforin and granzyme B on a Perkin Elmer/Cetus DNA thermocycler. After amplification, PCR products were separated by electrophoresis on 1.5% agarose gel containing ethidium bromide and visualized under ultraviolet light illumination.

Primer pairs used for the amplification of actin, IL-4, IL-6, IL-10, and IFN-γ cDNA have been described previously (Carayol *et al*, 1997). GM-CSF and TGF-β1 primers have been described previously (Asselin-Paturel *et al*, 1998). For IL-2 cDNA amplification, the 5′ primer was TAGAAGAAGAAC TCAAACCTCTG and the 3′ primer GTGAAACCATTTTAGAGCCCCT; for FasL the 5′ primer was ATAGGATCCATGTTTCTGCTCTTCCACCTACAGAAGGA and the 3′ primer ATAGAATTCTGACCAAGAGAGAGCTCAGATAC-GTTGAC; for perforin the 5′ primer was CGGCTCACACTCACAGG and the 3′ primer CTGCCGT-GGATGCCTATG; for granzyme B the 5′ primer was GGGGA-AGCTCCATAAATGTCACCT and the 3′ primer TACACACAAGAGGGCCCCCAGAGT.

Quantification of cytokine mRNA expression by RT-PCR To quantify the cytokine transcripts expressed in the tumor cell line and the T

