Reduced Number and Impaired Function of Circulating γδ T Cells in Patients with Cutaneous Primary Melanoma

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We studied the peripheral representation, in vitro expansion, cytokine production, and cytotoxicity of γδ T lymphocytes from 23 patients with cutaneous primary melanoma and 28 healthy subjects. We demonstrated that the absolute number and the percentage of circulating γδ T cells were significantly reduced in melanoma patients in comparison with healthy subjects. The decrease was due to a reduction of Vδ2 T cells, whereas the number of Vα1 T cells was not affected. As a consequence, the Vδ2/Vα1 ratio was inverted in melanoma patients. A lower percentage of γδ+ T cells producing tumor necrosis factor-α or interferon-γ was found in melanoma patients. After a 10 d in vitro culture, both the percentage and the expansion index of γδ T cells, and in particular of Vδ2 subset, were significantly reduced in melanoma patients in comparison with healthy subjects. The cytotoxicity of sorted γδ T cells against tumor cell lines and the percentage of γδ T cells producing perforins were preserved in melanoma patients. The numerical and functional impairment of γδ T cells could contribute to the inadequate immune response found in melanoma patients and offers the potentiality for the planning of new approaches of immune therapy of malignant melanoma. Key words: fluorescence-activated cell sorter/human/melanoma/tumor immunity/γδ T cells J Invest Dermatol 120:829—834, 2003

V arious clinical and experimental observations point to the existence of an immunologic host defense in cutaneous malignant melanoma. CD3+ T cell receptor (TCR) γδι/δi-expressing lymphocytes are considered the prevailing lymphocyte subset in primary as well as secondary malignant melanoma (Strohal et al, 1994). Immunomodulatory therapies with cytokines or adoptive transfer of T cells have accomplished complete or partial tumor regression in melanoma patients. Nevertheless, the immune response is in most cases inadequate to control tumor growth as tumor progression often occurs (Lee et al, 1999; Dudley et al, 2000). Hence, the coexistence of a cellular immune response in melanoma lesions (Kammla et al, 1999), demonstrated by the presence of elonally expanded T cells, and the inability of melanoma-specific killer T cells to arrest tumor growth, remains a major paradox of tumor immunology (Thor Straten et al, 1999; Nielsen et al, 2000).

T lymphocytes bearing the γδ TCR represent a minor population of human peripheral lymphocytes (1—10%) the majority of them expressing the Vδ9/Vδ2 TCR and the CD3+CD4−CD8− phenotype (Groh et al, 1988; Poccia et al, 1998). The second most frequent subset of peripheral blood γδ T cells expresses Vα1 in association with various Vγ elements. The ability of γδ T cells to respond to nonprocessed and nonpeptide phosphoantigens in a major histocompatibility complex (MHC)-unrestricted manner are important features distinguishing them from 2β T cells (Brenner et al, 1987; Bukowski et al, 1995). γδ T cells are activated by mycobacterial antigens, such as isopentenyl pyrophosphate (IPP) and related phenyl pyrophosphate derivatives (Costant et al, 1994; Tanaka et al, 1995; Burk et al, 1995), stress-associated heat shock proteins (Fisch et al, 1999), as well as by several cytokines, such as interleukin (IL)—2 (Kjeldsen-Kragh et al, 1993), IL-12 (Fujimiya et al, 1997), or tumor necrosis factor (TNF)—α (Ueta et al, 1996; Lahn et al, 1998). Other signals, such as MHC class I-related chains A and B, are able to engage the activating receptor, NKG2D, present on Vδ2 T cells, substantially enhancing the TCR-dependent Vδ2 T cell response to nonpeptide antigens (Das et al, 2001; Wu et al, 2002). Although little is known about the physiologic significance of γδ T cells, their marked reactivity toward mycobacterial and parasitic antigens, as well as tumor cells, suggests that γδ T cells play a part in the anti-infectious and anti-tumor immune surveillance (Poccia et al, 1998; Zheng et al, 2001). γδ T cells may contribute to the immune defense against cancer, having at least two important functions, i.e., reactivity to tumor cells, and regulatory interactions with γβ T cells (Kabelitz et al, 1999). γδ T cells strongly react against certain lymphoma cells, such as Daudi cells, suggesting a cross-reactivity between microbial and tumor-associated antigens (Kunzmann et al, 2000). Furthermore, γδ T cells have been identified among tumor-infiltrating lymphocytes in various cancer types (Kabelitz et al, 2000). Once activated, γδ T cells produce high levels of cytokines and, mainly, IFN-γ and TNF-α (Poccia et al, 1997). Mainly because of their cytokine production, γδ T cells have been proposed to be involved in co-ordinating the interplay between innate and acquired immunity and, in particular, to guide the establishment of acquired immunity contributing to select appropriate antigens and the strategies for their elimination, and to the definition of γβ T cell responses.
The role of γδ T cells may depend on their involvement in the early anti-tumor defense against melanoma, as suggested by the evidence that γδ T cells may constitute up to 25% of lymphocyte infiltrate in cutaneous primary melanomas, whereas they are not present in metastatic melanoma (Bachelez et al., 1992). These cells exert potent cytotoxic activity against autologous tumor cells (Bachelez et al., 1992; Nannoz et al., 1992). In another study, the survival of patients with necrotizing choroidal melanomas was increased with evidence of Vγ1 and Vδ1 TCR+ cells (Bialasiewicz et al., 1999).

