Melanoma-reactive Human Cytotoxic T Lymphocytes Derived from Skin Biopsies of Delayed-Type Hypersensitivity Reactions Induced by Injection of an Autologous Melanoma Cell Line¹

Gary A. Waanders,² Donata Rimoldi, Danielle Liénard, Stefan Carrel,³ Ferdy Lejeune, Pierre-Yves Dietrich, Jean-Charles Cerottini, and Pedro Romero

Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, CH-1066 Epalinges [G. A. W., D. R., D. L., S. C., J.-C. C., P. R.]; Centre Pluridisciplinaire d’Oncologie, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne [D. L., F. L.]; and Laboratoire d’Immunologie des Tumeurs, Division d’Oncologie, Université de Genève, 1211 Geneva 14 [P.-Y. D.], Switzerland

ABSTRACT

The expression by melanomas of multiple antigens that are recognized by specific MHC class I-restricted CTLs has been clearly demonstrated. The goal of many immunotherapy protocols being developed is, therefore, the induction and/or augmentation of CTLs specific for such antigens. One approach has been to immunize using irradiated autologous melanoma cells. Responses to this type of immunization and others are often subsequently measured by delayed-type hypersensitivity (DTH) reactions. The aim of this work was to characterize whether specific CTL responses occur at such DTH sites. Cutaneous DTH reactions were observed following injection of irradiated autologous melanoma cells expressing known tumor antigens. We isolated lymphocytes from biopsies of DTH reaction sites and could measure melanoma-specific CTL activity after 2–3 weeks of culture. The T-cell receptor-Vβ repertoire of the cultured lymphocytes, assessed by flow cytometry, was highly skewed in both the CD4⁺ and CD8⁺ T-cell subsets. The repertoires were different among cultures derived from independent biopsies of simultaneous or subsequent DTH reaction sites and very different to that of fresh peripheral blood lymphocytes (PBLs) or PBLs cultured under the same conditions. No particular T-cell expansions dominated several DTH reaction sites, nor could they be detected in PBLs. It appears that T-cell responses to this type of immunization may be limited to the local microenvironment. Establishing the value of DTH reactions in determining levels of systemic antitumor immunity requires further investigation; however, such reactions may indicate a patient’s competence to mount an antitumor immune response and enable the isolation of tumor-specific CTLs for use in tumor antigen identification.

INTRODUCTION

The recent description of numerous MHC class I-restricted peptides derived from genes expressed by human tumors either selectively (e.g., MAGE, GAGE, and BAGE) (1–3) or as cell lineage-specific antigens (e.g., tyrosinase, Melan-AMART-1, gp100, and gp75) (4–8) and recognized by human CTLs has prompted new interest in the potential of immunological interventions using specific epitopes of such antigens in the treatment of various cancers (reviewed in Refs. 9 and 10). The central aim of most such interventions is the induction or augmentation of CTL responses to antigens expressed by tumors. To this end, several approaches have been used including immunization with irradiated autologous or semiallogeneic melanoma cells with or without adjuvants (11–14) and/or transfected with cytokines such as granulocyte-macrophage colony-stimulating factor (15), immunization with antigenic extracts of melanoma cells either alone or in conjunction with adjuvants (16, 17), use of viral constructs engineered to express the antigenic peptides or proteins (18, 19), immunization using antigen-presenting cells pulsed with defined tumor antigens for expansion of specific CTLs (20), and intradermal injection of synthetic peptides alone (21).

DTH⁴ reactions in the skin appear to be frequently observed in many such immunotherapy protocols and are often used as an indicator of antitumor immunity and vaccine efficacy (16, 17, 21). However, very little is known about either the function, phenotype, specificity, or diversity of the lymphocytes participating in such DTH reactions. Here we have determined that diverse, functional CTLs specific for the immunizing cell line are indeed present at DTH sites. Tumor-reactive CTL could be derived from biopsies of such sites in a relatively short time. We demonstrate a remarkable degree of heterogeneity in the TCR repertoire of these tumor-reactive CTLs at different sites but no cumulative response over time (1 year). The rapid derivation of melanoma-specific CTLs from the skin may be im-

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² Present address: DiagnoCure, Inc., Sainte-Foy G1V 2K8, Canada. Phone: (418) 527-6100; Fax: (418) 527-0240. To whom requests for reprints should be addressed.

³ We gratefully announce the passing away of Dr. Stefan Carrel on March 18, 1996. We would like to take this opportunity to express our sincerest gratitude for his work.