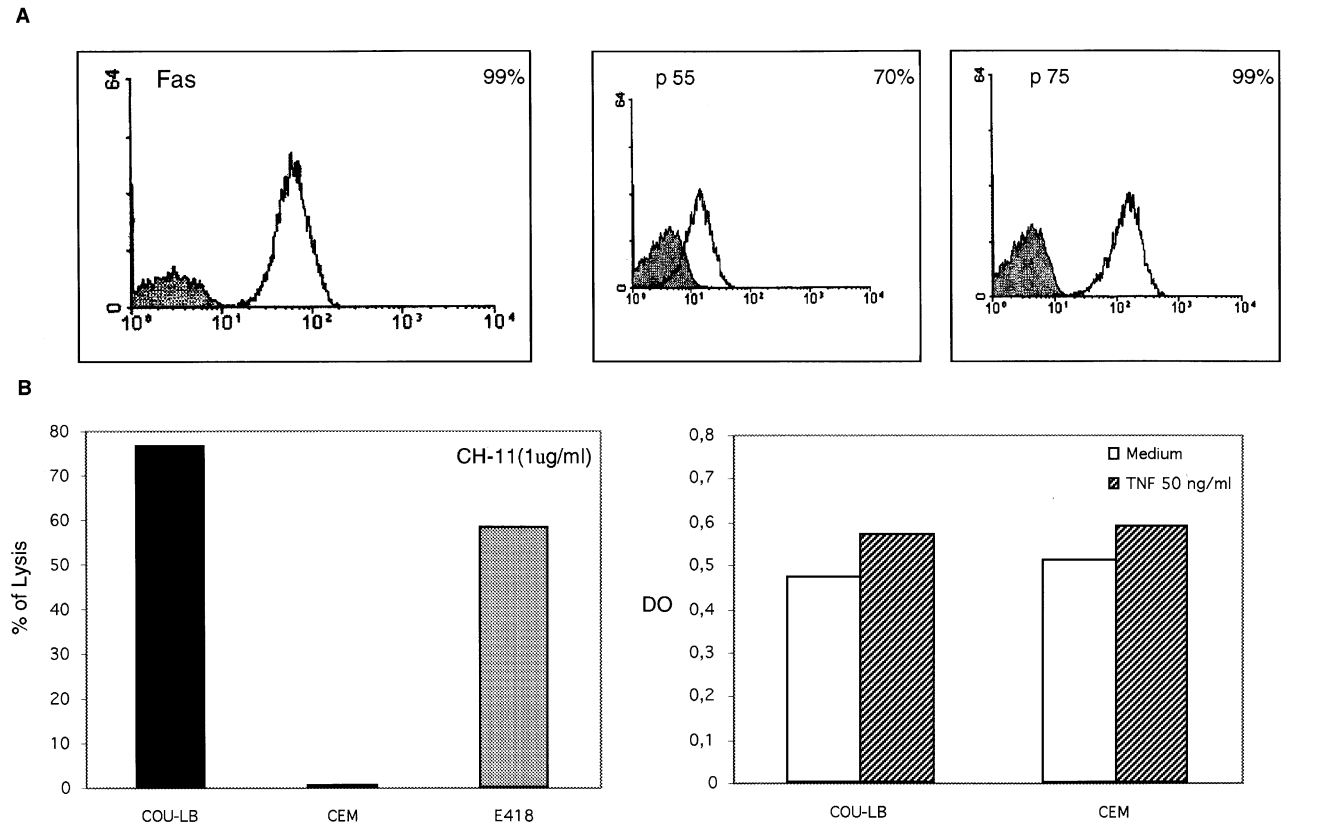

Figure 2. (*A*) Flow cytometric analysis of Fas and p55 and p75 TNF-R expression on Cou-LB cells. 3×10^5 cells were washed twice with PBS supplemented with 1% BSA and stained with UB2 anti-Fas MoAb and htr-9 and utr-1 MoAbs directed respectively against p55 and p75 TNF-R chains. (*B*) *Left panel*: Cou-LB-induced lysis by anti-Fas IgM MoAb (CH11). Cou-LB cells were ^{51}Cr-labeled for 1 h, washed three times with RPMI and then incubated for 4 h with 1 μg per ml of CH11 MoAb. Chromium release was then measured using an automated gamma counter. *Right panel*: Effect of TNF on the viability of Cou-LB tumor cells. Cells were incubated for 72 h with 50 ng per ml of recombinant TNF and cell viability was measured using the crystal violet assay as described in *Materials and Methods*. CEM and E418 cell lines were used as controls.

cell clones, a previously described methodology was used (Carayol *et al*, 1997). Briefly, a constant amount of cDNA corresponding to the reverse transcription of 50 ng of total RNA was mixed with 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, or 10 copies of the cytokine standard cDNA and then amplified to saturation (40 cycles) in 15 μl final volume. Aliquots (2 μl) of amplified material were copied in a one cycle runoff reaction (10 μl) primed with Fam fluorescent-labeled oligonucleotide specific for each cytokine gene. Runoff products were then subjected to electrophoresis on an ABI sequencer (Applied Biosystems, Foster City, CA). The software was devised to measure, for each detected DNA peak, both its length and its area. The ratio of the standard and wild type cDNA peaks provides a measurement of the ratios of the two initial molecular species, from which the number of cDNA copies in the sample can be calculated. The concentration of cDNA present in the original sample was obtained by determining the equivalent concentration of internal standard (Carayol *et al*, 1997).

Elispot assay Elispot assay was performed as described previously (Schmittel *et al*, 1997). Briefly, 96 well nitrocellulose-backed microtiter plates (Millipore, Vaucresson, France) were coated with 50 μl of mouse antihuman IFN-γ MoAb (4 mg per ml; Genzyme, Germany) and incubated overnight at 4°C. Wells were then washed with PBS and incubated with 200 μl of RPMI-1640 supplemented with 10% human AB serum for 2 h at 37°C. T cell clones were then added to the wells in triplicate at 2×10^5 cells per well in the presence or absence of irradiated Cou-LB T cell line (2×10^4 cells per well) and 100 U per ml of IL-2. Blocking experiments were performed in the presence of W6/32, anti-class I, MoAb at 30 μg per ml. After 24 h incubation at 37°C, plates were washed three times with PBS 0.05%, Tween 20 and wells were incubated overnight at 4°C with 100 μl of rabbit antihuman IFN-γ polyclonal antibodies (IP-500, 1:250 dilution; Genzyme, Germany). Plates were then washed with PBS, and 100 μl of alkaline phosphatase conjugate substrate (Bio-rad Laboratories, CA) were added. After 30 min incubation, the substrate solution was discarded and plates were washed under running water and air dried. Colored spots were counted using a dissecting microscope.