On the basis of the pivotal role that γδ T cells may have in the immune response against melanoma acting directly on tumor cells and, secondarily, by modulating the phenotype of T cell responses, we evaluated the peripheral representation and the in vitro expansion, cytokine production, and cytotoxicity of γδ T cells from melanoma patients, comparing the results with those obtained in healthy controls. This study demonstrated for the first time an alteration of circulating γδ T cells in melanoma patients, with a significant decrease of the number of γδ T cells in the peripheral blood, an altered pattern of cytokine production, and an impaired in vitro expansion of these cells. The knowledge about the deterioration of γδ T cells could account for the melanoma-related alterations of T cell-mediated adaptive responses and may be helpful for the planning of new approaches of immune therapy in melanoma patients.

MATERIALS AND METHODS

Cell preparation and stimulation

Human peripheral blood was obtained from 23 melanoma patients (mean age ± SD 56.3 ± 16.3 years; median: 64.5 y; range 32-80) and 25 healthy subjects (mean age ± SD 57.3 ± 18.9 y; median: 67.0 y; range 32-79). Healthy subjects were volunteers in good and stable clinical condition, and had laboratory parameters in the physiologic range. Melanoma patients have been admitted to the Dermatology Unit of the I.N.R.C.A. Hospital of Ancona (Italy). Melanoma patients were in good health other than for the existence of melanoma as checked on the basis of clinical and laboratory parameters. Melanoma patients and healthy subjects were equally distributed according to sex and the percentage of male and female inside each group was about 50%.

The purity of γδ T cells, assessed by flow cytometric analysis, was greater than 95%.

Cytotoxic assay was performed by a fluorimetric method as recently reported (Provini et al., 1992). The natural killer resistant cell line Daadi and the natural killer cell sensitive K562 cell line were used as target cells. Daadi is a human lymphoblastoid B cell line derived from a Burkitt lymphoma, which constitutively expresses antigens recognized by Vδ9 Vγ2 T cells. K562 is a human myeloid cell line derived from chronic myelogenous leukemia. The fluorescence was read with a H2O VICTOR® multilabel counter (Wallac, Turku, Finland). The percentage of specific lysis was calculated as follows:

% specific lysis = [(Fmed - Fexp)/Fmed]×100

where F represent the fluorescence of the solubilized cells after the supernatant has been removed; med=F from target incubated in medium alone; exp=F from target incubated with effector cells.

Lytic units (LU×10⁶ cells) were calculated by using a computational method (Bryant et al., 1992). One LU corresponded to the number of effector cells required to produce 20% of specific lysis.

Isolation of γδ T lymphocytes and cytotoxic assay

γδ T lymphocytes, in vitro expanded with IPP and IL-2 for 30 d, were isolated through cytofluorometric cell sorting (Vantage, Becton Dickinson). The purity of γδ T cells, assessed by flow cytometric analysis, was greater than 95%.