⁴ The abbreviations used are: DTH, delayed-type hypersensitivity; TCR, T-cell receptor; TCR-C, TCR constant domain; TCR-V, TCR variable domain; IL, interleukin; PBL, peripheral blood lymphocyte; HLA, human leukocyte antigen; RT-PCR, reverse transcription-PCR; mAb, monoclonal antibody; LCL, lymphoblastoid B-cell line.
portant when considering possible adoptive transfer of CTLs as an immunological intervention. Moreover, these skin-derived, autologous melanoma-specific CTL clones could be extremely useful as probes to identify novel tumor antigens.

Finally, whereas DTH reactions may be indicative of a patient’s ability to respond to tumor antigens, establishing whether the reactions adequately assess the level of systemic T-cell reactivity to the specific immunogen requires further investigation.

MATERIALS AND METHODS

Patient

Patient LAU63, a 65-year-old male (HLA-A1, A3, B8, Bx) with malignant melanoma (disease-free stage IIIAB, M. D. Anderson scale) was enrolled in immunotherapy studies with informed consent. This patient had previously undergone adjuvant therapy whereby he received up to 3 × 10^6 units of IFN-α three times per week for 1 year. This treatment was terminated approximately 9 months prior to this work.

Immunization

An autologous melanoma cell line (Me235) was derived from a tumor-infiltrated lymph node and maintained in RPMI 1640 supplemented with 10% FCS. These cells were transferred to culture medium containing 5% autologous or pooled human serum at least 2 days prior to injection. Before injection, the cells were irradiated with a dose of 15,000 rads. A total of no less than 2 × 10^7 (first immunization) and up to 10 × 10^7 cells (subsequent immunizations) were injected intradermally at multiple sites (3 to 5) at 5–6-month intervals.

Biopsy and Lymphocyte Recovery

Punch biopsies (measuring 5 mm in diameter and approxi-mately 5 mm in depth) of DTH reactions were performed at day 5 at some of the injection sites. Lymphocytes infiltrating the skin at the DTH reaction sites were harvested after mechanical dissociation of the biopsies into small fragments using scissors followed by treatment with 0.1% type I collagenase (Sigma Immuno Chemicals, Fluka Chemie, Buchs, Switzerland) and 0.02% DNase (Boehringer Mannheim) in complete tissue culture medium. The enzymatic digestion was performed for 2 h at 37°C. Lymphocytes were separated from the remaining nondigested tissue fragments by allowing the clumps in the suspension to settle at 1 × g for 5–15 min in normal medium and then harvesting the lymphocytes from the supernatant. Fragments remaining after the 2-h digestion were further incubated overnight in the enzyme solution. The lymphocytes from this digestion were then harvested as before. Immunization site biopsies series 2 and 3 were performed 5 and 11 months, respectively, after the first series.

Lymphocyte Cultures

The skin lymphocytes obtained from the biopsies of DTH reactions were cultured in 48-well plates in 1 ml of Iscove’s Modified Dulbecco’s Medium supplemented with 10% pooled human serum and penicillin and streptomycin (50 units/ml and 50 μg/ml, respectively), in the presence of recombinant IL-2 (200 units/ml, CTLT units) (a kind gift from Glaxo, Geneva, Switzerland) and recombinant IL-7 (20 ng/ml) (Sigma, LucernaChem). After this, all cultures were stimulated weekly with 5 × 10^5 irradiated Me235 cells and maintained in the presence of IL-2 (100 units/ml) and IL-7 (20 ng/ml), with the exception of cultures 1.2, 1.4, and 1.5, which were stimulated weekly with 2 × 10^5 irradiated Me235 cells and maintained in the presence of only IL-2 (10 units/ml) after day 7.

Cloning CTLs from Biopsy Sites

Lymphocytes were sorted using a FACStar Plus (Becton Dickinson, Mountain View, CA) from culture 1.5 on the basis of the expression of both CD8 and TCR-Vβ21.3. These cells were then plated into 96-well plates at a dose of three cells/well. These cultures were established with 1% phytohemagglutinin, 50 units/ml IL-2, irradiated Me235 cells (5 × 10^5 per well), and irradiated mixed autologous and allogeneic PBLs (1 × 10^5 per well). They were restimulated weekly with IL-2 (50 units/ml) and irradiated Me235 cells (5 × 10^5 per well) and irradiated mixed autologous and allogeneic PBL (1 × 10^5 per well).