RESULTS

Expression of Fas and TNF-R on CTL-specific Cou-LB target lymphoma cell line TC5 and TC7 T cell clones were isolated from lymphocytes infiltrating a human MHC class II$^-$ T cell lymphoma (Bagot *et al*, 1998). They displayed a CD4$^+$CD8dim$^+$ (TC5) and CD4$^+$CD8$^-$ (TC7) phenotype and mediated a specific MHC class I restricted cytotoxic activity (Bagot *et al*, 1998) toward the autologous tumor cell line, Cou-LB, established from patient peripheral blood (**Fig 1*A***). This cytotoxicity was inhibited by anti-CD3 (OKT3; data not shown) and anti-class I (W6/32) MoAbs but not by anti-CD4 (OKT4) and anti-CD8 (OKT8) MoAbs (Bagot *et al*, 1998). TC5 and TC7 clones were unable to kill K562, the Fas-sensitive Jurkat cell line (**Fig 1*A***) and the murine Fas$^-$ mastocytoma cell line P815 (**Fig 1*B***). In contrast, both CTL clones kill P815 in the presence of anti-CD3 (OKT3) MoAb, indicating that they are able to mediate a high redirected cytotoxic activity in the absence of Fas expression on target cells (Bensussan *et al*, 1994).

Immunofluorescence analysis, performed to further characterize Cou-LB tumor cells, indicated that they surface expressed Fas as well as p55 and p75 TNF-R (**Fig 2*A***). A significant FasL expression was also observed on the tumor cell membrane (data not shown). To determine whether Cou-LB lymphoma cell line was sensitive to Fas- and TNF-mediated cytotoxicity, cells were incubated in the presence of anti-Fas IgM MoAbs (CH11) or recombinant TNF (50 ng per ml). As shown in **Fig 2*B***, the tumor