RESULTS

Ex vivo analysis of γδ T lymphocytes

Peripheral blood lymphocytes from 23 melanoma patients and 28 healthy subjects were analyzed for the percentage and the absolute number of γδ T cells through double staining with anti-CD3 and anti-γδ TCR. As shown in Table 1 and Fig 1(A), the absolute number of γδ T cells was significantly reduced in melanoma patients in comparison with healthy subjects (66.1 ± 33.8 vs 92.9 ± 32.6 x 10⁶).
Table I. Absolute number of lymphocytes, γδ T cells, Vδ1 T cells, and Vδ2 T cells, in healthy subjects and melanoma patients

<table>
<thead>
<tr>
<th>Donors</th>
<th>Lymphocytes</th>
<th>γδ T Cells</th>
<th>Vδ1 T Cells</th>
<th>Vδ2 T Cells</th>
<th>Vδ2/Vδ1 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy subjects</td>
<td>2159 ± 118*</td>
<td>92.9 ± 126.6</td>
<td>38.5 ± 131</td>
<td>68.2 ± 358</td>
<td>1.8</td>
</tr>
<tr>
<td>Melanoma patients</td>
<td>2268 ± 539</td>
<td>66.1 ± 338*</td>
<td>331 ± 19.4</td>
<td>31.7 ± 137*</td>
<td>0.9</td>
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</table>

*Data are expressed as mean ± SD. 

DISCUSSION

In this study we have demonstrated for the first time the existence of numerical and functional alterations of γδ T cells from patients with cutaneous primary melanomas. The number of circulating γδ T cells, the percentage of cells producing IFN-γ or TNF-α, and their in vitro expansion, were all decreased in melanoma patients when compared with healthy age-matched subjects.

Both the percentage and the absolute number of circulating γδ T cells were significantly reduced in patients with cutaneous primary melanoma, thus suggesting a specific deficit for this lymphocyte population. The reduction of γδ T cell number well

p<0.01). The absolute number of peripheral lymphocytes was similar in the two groups of subjects (Table I). As shown in Fig 1(B), the percentage of CD3+ γδ T cells in peripheral blood was significantly lower in melanoma patients than in healthy donors (mean ± SD, 3.2 ± 2.1 vs 4.8 ± 1.7; p<0.01). As shown in Table I, the absolute number of Vδ1 T cells did not show significant difference in the two groups of donors (38.5 ± 131 and 331 ± 19.4, in healthy subjects and melanoma patients, respectively). Differently, the absolute number of Vδ2 T cells was significantly reduced in melanoma patients in comparison with healthy subjects (31.7 ± 13.7 vs 68.2 ± 35.8; p<0.01). The Vδ2 and Vδ1 subsets were differently represented in the two groups: in healthy controls the Vδ2 subset was predominant (Vδ2/Vδ1 ratio = 1.8), whereas in melanoma patients the proportion of Vδ2 and Vδ1 subsets was similar (Vδ2/Vδ1 ratio = 0.9) (Table I). No significant correlation was found plotting the number of γδ T cells and the tumor thickness of the respective patient.

Cytokine production by γδ T lymphocytes As it has been demonstrated that activated γδ T cells produce IFN-γ and TNF-α, we studied the intracellular production of these lymphokines in 1 d stimulated γδ T cells from healthy subjects and melanoma patients. As shown in Figs 2 and 3, the percentage of γδ T cells producing IFN-γ was significantly reduced in melanoma patients in comparison with healthy controls (mean ± SD, 9.3 ± 8.5 vs 18.3 ± 9.5, p<0.01). In a similar way, the percentage of γδ T cells producing TNF-α was lower in melanoma patients than in healthy controls (11.5 ± 3.8 vs 17.9 ± 7.7 for melanoma patients and healthy subjects, respectively; p<0.02).

Expansion of γδ T lymphocytes The expansion of γδ T cells from healthy controls and melanoma patients was evaluated after 10 d of culture in the presence of IPP and low-dose IL-2. Both the proportion of γδ T cells, evidenced by double staining fluorescence-activated cell sorter analysis, and their relative increase in comparison with the γδ T cell number found on day 0 (expansion index) were evaluated. As shown in Fig 4, the proportion of γδ T cells reached on day 10 was significantly lower in melanoma patients than in healthy donors (mean ± SD, 11.9 ± 8.4 vs 36.5 ± 17.6; p<0.01). In a similar way, the expansion index of γδ T cells on day 10 vs day 0 was significantly reduced in melanoma patients in comparison with healthy controls (4.4 ± 3.8 vs 8.6 ± 6.7; p<0.02) (Fig 4). As shown in Table II, the expansion index of the Vδ2 subgroup was significantly lower in melanoma patients than in healthy donors (3.3 ± 3.6 vs 58 ± 4.4; p<0.03).