Melanoma Cell Lines

Melanoma cell lines were derived from various patients following mechanical dissociation of resected tumor metastases. The autologous line Me235 as well as Me190 (HLA-A3, Ax, B7, Bx), Me200 (HLA-A3, A28, B39, Bw62), and Me243 (HLA-A24, A29, B44, Bx) grew directly in vitro in RPMI 1640 supplemented with 10% FCS. Me197 (HLA-A1, A29, B7, Bw52) and Me241 (HLA-A1, A33, B8, B14) were established from tumors transplanted s.c. into nude mice.

Expression of Genes Encoding Tumor Antigens

The expression of various tumor antigens and β-actin was analyzed at the mRNA level using RT-PCR. Briefly, total mRNA was isolated using Trizol (Life Technologies, Inc.), and cDNA was synthesized as described previously (22). RT-PCR was then carried out as described elsewhere (23) using the following primer pairs for the indicated genes: (a) MAGE-1, sense 5'-CGGCCGAAGGAACCTGACCCAG and antisense 5'-GGAA- GATfATCAGGAGGCCTGC; (b) MAGE-3, sense 5'-TGAGGAGCAAGGCGCTCACTGCGTCGCC; (c) Melan-A, sense 5'-CTGAC- CTTACAGATGGCCAGAG and antisense 5'-ATCATGCA- TTTGCAACATTATTTGATAGAG; (d) tyrosinase, sense 5'- GGATAGCCTGATGCTCTTCAAG and antisense 5'-CC- AAGAGGCAATCGACCCAGAT; (e) gp100, sense 5'-GGAA- GACCAAAATGTCTGAGTGG and antisense 5'-CACAGCAT- CATATCAGGTAC; and (f) β-actin, sense 5'-GGCATTGGT-GATGACTCCCG and antisense 5'-GCTGGAAAGGTGGA- CAGCGA.

Immunohistology

Skin biopsies of injection sites were snap frozen using a liquid N2/isopentane slurry. Thin sections were cut from frozen tissue samples, labeled with nonconjugated mAbs against CD3, CD4, CD8, or CD69, followed by a biotinylated anti-mouse immunoglobulin, and finally with avidin-peroxidase and processed, as described previously (24).
**Immunofluorescence Staining**

Lymphocytes from either peripheral blood or skin biopsies were stained for two- or three-color flow cytometric analysis using protocols described previously (25) with slight modifications. Briefly, any mAb labeling involving an indirect step (e.g., anti-TCRB-V3 mAbs plus anti-mouse immunoglobulin-FITC) was followed by a blocking step using mouse immunoglobulin prior to any further labeling steps with directly conjugated mAbs. All mAbs were diluted in PBS-5% FCS, and titrations were tested on fresh PBLS to give optimal labeling. All staining was performed in a volume of 10 μl using a minimum of 5 × 10^6 cells and a maximum of 10^8 cells in either V-bottomed tubes or V-bottomed 96-well plates. Washing steps involved the addition of at least a 10-fold larger volume of PBS-5% FCS. Cells were resuspended in final volume of 300–400 μl for analysis using a FACScan (Becton Dickinson). Analysis was performed using the Lysys II software (Becton Dickinson).

mAbs used in this report were as follows. mAbs reactive with TCRBV2 (E22E7.2), TCRBV3 (LE-89), TCRBV5S1 (IMMU 157), TCRBV5S2 (36213), TCRBV6S1 (CRI304.3), TCRBV13S6 (JU74), TCRBV14 (Ca 243), TCRBV16 (Ta37), TCRBV17 (E17.5F3), TCRBV18 (BA62), TCRBV21S3 (IG 125), and TCRBV22 (IMMU 546) were all obtained from Immunotech SA (Marseille, France); anti-TCRBV6S7 (OT145) was obtained from T Cell Diagnostics Inc. (Cambridge, MA); anti-TCRBV9 (MKB-1 P1.2) and anti-TCRBV23 (Hut 78) was a gift from Dr. O. Kanagawa; anti-TCRBV13S1 (H131.21.5.14.2) and anti-TCRBV13S2 (H13.2) was a gift from Dr. J. Kappler (Howard Hughes, Denver, CO); anti-TCRBV5S3 (3D11) and anti-TCRBV8 (MX6) were both produced by Dr. S. Carrel (Ludwig Institute for Cancer Research, Lausanne Branch); anti-CD4, anti-CD8, and anti-CD28 mAbs were obtained from Becton Dickinson.

