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Presence of γδ TcR+ Tumor Infiltrating Lymphocytes (TILs) in One Case of Pediatric Osteogenic Sarcoma

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Purpose: To characterize αβ and γδ TcR expression in cultured TILs from a metastatic osteogenic sarcoma.

Patient and methods: TILs were isolated from a lung metastasis of an osteogenic sarcoma in a 16-year-old female patient. Culture conditions were IL-2 and anti-CD3 MoAb+IL-2. TcR expression and surface phenotype were studied by flow cytometry. 4h-chromium release assays were used to assess cytotoxicity.

Results: IL-2-expanded tumor infiltrating T-lymphocytes contained 50% γδ TcR+ cells whereas γδ TcR+ cells represented 20% of T-cells in cultures expanded with anti-CD3 MoAb+IL-2. The phenotype of γδ TcR+ cells was CD3+, CD16−, CD4−, CD8−, and CD29+. Anti-CD3 MoAb + IL-2-expanded TILs mediated a significant lysis of the autologous tumor and of an allogeneic lymphoma and displayed a higher activity against the NK-sensitive target K562. In cold target experiments, unlabeled autologous tumor cells blocked the lysis of K562, indicating that MHC-unrestricted effectors were involved in the lysis of both targets.

Conclusions: This report demonstrates the expansion of large numbers of γδ TcR+ T-cells after short-term culture of TILs from a metastatic osteosarcoma. Further studies are needed to determine the role of γδ TcR+ cells in host immune responses directed against this tumor.

Key words: Tumor infiltrating lymphocytes (TIL), gamma/delta T-cell receptor, osteogenic sarcoma

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INTRODUCTION

OLIGOCLONALITY OF TUMOR infiltrating lymphocytes (TILs) has been demonstrated in several human tumors including lung cancer,[1] melanoma,[2] and ovary adenocarcinoma.[3] It is a strong argument that TILs may be part of a specific immune response of the host against the tumor. Therefore, TILs could be viewed as tools to identify the tumor antigens recognized by the immune system. Animal models also suggest that *in vitro* expanded TILs could be used in adoptive cellular immunotherapy protocols with a higher efficiency than lymphokine activated killer (LAK) cells generated from peripheral blood lymphocytes.[4] In most cases, TILs are CD3+ T-cells expressing the αβ T-cell receptor (TcR), while γδ TcR+ lymphocytes usually represent a minor subset of TILs (less than 5%).[5,6]

Despite their low frequency in TILs and peripheral blood, T-cells expressing the γδ TcR are of major interest in the field of tumor immunity. After short-term culture *in vitro*, these lymphocytes develop MHC-unrestricted cytolytic activity against a variety of tumor cells of distinct histologic origin.[7,8] Recent reports have suggested that molecules related to the 65-kDa heat shock protein found in mycobacterial extracts could be involved in the non-MHC restricted lysis of some tumor cells by γδ TcR+ lymphocytes.[9,10]

We have characterized the phenotype and function of TILs from a lung metastasis of an osteogenic sarcoma in a 16-year-old patient. We observed a high frequency of γδ TcR+ T-cells in the cultured TILs from this tumor. TILs displayed a significant cytolytic activity of the autologous tumor but also mediated non-MHC restricted cytotoxicity of K562 and of CESS, an allogeneic B-cell lymphoma.

PATIENT AND METHODS

Tumor. The tumor was a pulmonary metastasis of an osteogenic sarcoma. The patient was a 16-year-old girl who had developed an osteogenic sarcoma of the

metaphyseal region of the proximal tibia two years earlier. Several pulmonary metastases were present when the primary lesion was diagnosed. The patient received a preoperative chemotherapy regimen including high dose methotrexate with citrovorum factor rescue and a combination of bleomycin, cyclophosphamide, and dactinomycin. Complete regression of the pulmonary metastases was obtained. The primary tumor was resected, and the biopsy specimen showed less than 25% necrosis. Therefore, the patient received several postoperative chemotherapy treatments with cisplatinum and adriamycin. She finally developed a large pulmonary metastasis, invading the pleura and occupying the anterior mediastinum. The metastasis was resected by anterior thoracotomy and sent without fixation to the pathology department under aseptic conditions.