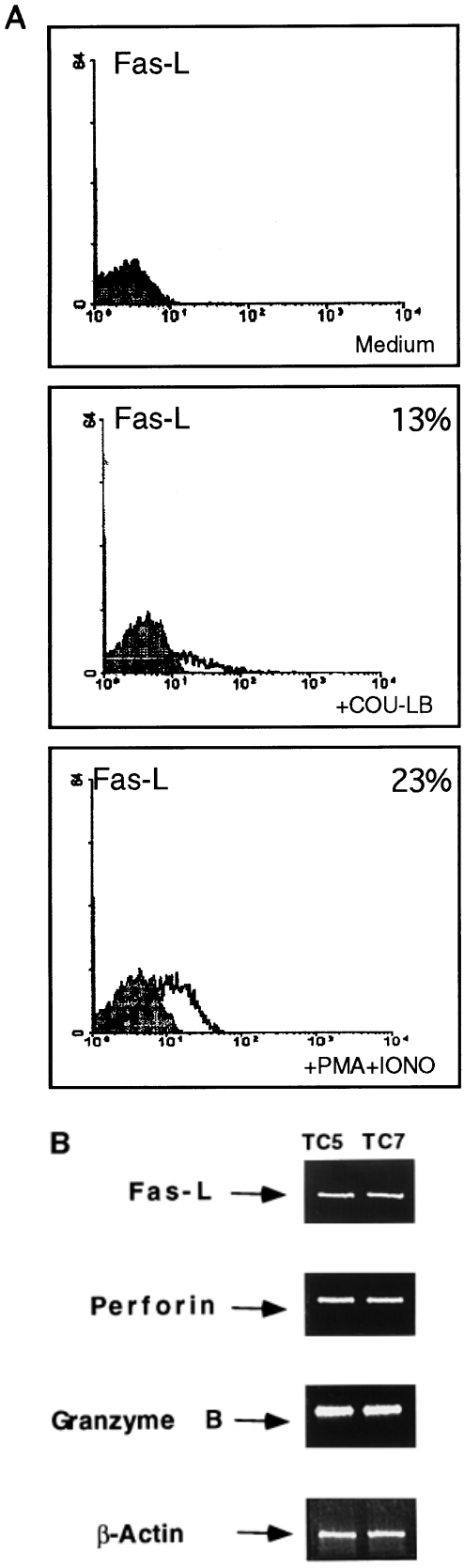

Figure 3. (*A*) Surface expression of FasL by TC5 T cell clone. FasL expression was evaluated by flow cytometry using the 4H9 anti-FasL MoAb on CTL clone activated for 2 h in the presence of Cou-LB cells or PMA+ ionophore. (*B*) RT-PCR analysis of FasL, perforin, granzyme B and β-actin in TC5 and TC7 T cell clones. Total RNA was extracted from T cell clones, reverse transcribed and amplified by PCR using specific primer pairs.

cell line was sensitive to Fas-induced (*left panel*) but not to TNF-induced (*right panel*) lysis, indicating that the Fas/FasL alternative cytotoxic pathway was functional and that the T cell lymphoma was resistant to TNF.

CD4+ CTL clones do not use the Fas/FasL alternative cytotoxic pathway to lyse their specific target CTL clones use two distinct mechanisms to kill target cells, the calcium-dependent, perforin–granzyme-mediated pathway and the calcium-independent Fas/FasL-mediated pathway. Initial immunofluorescence experiments were performed to analyse FasL expression on TC5 and TC7. Both CTL clones were found to surface express FasL, following stimulation with Cou-LB autologous tumor cells or in the presence of PMA+ ionomycin (**Fig 3*A*** and data not shown). RT-PCR analyses, undertaken with the use of specific primer pairs, confirmed FasL expression in the CTL clones and, as expected from functional studies presented in **Fig 1*B***, revealed intense bands corresponding to perforin and granzyme B mRNA expression (**Fig 3*B***).

In order to determine the mechanisms implicated in TC5 and TC7 CD4+ T cell clone-mediated lysis toward autologous tumor cells, cytotoxic experiments were performed in the presence of anti-Fas IgG-1 blocking MoAbs (ZB4) or $MgCl_2$ and EGTA. No inhibition of TC5 and TC7 cytotoxicity was observed when target cells were preincubated with ZB4 MoAbs (**Fig 4*A***). In contrast, EGTA blocked TC7-mediated lysis toward Cou-LB tumor cells. A slight inhibition was observed when TC5 was used as effector cells (**Fig 4*A***). The control experiment indicated that soluble FasL (sFasL) induced a high lysis of Cou-LB target which was blocked in the presence of ZB4 MoAb, confirming its inhibitory potential (**Fig 4*B***). These results indicate that despite FasL expression on TC5 and TC7 and Fas on Cou-LB cells, this cytotoxic pathway is not implicated in CTL clone lysis of autologous target. Therefore, TC7 clone most probably uses a perforin–granzyme-mediated cytotoxic pathway.

Cou-LB tumor cell line expresses a Th2-type cytokine profile The basis of cellular and molecular cross-talk between tumor cells and immune cells remains a crucial issue for an effective use of cytokines in the biotherapy of cancer. Immunosuppression at the tumor site appears to be one of the major obstacles in cancer immunotherapy. Therefore, identifying the factor secreted by lymphoma T cells in order to overcome such immunosuppression may be of great interest. For this purpose, we have investigated, using a highly sensitive quantitative RT-PCR analysis (Carayol *et al*, 1997), both Th1, Th2 and TGF-β1 cytokine mRNA expression in Cou-LB T cell lymphoma. This tumor cell line was found to express IL-4, IL-6, IL-10, and TGF-β1 mRNA (**Table I**). TGF-β1 protein expression was also observed in Cou-LB before and after stimulation with PMA (36 pg per ml *versus* 400 pg per ml in supernatant from 10^6 cells per ml cultured for 24 h). In contrast, no IFN-γ, IL-2, and GM-CSF mRNA was detected. These results indicate that Cou-LB lymphoma possesses a Th2-type cytokine pattern.