**Molecular Analysis of TCR-V3**

**Sequencing of TCR-V3 Expressed by Clone 2C4.** This Me235-reactive CTL clone was derived from biopsy culture 1.5 after sorting cells expressing CD8 and TCR-V321 using a FACStar Plus (Becton Dickinson) and culturing at limiting dilution (three cells/well). Total mRNA was isolated from the expanded clone using Trizol (Life Technologies, Inc.), and cDNA was synthesized as described (22). RT-PCR specific for TCR-V321 was then performed (35 cycles) using 2 μl of cDNA as template in a final volume of 100 μl using the following primer pair: TCR-V321 sense 5'-ATTCCACAGTGGCTAAG-GATCGA and TCR-C3 antisense 5'-TGCTGACCCCACTGT-GCCACCTCCCTCCATT. PCR products were purified using QIAquick PCR Purification kits (Qiagen, Basel, Switzerland), and the purified products were directly sequenced using the
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Sequenase Version 2.0 DNA Sequencing kit (United States Biochemical, Cleveland, OH) with slight modifications (26). The TCR-Vβ21 PCR primer used above for PCR amplification was used here to sequence the sense strand, whereas the anti-sense strand was sequenced using an internal TCR-Cβ primer 5'-GCTTCTGATGGCTCAAACAC.

CDR3 Size Analysis of TCR-Vβ Transcripts. The size of the CDR3 region of selected PCR-amplified TCR-Vβ transcripts was analyzed using a run-off procedure, as previously described (27). Briefly, total RNA was prepared from the T cell clone and skin biopsies (1 to 5 × 10⁶ cells or approximately 100 mg tissue) using Trizol (Life Technologies, Inc.), and converted to cDNA by standard methods using reverse transcriptase and an oligo dT primer. These cDNAs were amplified using an experimentally validated TCR-Vβ21 specific sense primer paired to one TCR-Cβ anti-sense primer in a 40 cycle RT-PCR (28). Then, 2 μl aliquots of the PCR products were subjected to 1 to 5 cycle run-off reactions using dye-labeled oligonucleotide primers, specific either for TCR-Cβ or one of the 13 human junctional TCR-Jβ segments. In the case of clone 2C4, an

Fig. 2  T-cell infiltration at cutaneous DTH reactions following injection of irradiated autologous melanoma cells. Frozen sections were labeled with mAbs against CD3 (A), CD4 (B), CD8 (C), and CD69 (D) and developed using anti-mouse immunoglobulin-biotin and avidin-peroxidase. Positive labeling is indicated by a red-brown color. E, negative control-labeling in the absence of a first-layer mAb. ×150.

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oligonucleotide primer specific for the N region was synthesized and used to probe for the presence of this clone in peripheral blood and other injection site skin biopsies. The run-off products were separated on an automated sequencer (Applied Biosystems), in the presence of fluorescent size markers. The length of DNA fragments and the fluorescence intensity of the bands were analyzed with the Gene Scan Analysis software (Applied Biosystems).

### CTL Assay

Specific CTL activity was assessed using a conventional 51Cr release assay as described previously (29). All assays were performed in the presence of a 50-fold excess of K562 cells as cold-target inhibitors. Target cells (1000 per well), K562, and varying numbers of effector cells (to give effector:target ratios ranging from 1:1 up to 100:1 in some experiments) were incubated in V-bottomed microwells for 4 h at 37°C, and chromium release was measured thereafter.

### RESULTS

#### Characterization of the Human Melanoma Cell Line Used for Immunization.

A melanoma cell line (Me235) was derived from a surgically excised tumor-infiltrated lymph node of the patient. This cell line expressed high levels of MHC class I molecules, which were up-regulated by treatment with IFN-γ (Fig. 1A). The majority of these cells did not constitutively express MHC class II but were positive following treatment with IFN-γ (Fig. 1A). An unrelated antigen, CD2, showed no increase following IFN-γ treatment. Analysis at the mRNA level of the expression of genes encoding various antigens by RT-PCR demonstrated that Me235 expressed the tumor antigens MAGE-1 and MAGE-3, and the melanocyte lineage-specific antigens gp100, tyrosinase, and Melan-A (Fig. 1B). Me235 appeared, therefore, to be a good vaccine candidate and was irradiated and injected intradermally to evaluate its potential immunogenicity.