Cytotoxicity of γδ T lymphocytes In order to evaluate the cytotoxic potential of γδ T cell cultures after in vitro expansion, we tested the cell activity of γδ T cells against Daudi and K562 tumor cell lines and their perforin content through flow cytometry. Purified cultures of activated γδ T cells (10 d incubation), obtained through cytofluorimetric cell sorting, were cytotoxic against Daudi tumor cells. A great heterogeneity of cytotoxic activity was found in both groups considered. Mean levels of cytotoxicity were similar between healthy subjects and melanoma patients (438.2 ± 141.7 vs 402.5 ± 359.2, data not shown). The cytotoxic activity of γδ T cells was also tested against the K562 tumor cell line, with a similar distribution of cytotoxicity in normal donors and melanoma patients (data not shown).

The percentage of γδ T cells producing perforins among total γδ T cells was not significantly different in melanoma patients in comparison with healthy subjects (428 ± 37.6 vs 51.4 ± 8.8, data not shown).
correlated with the decrease of the Vδ2 T cell subset, i.e., the most frequent subset of circulating γδ T cells (Groh et al, 1989; Poccia et al, 1998). The Vδ2 population is involved in the reactivity toward microbial antigens and tumor cell antigens (Poccia et al, 1998; Zheng et al, 2001). The role of Vδ2 T cells in the immune defense against cancer has been demonstrated on the basis of their reactivity against certain lymphoma cells, such as Daudi cells (Kunzmann et al, 2000), and for their presence among tumor infiltrating lymphocytes in various cancer types (Kabelitz et al, 2000). On this basis, our data strongly suggest that an impaired γδ T cell potential may contribute to a lower immune defense against melanoma. Both Vδ1 and Vδ2 T cell populations have been reported to contribute to anti-tumor immunity because of their cytotoxic and Th1-type cytokines producing activities (Wu et al, 2002). Furthermore, both γδ T cell subsets have been found able to respond to stress-induced expression of the MHC class I-related chains A and B (Wu et al, 2002). MHC class I-related chains function as ligands for NKG2D, an activating receptor complex that triggers natural killer cells, costimulates CD8+ T cells, and is required for stimulation of Vδ1 T cells (Groh et al, 1999; Das et al, 2001; Girardi et al, 2001; Wu et al, 2002). The role of γδ T cells in the early anti-tumor defense against melanoma has been previously suggested by the evidence that a significant accumulation of Vδ1 T cells was found in three of 11 primary cutaneous melanomas, with proportions of γδ T cells ranging from 15 to 25% of lymphocyte infiltrate, whereas γδ T cells were not present in eight metastatic melanomas (Bachelez et al, 1992). In our study, we did not find numerical changes of the Vδ1 T cell population in melanoma patients in comparison with controls, whereas the Vδ2 subset was significantly reduced in melanoma patients. Almost half of our melanoma patients had an absolute number of circulating γδ T cells similar or lower than half of the mean number found in controls (Fig 1). Phenotypic analysis of the tumor infiltrating γδ T cells revealed that they use the products of the Vδ9 and Vδ2 genes in a way similar to most of circulating TCR γδ. It is noteworthy that γδ lines or clones developed from lymphocytes infiltrating primary melanoma display a potent autologous MHC-unrestricted tumor cell cytotoxicity (Bachelez et al, 1992; Nanno et al, 1992). In a more recent study, the presence of γδ T cells well correlated with the survival of patients with necrotizing choroidal melanoma (Białasiewicz et al, 1999). In our study, not only the number but also the function of γδ T cells was altered in melanoma patients. The in vitro expansion of γδ T cells, that represent one of the most relevant functional parameters for γδ T cells, was significantly reduced in patients with cutaneous primary melanomas. This would imply that γδ T cells from melanoma patients have a decreased proliferative capacity in comparison with healthy subjects. Under normal conditions, γδ T cells respond to antigen challenge by secreting large quantities of TNF-α and IFN-γ (Poccia et al, 1997; Kabelitz et al, 2000), which contribute to the activation of both specific and aspecific immune responses. In this study, we show that the percentage of γδ T cells producing either TNF-α or IFN-γ is significantly reduced in melanoma.