TC5 and TC7 CTL clones express a Th1-type cytokine profile To investigate whether TC5 and TC7 express a Th1- or a Th2-type cytokine and whether the tumor cells may alter their lymphokine secretion profile, we have investigated IL-2, IFN-γ, GM-CSF, IL-4, IL-6, IL-10, as well as TGF-β1 mRNA expression in TIL clones before or after coculture with Cou-LB cells. In contrast with the tumor cell line, no Th2-type cytokine mRNA (i.e. IL-4, IL-6, and IL-10) was detected in the two CTL clones (**Table I**). mRNA encoding for IL-2, IFN-γ, and GM-CSF, however, was expressed in both clones with a high expression level of IFN-γ in TC7 clone (**Table I**). Surprisingly, a high copy number of TGF-β1 mRNA was expressed in both clones. We then tested IFN-γ protein secretion in the supernatant of TC5 clone cultured at 10^6 cells per ml in the absence or presence of Cou-LB. Our results indicated a significant enhancement of IFN-γ production when the T cell clone was cocultured with the

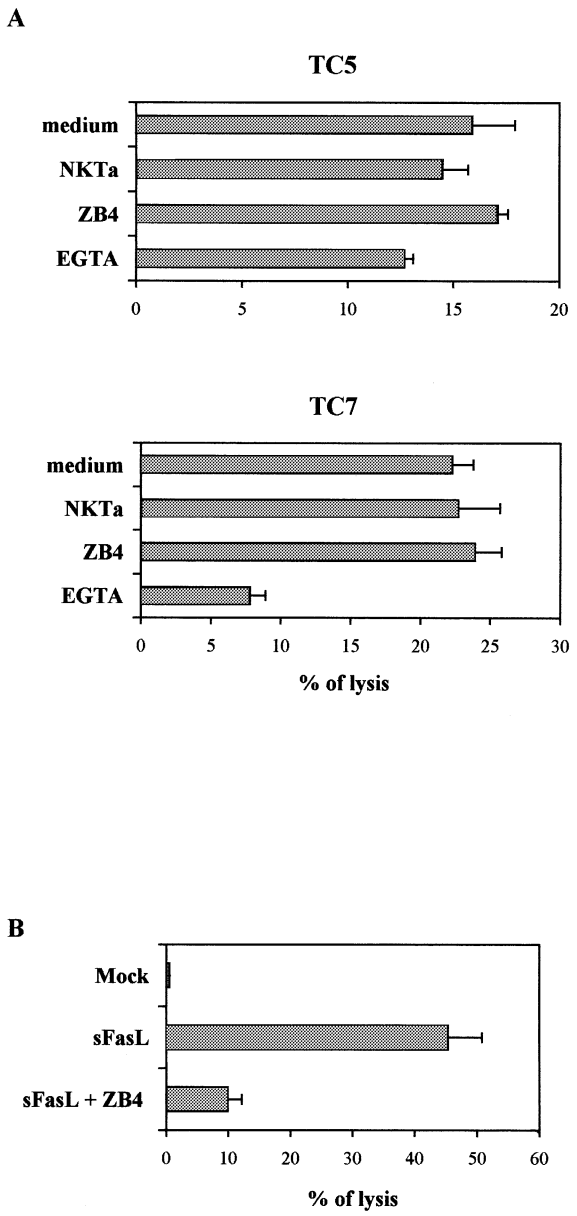

Figure 4. (*A*) Cytotoxic activity of TC5 and TC7 T cell clones toward Cou-LB target cells. Experiments were performed either in media or after preincubation of target cells for 1 h in the presence of anti-Fas ZB4 MoAb, NKTa (isotypic control), or in the presence of EGTA (4 mM) + $MgCl_2$ (3 mM). The E:T ratio was 40:1. (*B*) Cou-LB-induced lysis by soluble FasL (sFasL) was inhibited by ZB4 blocking MoAb. Mock empty vector was used as control. ^{51}Cr-labeled Cou-LB cells were preincubated in media in the presence of ZB4 and then sFasL was added.

autologous tumor cells (1.5 pg per ml *versus* 75 pg per ml). As expected from RT-PCR analysis no IFN-γ protein was detected in the supernatant of the Cou-LB cell line.