#### T Cells Are Present among Lymphocytes Infiltrating DTH Sites.

The sites of injection of the autologous melanoma cell line Me235 showed evidence of local inflammation, erythema, and induration typical of DTH reactions. To analyze the response occurring at these sites, punch biopsies were performed at day 5 after immunization. Immunohistological examination of biopsies of the DTH sites revealed a large proportion of T cells among the cells infiltrating the injection sites (Fig. 2A). Both CD4- and CD8-positive cells were present (Fig. 2, B and C, respectively), and many of these expressed classical T-cell activation markers such as CD69 (Fig. 2D).

#### Lymphocytes Harvested from Biopsies of DTH Reaction Sites Proliferate in the Presence of Autologous Melanoma Cells.

Lymphocytes were recovered from the biopsies by mechanical dissociation and collagenase digestion. These lymphocytes, usually not more than approximately $5 \times 10^5$ per biopsy, were cultured with IL-2, IL-7, and $5 \times 10^5$ irradiated Me235 cells. In parallel, an equivalent number of PBLs were placed into culture under the same conditions. As shown for the second series of biopsies (Table 1), after 15 days the number of lymphocytes had increased in all cultures, but the most dramatic increase was seen in the lymphocytes derived from the biopsies of the DTH sites. In contrast to the injection-site biopsies, we were unable to obtain enough cells for further analyses from a similar skin biopsy taken at a distant, noninjected site (data not shown).

#### Selection of Melanoma-reactive CTLs from Biopsy Sites.

We carried out phenotypic and functional analyses of day 15 cultures derived from the first series of biopsies. At this time point, the percentage of CD3+ cells in each culture was 85% or more (Fig. 3A). In cultures 1.2 and 1.4, CD4+ T cells comprised the vast majority, whereas not more than 5% of the cells expressed CD8 (Fig. 3B). In contrast, the overall phenotypic profile was more heterogeneous in culture 1.5 (these lymphocytes came from an overnight digestion of pooled fragments remaining after the initial 2-h collagenase digestion of two biopsies), with approximately 20% of the cells expressing CD8. As can be seen in Fig. 3C, the vast majority of CD8+ T cells in these cultures, as in all other cultures of lymphocytes, expressed CD28, with the proportion of CD8+ cells expressing CD28 being roughly equal to those belonging to the CD4 subset in the panel above. The CD4+ subset in PBLs usually expresses slightly higher levels of CD28 than CD8+ T cells (30). In the presence of Me235 cells, the lymphocyte populations in each culture became increasingly skewed toward the CD8+ T-cell subset (Fig. 3, D and E).

In parallel with the phenotypic studies at days 15 and 28, we assessed the levels of CTL activity against the Me235 line or an autologous EBV-transformed lymphoblastoid cell line (Fig. 4). In addition, CTL activity was measured at day 28 against a panel of semiallogeneic or allogeneic melanoma cell lines (Fig. 4). The CTL activity in the cultures at day 15 against either the autologous Me235 cells or the EBV-transformed cells was at best only very low. However, on day 28, CTL activity against Me235 was easily detected, with the highest activity seen clearly in culture 1.5. At no time were the CTLs in these cultures specific for either MAGE-1.A1 or MAGE-3.A1 peptides restricted to HLA-A1 because pulsing targets (particularly the autologous EBV line) with either peptide did not significantly alter the levels of lysis (data not shown). It should be noted that the Me235 cells express both MAGE genes, as determined by RT-PCR (Fig. 1), and they are efficiently recognized by both HLA-A1-restricted MAGE-1- and MAGE-3-specific CTL clones (data not shown).

### Table 1 Lymphocyte recoveries from skin biopsies following mechanical dissociation, collagenase digestion, and in vitro stimulation

<table>
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<tr>
<th>Biopsy no.</th>
<th>Seeded cell no.*</th>
<th>Day 0</th>
<th>Cell no.</th>
<th>Day 7</th>
<th>Cell no.</th>
<th>Day 15</th>
<th>Fold increase Days 0–15</th>
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* x 10^6.
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Fig. 3 Phenotypic profiles of cultured lymphocytes derived from biopsies of DTH reactions induced by injection of irradiated autologous melanoma cells. A. CD3 expression by lymphocytes following 2 weeks of culture. B. CD4 versus CD8 expression at 2 weeks. C. CD28 versus CD8 expression showing that all of the CD8+ cells expressed CD28 at 2 weeks. D. CD28 versus CD8 expression at week 3, showing the increasing percentage of CD8+CD28+ cells. E. week 4 profile of CD28 versus CD8 with increased skewing toward CD8+CD28+ cells. Viable lymphocytes were selected by gating using forward and side light scatter. Each plot represents at least 10,000 cells.