Preparation of cells. Cell suspensions were prepared by mechanical and enzymatic dissociation of the tumor specimen using an enzyme mixture containing collagenase and hyaluronidase (Sigma). Cell suspensions containing a mixture of tumor cells, lymphocytes, and monocytes were incubated with complete medium (CM) at 37°C in a humidified atmosphere of 5% CO_2. Culture medium consisted of DMEM (Gibco), 30 U/ml penicillin-streptomycin (Gibco), 1% nonessential amino acids (Gibco), and 1 mM sodium pyruvate (Gibco). Heat-inactivated fetal calf serum (FCS) (30G, Gibco) was added at 10% to obtain complete medium (CM). Recombinant human IL-2 (rIL-2) was kindly provided by Glaxo Institute for Molecular Biology (AG, Genève, Switzerland) and used at 50 U/ml. For anti-CD3 + rIL-2 activated cultures, anti-CD3 MoAb (purified mouse IgG_{2a} clone BMA030, Becton Dickinson, Erembodegem, Belgium) was added once at the beginning of the cultures at 10 ng/ml.

Antibodies and flow cytometry. FITC-conjugated-anti-CD4 (Mouse IgG_1 clone SK3). PE-conjugated-anti-CD8 (mouse IgG_1 clone SK1), FITC-conjugated anti-CD45RA (Mouse IgG_1 clone L48), FITC-conjugated and biotinylated-anti-CD3 (Mouse IgG_1 clone SK7), FITC-conjugated-anti-TcR-γδ1 (Mouse IgG_1 clone 11F2), purified anti-TcR-αβ (Mouse IgG_1 clone WT31), and PE-conjugated anti-CD16 (Mouse IgG_1 clone B73.1) were purchased (Becton Dickinson, Erembodegem, Belgium). PE-conjugated anti-CD29 (Mouse IgG_1 clone 4B4) was purchased (Coulter, distributed by Analis, Namur, Belgium). Streptavidin-PE (Dako) was used to reveal biotinylated-anti-CD3. FITC-labeled-goat anti-mouse IgG (Dako) was used to reveal purified anti-TcR-αβ. For flow cytometry analysis, cell suspensions were layered over Ficoll Hypaque and spun at 2000 rpm for 20 min. Viable lymphocytes were collected, washed, and incubated for 30 min. on ice with appropriate amounts of antibodies and second steps in CM with sodium azide 0.1%. Cells were washed twice and analyzed for fluorescence intensity on a FACScan (Becton Dickinson).

Cytotoxicity assays. Effector cells were washed, counted, and resuspended in CM at a concentration of 2.5×10^5 cells/100 µl. Four serial dilutions in triplicates were made in 96-well U-bottom plates (Nunc). Target cells were incubated with $Na^{51}CrO_4$ (Medgenix, Fleurus, Belgium) for 1 h at 37°C, washed three times, counted, and adjusted to obtain 10×10^3 cells/100 µl. This suspension was added to the serial dilutions of effector cells. After centrifugation, the microplates were incubated for 4 h at 37°C, 100 µl of supernatant and were then recovered from each well; radioactivity was measured in a γ-counter (Cobra auto-gamma Packard, Downers Grove, IL). Maximal chromium release was obtained by adding 100 µl of detergent (RBS 10% Chemical products) to 100 µl of target cells suspension, and spontaneous release was given by the incubation of target cells without effectors cells. Percent cytotoxicity was calculated according to the following formula:

$$\frac{\text{Experimental mean CPM} - \text{Spontaneous mean CPM}}{\text{Maximal mean CPM} - \text{Spontaneous mean CPM}} \times 100$$