To determine whether tumor cells may activate CTL clones and induce an alteration of their cytokine expression profile, we incubated TC5 and TC7 in the presence of Cou-LB for 12 h, and Th1 and Th2 cytokine mRNA expression was determined after removal of tumor cells with anti-TCRVβ13 MoAb and magnetic beads. **Table I** shows that CTL clone activation with specific tumor target enhances IFN-γ, GM-CSF, and IL-2 expression levels. A dramatic increase in TGF-β1 mRNA expression was also detected in both CTL clones. In contrast, a weak increase in IL-4 and IL-10 mRNA expression was observed following stimulation with autologous tumor cells (**Table I**). Activation of TC5 and TC7 lymphocytes by specific target was confirmed by Elispot analysis demonstrating an increase in IFN-γ spot number following preincubation of the CTL clones with Cou-LB (**Fig 5**). This IFN-γ secretion was inhibited by W6/32 anti-class I MoAbs in a similar manner as described previously in cytotoxic activity experiments (Bagot *et al*, 1998). These results indicate that the MHC class I restricted CTL clones express a Th1-type cytokine mRNA profile with an expression level sensitively increased in the presence of the specific Th2-type autologous T cell lymphoma.

DISCUSSION

TC5 ($CD4^+CD8dim^+$) and TC7 ($CD4^+$) CTL clones mediate a specific cytotoxic activity toward an autologous MHC class II^-/$class^+$, $CD4^+$ CTCL (Bagot *et al*, 1998). In this study we investigated the cytotoxic mechanisms involved in autologous tumor cell lysis by the two $CD4^+$ TIL clones. We showed that, despite Cou-LB surface expression of p55 and p75 TNF-R, the tumor cell line was resistant to TNF-induced lysis. Resistance to TNF, frequently developed by several tumors of different histologic types, is of major concern in cancer therapy and the mechanisms underlying it have yet to be fully elucidated. In addition, a correlation between *in vitro* resistance to TNF-induced apoptosis and high *in vivo* tumor metastatic capacity has been reported (Korobko *et al*, 1999). It is now clearly established that cell-surface expression of TNF-R is necessary but not sufficient to induce a biologic response, and post-receptor mechanisms are important in controlling susceptibility to the cytotoxic action of TNF. Recent data suggest that alteration of sphingomyelinase activation and loss of p53 function may correspond to mechanisms by which human tumor cells escape TNF-mediated apoptosis (Cai *et al*, 1997a,b). Furthermore, it has been reported that constitutive activation of NF-kB may cause resistance to apoptosis induced by this cytokine in human HuT-78 CTCL cells (Giri and Aggarwal, 1998).