TCR-Vβ Repertoire Skewing among Cultured Lymphocytes from Biopsy Sites. Although the TCR-Vβ repertoire was not characterized at the time of biopsy because of the low lymphocyte recovery, the proliferation that occurred in the following weeks allowed us to perform quantitative TCR-Vβ repertoire analyses using a panel of anti-TCR-Vβ mAbs.

The various cultures demonstrated diverse TCR-Vβ repertoires with a relatively large amount of variation among biopsy sites (Table 2). For example, CD8+ T cells from culture 1.2 expressed mainly Vβ16; in culture 1.4, those expressing Vβ2 dominated, and in culture 1.5, CD8+ cells expressing Vβ21.3 were the most frequently detected. In the other series, CD8+ T cells expressing Vβ2, Vβ5.1, or Vβ13.2 were also present at elevated frequencies relative to either fresh or cultured PBLs. In some cultures (e.g., 2.2) dominant Vβ expression could not be determined because of the limited nature of the panel of anti-TCR-Vβ mAbs. The skewing in the CD8 subset in each culture became more pronounced with each restimulation with the Me235. Stimulation of each culture with anything other than Me235 resulted in drastic reductions in both the fraction of CD8+ T cells and the frequency of cells expressing the Vβ domains mentioned above (data not shown). Interestingly, we also observed significant biasing of the repertoire in the CD4+ T-cell subset in the cultures relative to PBLs.
C1R-Al is a C1R cell line stably transfected with HLA-Al. HLA-A or -B alleles with Me235 (HLA-C was not determined).

any one or more HLA class I alleles with Me235. Me243 does not share Mel9O, Me197, Me200, and Me241 are all melanoma cell lines sharing an autologous EBV-transformed lymphoblastoid B-cell line; and cells expressing previously elevated V.3 domains.

The first series of biopsies demonstrated the expansion of a different repertoire of tumor-reactive cells from each immunization site. In the two subsequent series of injection-site biopsies, the development of independent repertoires in different sites was again observed. With the exception of Vβ2* cells from cultures 1.4 and 2.1 and Vβ13.2 in cultures 1.4 and 3.1, we did not repeatedly see the preferential expansion of CD8* T cells expressing previously elevated Vβ domains.

A single biopsy (2.3) was divided into two pieces before lymphocyte isolation and culture (cultures 2.3.1 and 2.3.2) to determine whether the limiting cell numbers present in biopsies would give rise to different T-cell populations. As shown in Table 2, the two cultures demonstrated both very different T-cell phenotypic profiles (CD4:CD8 ratio) and TCR-Vβ repertoires, suggesting that the T-cell pool present before culture is limiting, giving rise to a randomly selected pool from which tumor-specific cells are derived. As before, we detected specific CTL activity against the Me235 cell line in each culture (data not shown).

**CTL Activity of Cells Expressing Defined TCR-Vβ Domains.** To identify which cells were responsible for the specific CTL activity against the Me235 tumor line, cells from biopsy series 1 cultures were sorted by fluorescence-activated cell sorting according to the expression of CD8 and the various Vβ that were expanded. Thus, from cultures 1.2, 1.4, and 1.5, we sorted CD8* Vβ16* cells, CD8* Vβ2* cells, and CD8* Vβ21.3* cells, respectively. After several weeks of restimulation and proliferation induced by continuous culture with Me235, high levels of CTL activity against Me235 were demonstrated by the Vβ2* cells and Vβ21.3* cells, whereas the cells expressing Vβ16 from culture 1.2 had a lower activity (Fig. 5). The latter cells could not be tested against the auto EBV LCL target because they proliferated poorly after sorting.

**Functional and Molecular Analysis of an Me235-reactive CTL Clone.** The population of sorted CD8* Vβ21.3* cells from culture 1.5 was plated at limiting dilution in the presence of Me235 cells and IL-2. One of the clones, 2C4, selected on the basis of rapid growth, was shown to be cytolytically active specifically against Me235 (Fig. 6A). Total RNA from clone 2C4 was prepared, and following cDNA synthesis, a TCR-Vβ21-specific Vβ/Cβ TCR fragment was amplified by PCR. The PCR product was directly sequenced and revealed a Vβ21.3, DB1.1, JB2.7, CB2 rearrangement (Fig. 6B).