Target cells for cytotoxicity assays. K562 is an MHC-negative erythroleukemic cell line commonly used to study natural killer (NK) activity. CESS is an EBV-induced B-cell lymphoma and is resistant to NK activity. Both cell lines were cultivated in RPMI-1640 with 10% FCS. Fresh tumor cells from the biopsy specimen did not grow in culture, therefore small tumor fragments were frozen after dissection. A few hours before the cytolytic assay, tumor fragments were thawed and enzymatically dissociated to obtain cell suspensions. These cells were washed and incubated at 37°C a few hours before radioactive labeling. This procedure allowed an optimal viability of the tumor cells. In the cytotoxicity assay showed in Figure 1, the spontaneous release was 1891 ± 98 cpm and the maximum release was 10367 ± 1453 cpm (n=3). These tumor cell suspensions were not depleted in lymphocytes and monocytes, however the contribution of nontumor cells to $Na^{51}CrO_4$ uptake was considered to be negligible.

Cold target inhibition. Cytolytic specificity was evaluated by adding unlabeled competing targets to the ^{51}Cr release assay. Varying ratios of cold targets were mixed with ^{51}Cr-labeled targets (5×10^3/well) in 100 µl CM. Effector lymphocytes (1×10^5 cells/well) were then added and the plates incubated for 4 h at 37°C. The percent inhibition of cytotoxicity (i) was calculated according to the formula:

$$i = \left(\frac{\text{exp.}}{\text{cont.}} - 1\right) \times 100$$

where exp. is the specific lysis observed in the presence of cold targets and cont. is the specific lysis observed without cold targets.

TABLE I
Sequential Surface Phenotypes of TILs from Osteogenic Sarcoma in Culture with rIL-2 (50 U/ml) or rIL-2 + BMA030 (10 ng/ml).

Culture Condition	Day of Culture	Percentage of Cells Expressing[a]							
		CD3	αβTcR	γδTcR	CD16	CD4	CD8	CD29	DN[b]
rIL-2	10	63	34	30	34	11	23	NT	29
rIL-2+anti-CD3	10	81	67	18	15	31	32	NT	18
rIL-2	28	98	48	46	2	6	40	97	52
rIL-2+anti-CD3	28	98	75	21	1	3	75	96	20
rIL-2+anti-CD3	49	99	73	28	1	4	69	99	26

[a]Reactivity of cultured cells with specific MoAb was assessed by flow cytometry analysis. NT, not tested.
[b]Calculated proportion of CD3+ T-cells negative for CD4 and CD8 (double negative: DN) obtained with the formula CD3 – (CD4+CD8).

RESULTS

1. *Presence of γδTcR + T-cells in Cultured TILs*

After enzymatic dissociation of the tumor and culture with rIL-2 (50 U/ml) or rIL-2 (50 U/ml) + anti-CD3 MoAb (10 ng/ml), tumor cells rapidly disappeared and activated lymphocytes were visible in the wells. Viable lymphocytes were isolated and flow cytometry analysis was performed (Table I). After 10-day culture with IL-2, 63% of TILs expressed CD3 but only 34% were recognized by the MoAb WT31 specific for an epitope associated with the αβ TcR. The CD3+ αβ TcR- T-cells, representing 47% of CD3+ T-cells, expressed the γδ TcR. After initial stimulation with anti-CD3 MoAb, the frequency of γδ TcR+ cells was only 18%, representing 22% of CD3+ T-cells. After 28 days, natural killer (NK) cells expressing CD16 had disappeared in both culture conditions and all remaining lymphocytes expressed CD3. The proportion of CD3+ T-cells expressing the γδ TcR remained around 50% in IL-2-expanded cultures and around 20% in anti-CD3 MoAb+IL-2-expanded TILs (Table I). Anti-CD3 MoAb expanded cultures displayed a 11.50-fold expansion of total lymphocyte counts between day 10 and day 28, whereas IL-2 alone only induced a modest 1.70-fold expansion. Furthermore, the viability of IL-2-expanded cultures decreased after day 28 while further expansion was observed in the anti-CD3 MoAb+IL-2-expanded TILs. Therefore, although anti-CD3 MoAb activation was associated with a lower proportion of γδ TcR+ cells, this culture condition allowed a higher absolute expansion of these cells in comparison with IL-2-expanded cultures.