Data generated using murine T cells have suggested a critical role for Fas in $CD4^+$ T-cell-induced apoptosis of target cells (Hanabuchi *et al*, 1994; Ju *et al*, 1994; Stalder *et al*, 1994; Zajac *et al*, 1996; Lewinsohn *et al*, 1998). More recent data, however, including in human models, support a role of perforin-granzymes in $CD4^+$-mediated cytotoxicity (Gagnon *et al*, 1999; Sun *et al*, 1999; Yasukawa *et al*, 1999). Indeed, it has been reported that $CD4^+$ lymphocytes exhibited CD95-independent antigen-specific cytotoxicity that was diminished in perforin-deficient mice (Williams and Engelhard, 1996). So far little is known about the role of $CD4^+$ CTL effector mechanisms in CTCL. Therefore, we have investigated the relative contribution of CD95 and perforin–granzyme cytolysis pathway in the TC5 and TC7 killing of Cou-LB lymphoma. Initial studies indicated that the malignant T cells were sensitive to Fas-induced lysis and that both CTL clones expressed perforin and granzyme B as well as FasL following stimulation by autologous tumor cells. Our results indicate that the cytotoxicity of TC5 and TC7 did not involve the Fas-mediated pathway, since no inhibition of their specific activity was observed using anti-Fas blocking MoAb. In contrast, this lysis, namely that mediated by TC7, was Ca^{2+} dependent and was inhibited in the presence of EGTA + $MgCl_2$. TC5 cytotoxicity toward the Cou-LB tumor cell line was inhibited in the presence of anti-TRAIL blocking MoAb, suggesting that TC5 may involve the TRAIL pathway in its lytic activity (data not shown). $CD4^+$ CTL clones, able to kill their specific target in a Fas-independent fashion, were previously described in melanoma and glioma solid tumors as well as in anti-acute myelogenous leukemia-associated DEK-CAN fusion peptide response (Rivoltini *et al*, 1998; Thomas and Hersey, 1998; Hishii *et al*, 1999; Ohminami *et al*, 1999). These data fit with ours and suggest that the predominant mechanism in tumor killing by TIL is perforin–granzyme dependent.

The lack of Fas-dependent lysis in our CTCL model suggests that this is unlikely to play a major role in tumor destruction, but does not preclude another, perhaps counterproductive, role for Fas. Indeed, it has been observed in several human cancer models that tumor cells can express FasL and it has been proposed that they can

Table I. Quantification of cytokine mRNA by RT-PCR[a]

	Th1			Th2			
	IL-2	IFN-γ	GM-CSF	IL-4	IL-6	IL-10	TGF-β1
Cou-LB	<10	<10	10	1000	2,370	60	92,000
TC5	230	7,120	340	<10	ND	<10	527,000
TC7	660	61,480	350	<10	10	<10	832,000
TC5+Cou-LB	3,130	71,150	2,310	270	ND	90	7,440,000
TC7+Cou-LB	1,100	320,000	1,390	20	ND	40	6,600,000

[a]Th cytokine mRNA pattern in Cou-LB tumor cell line and CTL clones before and after coculture, at 1:1 ratio, with the autologous tumor cells for 12 h and removal of Cou-LB cells using an anti-TCRVβ13-specific MoAb and magnetic beads. Cytokine mRNA expression was measured by quantitative PCR (see *Materials and Methods*). Results are expressed as copy numbers of cytokine transcripts, determined with a fragment size analysis software after migration of the PCR products on a DNA sequencer (Applied Biosystems).

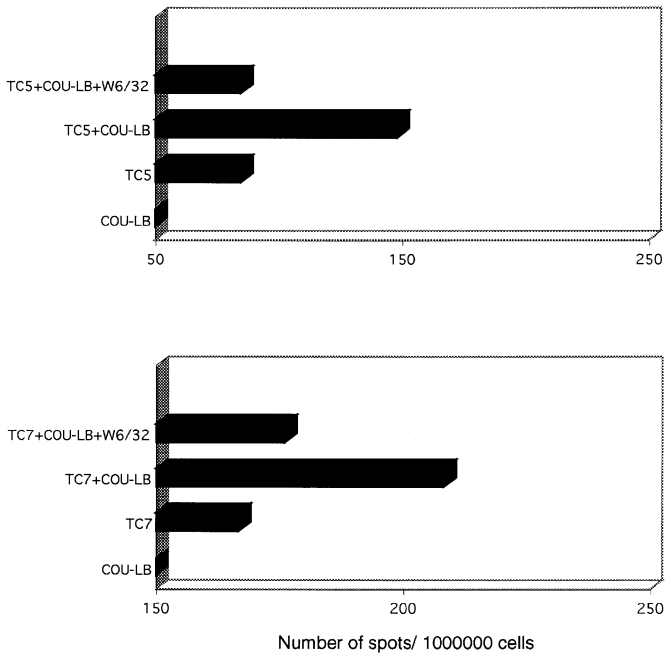

Figure 5. Numbers of T cell clones secreting IFN-γ. TC5 and TC7 were seeded in a concentration of 2×10^5 cells per well and were cultured for 24 h in the presence or absence of 2×10^4 cells per well of the T cell lymphoma Cou-LB and 100 U per ml of IL-2. Blocking experiments were performed in the presence of anti-class I MoAb (W6/32 at 30 μg per ml).