We used a PCR assay that takes advantage of the TCR β chain CDR3 size heterogeneity of different T-cell clones to determine the presence of clone 2C4 in vivo. This assay has previously been shown to be a very useful tool for detecting in vivo oligoclonal expansions in T-cell populations and to search in vitro-derived CTL clones using N region-specific primers (31). TCR-Vβ/Cβ PCR products were further amplified with a nested fluorescent Cβ or Jβ primer. The sizes of the fluorescent run-off products thus obtained were determined by electrophoresis on an automated DNA sequencer. The size variations of the run-off products are only due to differences in the lengths of CDR3 regions, reflecting the imprecise V/DJ joining mechanism. In PBLs from healthy donors, the repertoire of any Vβ-Cβ and Vβ-Jβ combination is usually represented by a bell-shaped profile with peaks spaced 3 nucleotides apart corresponding to in-frame sequences (27).

As expected from its sequence and the position of the respective primers (Cβ, JB2.7, and N), clone 2C4 was characterized by a single peak at 143 nucleotides when its cDNA was analyzed using the fluorescent Cβ primer (Fig. 6C), at 102 nucleotides using the JB2.7 primer (Fig. 6C), and at 66 nucleotides with the N primer (data not shown). There was no dominant peak in TCR-Vβ21 transcripts extracted from biopsies 2.2 and 4.1, where the profiles obtained suggested a polyclonal population (Fig. 6C). The absence or very low frequency of clone 2C4 in both these skin biopsies was confirmed using the JB2.7 primer because no peak was observed at the size of 102 nucleotides. In contrast, a predominant peak was noted in biopsy...
Table 2  TCR-Vβ repertoires of lymphocytes from either fresh PBLs, cultures of PBLs, or skin-infiltrating lymphocytes derived from injection site biopsies

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<th>Fresh PBLs</th>
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<td>46.6</td>
<td>79.9</td>
<td>28.3</td>
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Cultures were maintained under the conditions given in Table 1, with the exception that culture 1.5 contained IL-2 (100 units/ml) and IL-7 (10 ng/ml) in the first 7 days and that all cultures from series 1 were maintained after day 7 with IL-2 only (10 units/ml).

a Numbers represent the percentage of cells in the cultures with the given phenotype.
b Numbers correspond to the percentage of cells within the indicated subset expressing the given TCR-Vβ domain. Blank space indicates <0.2%.
c Numbers in boldface correspond to those TCR-Vβ domains expressed at a significantly higher percentage than in fresh PBLs.
d ND, not determined.

3.2 but at a different size (149 nucleotides), suggesting the presence of a different clonal population. This peak was due to a rearrangement to the Jβ2.5 gene segment, whereas no detectable VB21 transcripts were rearranged to Jβ2.7. Using the primer specific for its N region, clone 2C4 was not detected in any of the skin biopsies or in pre- and postimmunization PBLs, even after five cycles of run-off (data not shown). This result suggests that clone 2C4 was either absent or present at a very low frequency (<1 in 10⁴ T cells; Ref. 27).

DISCUSSION

Immunological interventions for the treatment of malignant melanoma (and other cancers) aimed at generating effective T-cell immunity require the in vivo induction and expansion of CTL clones specific for antigens expressed by the melanoma. The characteristics of interactions between tumors and the immune system that lead to the development of clinically significant CTL responses are not clearly defined. For example, the DTH reaction has been reported in various cancer immunotherapy protocols (16, 17, 21), but the relevance of this response to overall tumor immunity is controversial. We have determined that specific CTLs can be isolated from the lymphocytes participating in DTH reactions following intradermal injection of irradiated autologous tumor cells into the skin over a period of 1 year. We found different, highly skewed repertoires among T cells localized at the different injection sites. Similar results have recently been obtained in a mouse experimental model (32) and from injection site DTH biopsies in another patient under study in this group (data not shown). Within these T-cell populations we observed diverse CTLs specific for as yet unknown tumor peptides but minimally not the HLA-A1-restricted peptides of MAGE-1 or MAGE-3. The presence of CTL specific for the autologous melanoma in the biopsy sites in the skin was consistently observed in each series of injections and biopsies over the 1-year period.