The CD4/CD8 ratio of TILs decreased in both culture conditions reaching 0.15 and 0.04 on day 28 for IL-2- and anti-CD3 MoAb+IL-2-expanded cultures respectively. Interestingly, the number of CD3+ T-cells was higher than the number of cells expressing either CD4 or CD8 indicating that a large fraction of CD3+ T-lymphocytes was negative for CD4 and CD8. At all time points and in both culture conditions, there was a perfect correlation between the frequency of double negative CD3+ T-cells (CD3 - (CD4 + CD8)) and the frequency of γδ TcR+ T-cells. Although three-color staining was not performed, this correlation indicated that most γδ TcR+ T-cells present in the cultures were CD4- CD8- as previously described for γδ TcR+ T-cells found in the peripheral blood.[7,8] At day 28, all lymphocytes displayed a memory activated phenotype: CD45RA-CD29+. Taken together these results showed that the γδ TcR+ T-cell population was CD3+, CD16-, CD4-, CD8-, and CD29+.

2. *Cytotoxic Activity of TILs Expanded in Anti-CD3 MoAb+ IL-2*

TILs cultured for 19 days in the presence of anti-CD3 MoAb + IL-2 displayed a moderate but significant cytolytic activity against the autologous tumor (25% specific lysis E:T 25:1) and against CESS, an EBV-induced B-cell lymphoma (20% specific lysis E:T 25:1) (Fig. 1). A strong activity was demonstrated against K562 (50% specific lysis E:T 25:1). The cytolytic activity of TILs cul-

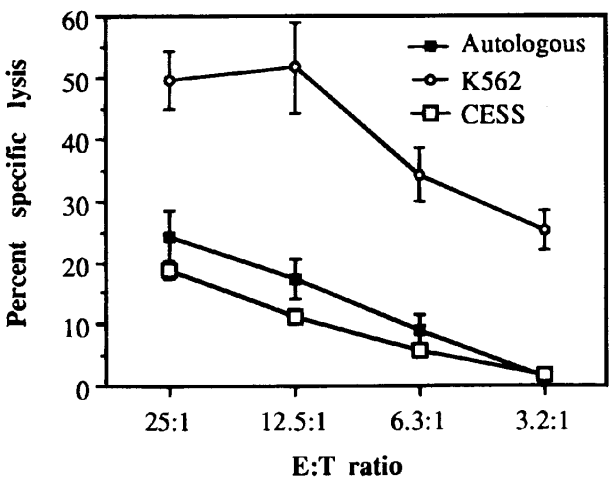

FIGURE 1 **Cytotoxic activity mediated by anti-CD3 MoAb + IL-2-expanded TILs (day 19) against (—■—) autologous tumor cells, (—○—) K562, (—□—) CESS.** Results represent mean specific lysis ± SD from the triplicates of a 4 h-chromium release assay.

tured in IL-2 alone was not tested because of insufficient expansion in this condition. Afterward, the cytolytic activity against the autologous tumor declined while the activity against K562 remained high (not shown). We used cold target inhibition experiments to determine if the effectors responsible for the lysis of K562, assumed to be non-MHC restricted, could bind to the autologous tumor cells. On day 33, the addition of unlabeled autologous tumor cells strongly inhibited the lysis of K562 although less efficiently than unlabeled K562 cells (Fig. 2). This inhibition indicated that a large proportion of the non-MHC restricted cytolytic effectors present in the anti-CD3 MoAb + IL-2-expanded cultures could bind to the autologous tumor cells.