destroy TIL by this mechanism, contributing to the immune privilege of tumors (Hahne *et al*, 1996; O'Connell *et al*, 1996; Williams, 1996; Walker *et al*, 1997). The Cou-LB cell line constitutively expresses FasL on its surface; thus the killing of TIL by FasL-expressing lymphomas may have contributed to tumor escape from immune surveillance. Further studies are now under way to elucidate this mechanism.

It is clearly established that the outcome of the antitumor response is governed by a network of cytokines and that understanding the cytokine repertoire within the tumor microenvironment is crucial for the development of reactive T cell effectors. In this context, it has been described that CTCL patients show a predominant type-2 immune response that might be responsible for several clinical and immunologic abnormalities (Dummer *et al*, 1993). In order to investigate the respective parts of the reactive and the malignant T lymphocytes in the CTCL Th2-type cytokine profile, we determined their cytokine mRNA expression pattern. We showed that Cou-LB tumor cells expressed Th2-type cytokines (IL-4, IL-6, and IL-10) as well as TGF-β1 mRNA. In contrast, no GM-CSF, IL-2, and IFN-γ Th1-type cytokine mRNA was detected. These results strongly suggest that the lymphoma cells may correspond to the major source of the Th2-type cytokines reported in skin biopsies and peripheral blood of CTCL patients (Dummer *et al*, 1993, 1996; Saed *et al*, 1994; Vowels *et al*, 1994; Rook *et al*, 1997). IL-4 and IL-10 are key cytokines involved in inhibition of Th1-cytokine response and promote the development of Th2-type response (Mossman and Coffman, 1989; de Waal Malefyt *et al*, 1992). This response is probably associated with profound defects in the cell-mediated immunity observed in CTCL patients and may contribute to downregulation of the local antitumor reactivity and thus to tumor escape.

It is now well established that CD4+ T cells can mediate a cytotoxic activity and may correspond to a critical component in antitumor immune response (Hahn *et al*, 1995). This type of cell-induced cytotoxicity is generally associated with a Th1, but not Th2, phenotype. The cytokine expression profile of TC5 and TC7 cells, determined before and after stimulation with Cou-LB tumor cells, indicated that both CTL clones express Th1 (GM-CSF, IL-2, and IFN-γ) but not Th2(IL-4, IL-6, and IL-10) cytokine mRNA. Incubation of TIL clones with Cou-LB induces their activation and an amplification of Th1-type cytokine and TGF-β1 mRNA expression. The immunosuppressive effect of TGF-β1 on the proliferation, differentiation, and activation of the CTL response has previously been reported in several tumor models (Ranges *et al*, 1987; Torre-Amione *et al*, 1990; Maeda and Shiraishi, 1996; Chouaib *et al*, 1997; Groux *et al*, 1997; Asselin-Paturel *et al*, 1998). The functional inhibitory capacity of TGF-β1 involves inhibition of cytokines essential in CTL generation and potentiation, namely IL-12 (Pardoux *et al*, 1997) and its signaling (Pardoux *et al*, 1999). Clinical trials based on rhIL-12 administration in CTCL patients induced lesion regression and increased antitumor CTL response (Rook *et al*, 1999). Therefore, understanding the functional interactions between immunosuppressive and immunoregulatory cytokines may ultimately help provide new approaches to intervene in specific ways in cancer therapy and will help in the optimization and elaboration of new immunotherapeutic strategies in CTCL.

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