In the absence of any molecular or functional characterization of the TCR repertoire in situ before injection and at the time of biopsy, we are not able to say how these CTLs arose, i.e., through antigen-specific or nonspecific recruitment or by induction of proliferation of resident skin lymphocytes. Given the low levels of specific CTL activity observed at the early in vitro time points, we assume that the majority of cells present at the time of biopsy were most likely not specifically reactive with the immunizing autologous melanoma cells (using conventional CTL assays to measure their function), consistent with previous measurements of specific T-cell frequencies in inflamed skin lesions (33). Nonetheless, the number of melanoma-reactive CTLs was probably greater among DTH lymphocytes than among the same number of PBLs because proliferation following stimulation with
Me235 was greater, and CTL activity could be easily detected in DTH lymphocytes.

After coculture with the autologous tumor, we observed a pronounced skewing of the TCR-Vβ repertoire in each culture and a gradual increase in the percentage of CD8+ T cells. The TCR repertoire skewing in the CD8+ subset was not due to the presence of CD28 cells, shown previously to biased TCR-V3 repertoire (34). In each culture, the TCR repertoire skewing in the CD8 subset was highly skewed in the autologous EBV LCL of T-cell main. Sorted cells were cultured with Me235 for 4-6 weeks to allow TCR-V3 repertoire skewing was different. We could imagine this selection of CD8+ T cells through the induction of dramatic proliferation in the presence of the Me235 line suggested specific recognition of MHC class I-restricted antigens expressed by the cell line. Indeed, CTL activity could be inhibited efficiently by the anti-MHC class I mAb W6/32 (data not shown).

Interestingly, in the earlier cultures significant repertoire skewing was also seen in the CD4+ T-cell subset, consistent with other reports showing CD4+ T cells reacting with melanomas (36-38). The reason for such selection in this subset is not clear, but it should be noted that a minor fraction (1-3%) of the stimulating cell line Me235 constitutively expresses MHC class II molecules at the cell surface and that following exposure to IFN-γ, the majority of cells express MHC class II. In this regard, we have observed IFN-γ production by CD8+ T cells recognizing Me235 (data not shown).

Tumor-specific CTLs derived from biopsies of DTH reactions may be useful in screening genomic or cDNA libraries to identify genes encoding new target antigens in immunizing tumor cell lines. This approach has proved successful using CTLs derived from circulating lymphocytes or tumor-infiltrating lymphocyte cultures (2-6, 8, 9). In this context, although the biopsies were from an HLA-A1 individual, it is interesting that thus far, none of the CTLs obtained have shown any specificity for the known HLA-A1-restricted epitopes from MAGE-1 or MAGE-3. In any case, new peptide epitopes from any of the known tumor genes or from new genes may prove to be important as immunogens for the induction of CTL.

The relevance of the CTLs derived from each biopsy site to the overall level of specific CTL immunity was investigated using a PCR-based approach (27, 31) because the exact antigenic specificity of the clones was not known. After determining the nucleotide sequence of the TCR-β chain of clone 2C4, we probed PBL and subsequent immunization-site biopsies (before in vitro expansion) using a clonotypic N-region primer. We were unable to detect any T cells using the same TCR-β chain as the clone (i.e., using the same rearrangement with similar CDR3 length or using the same N-region), suggesting that this clone may not have expanded sufficiently in vivo to allow subsequent detection. Indeed, a large degree of proliferation would have been required to detect the clone in the PBLs (by PCR) or to have homing of the clone to subsequent immunization sites (where it might be re-isolated and expanded in vitro). In fact, the CTLs isolated from each DTH biopsy and expanded in vitro may not have produced any progeny in vivo simply.
because the conditions in vivo were not appropriate (e.g., limited antigen or cytokines) or because the precursors were removed from the site in the biopsy. Therefore, the failure to detect in vivo the clones expanded in vitro does not necessarily imply that no systemic antitumor immunity resulted from the immunization. The kinetics of the response and any clonal dominance that may ensue in vivo and its relevance to that seen in vitro require further study.

In summary, the T cells found participating in DTH reactions induced by injection of autologous melanoma cells appear to be randomly sampled from a T-cell pool of limited size. Nonetheless, a portion of the CTLs present at each of the sites showed specificity for the immunizing cell line. Thus, the DTH reactions may indicate the potential of the patient’s immune system to mount a response to particular immunogens. Indeed, specific CTLs derived from these sites could eventually be useful as probes for detecting new tumor antigens or as effectors in adoptive immunotherapy protocols.

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