DISCUSSION

This report demonstrated the presence of a large proportion of γδ TcR+ T-cells within the cultured TILs from a metastatic osteogenic sarcoma. To our knowledge, only two published reports have described large numbers of γδ TcR+ T-cells in TILs.[11,12] Bachelez *et al.* reported frequencies of γδ TcR+ T-cells between 15 and 25% in three cases of primary cutaneous melanoma.[11] γδ TcR+ T-cells were not found in metastatic lesions even in subcutaneous sites, therefore it was postulated that the melanoma-associated γδ TILs could derive from the large pool of γδ TcR+ T-cells present in normal epidermis.[11] Rivoltini *et al.* studied the frequency of γδ T-cells in IL-2-expanded TILs from 20 pediatric tumors including osteosarcomas, Wilms' tumors, neuroblastomas, ovarian teratocarcinomas and melanoma.[12] Interestingly, the only tumors in which large numbers of γδ TILs were observed were two cases of metastatic osteogenic sarcoma with respectively 52% and 61% of T-cells expressing the γδ TcR.[12] Unfortunately, no other data are available about TcR expression by TILs from osteogenic sarcoma.

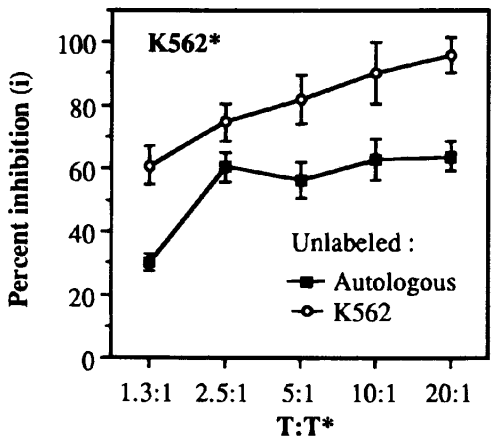

FIGURE 2 **Cold target inhibition of the lysis of K562.** Effectors were anti-CD3 MoAb+ IL-2 expanded TILs (day 33). ^{51}Cr labeled K562 cells were mixed with effectors at E:T 20:1 with addition of competing unlabeled: (—■—) autologous tumor cells, and (—○—) K562 as indicated. T:T* represents the ratio between unlabeled targets and ^{51}Cr labeled K562 cells. The data represent mean percent inhibition (i) ± SD from triplicates. i was calculated according to the formula described in the methods section.

We did not study the frequency of γδ TcR+ T-cells *in situ*, before tumor dissociation and *in vitro* activation. Therefore, the large number of γδ TcR+ T-cells could be viewed as a preferential expansion due to *in vitro* culture. Although we cannot formally rule out this hypothesis, it is important to note that using the same protocol of isolation and *in vitro* expansion, we never detected any increase of CD3+ γδ TcR+ cells in TILs from a large number of breast and gastrointestinal tumors.

The cultured TILs demonstrated a moderate lytic activity against the autologous tumor which was significantly lower than that against the NK sensitive target K562. A comparable autologous cytotoxicity and an absence of tumor specificity were also found in most TILs cultures from pediatric tumors in Rivoltini's study, although these authors did not study autologous cytotoxicity in the cases of osteosarcomas with γδ TcR+ TILs.[12] Since our cytolytic assays were performed with non-sorted effector cells, our data are not sufficient to prove that the cytolytic activity observed was actually due to γδ TcR+ T-cells.

Recent evidence has shown that γδ TcR+ lymphocytes may recognize a heat shock protein in human tumors.[9,10] Therefore, the presence of γδ TcR+ TILs in three cases of metastatic osteogenic sarcoma could be due to the expression of heat shock–related proteins by these tumor cells. It remains to define if such an expression would be intrinsic to this type of tumor or mostly related to the intensive preoperative chemotherapy or radiotherapy regimens received by the patients. Since MoAb directed against hsp60 have been recently developed,[13] our findings together with Rivoltini's report[11] advocate for a careful analysis of hsp60 surface expression in metastatic osteogenic sarcoma.

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