Figure 2. Analysis of cytokine production by γδ T cells in melanoma patients and healthy donors. PBMC from patients with cutaneous primary melanomas or healthy subjects, were stimulated for 18 h in the presence of IPP (30 mg per ml) and IL-2 (100 U per ml). The last 12 h of culture were performed in the presence of GolgiPlug, a protein transport inhibitor containing brefeldin. Single-cell analysis of cytokine synthesis in γδ T cells from a representative healthy subject or melanoma patient was performed following dual staining with cell surface anti-γδ (FITC) MoAb and intracellular anti-IFNγ or anti- TNF-α (PE) MoAb. Numbers in brackets indicate the percentages of γδ cells synthesizing a given cytokine among total γδ T lymphocytes.
to the ineffectual immune defense against melanoma with at least two different mechanisms. One of these is based on the lower γδ T cell potential, determined by the reduced number and expansion of γδ T cells, and particularly of Vδ2 subset, found in melanoma patients. In this case, the impairment of γδ T cells may represent a new mechanism by which melanoma tumor cells escape an efficacious immune response. The second mechanism is based on the regulatory interactions between γδ T cells and Vβ T cells. Several studies have demonstrated that the type and the functioning of the specific lymphocyte response is dependent on signals provided by the innate recognition system (Fearon and Locksley, 1996; Medzhitov and Janeway, 1997). Indeed, even if the recognition of foreign or nonself proteins is controlled by the rearranging genes that encode specific receptors, the functional outcome of most responses to pathogens and the initiation of the response itself is determined by the type of innate immune response they elicit (Fearon and Locksley, 1996; Medzhitov and Janeway, 1997). Recent evidence has suggested that one of the crucial players involved in the regulation of innate and acquired immunity is the γδ T cell population (Mak and Ferrick, 1998). There is substantial cross-talk between γδ and αβ T cells. It has been demonstrated that the proliferative response of human peripheral blood γδ T cells towards microbial antigens or Daudi tumor cells, depends on helper signals provided by CD4+ αβ T cells (Burns et al, 1996a,b). Moreover, some γδ T cell function may depend on αβ T cells, as, for example, the γδ T cell proliferation in response to activated CD4+ αβ T cells (Burns et al, 1996a,b), and the antigen presentation by CD4+ αβ T cells to γδ T cells (Vila et al, 1995; Collins et al, 1998). On the other hand, substantial evidence has suggested that γδ T cells regulate certain αβ T cell-mediated immune responses, pointing the definition of αβ T cell responses toward a Th1 or Th2 phenotype (Fearon and Locksley, 1996; Mak and Ferrick, 1998; Kabelitz et al, 2000). This fact is particularly related by the cytokines secreted by γδ T cells that, in turn, are able to mediate both innate and acquired immunity (Ferrick et al, 1995). We propose that, in analogy with what has been recently demonstrated by us in aged people and centenarians (Argentati et al, 2002), the numerical and functional impairment of γδ T cells demonstrated in this study could determine a derangement in the establishment of acquired immunity, with consequent difficulties in selecting appropriate antigens and the strategies for their elimination. Further studies will be performed to investigate the specific causes involved in the impairment of γδ T cells in primary cutaneous melanoma patients. At present, we may only suggest that an impairment of γδ T cells may determine a lower immune defense against melanoma which, in turn, may predispose to melanoma. The healthy and stable conditions of melanoma patients examined in this study make improbable that the γδ T cell defect is secondary to the malignancy; even if this possibility may not be definitively excluded. Finally, the fact that approximately 80% of patients with melanoma have family histories of the disease, suggesting a genetic susceptibility (Platz et al, 1997), raises the question on whether the differences we have found might be attributable to the genetic background of the melanoma patients. We do not think this is the case, as melanoma patients and controls enrolled in our study were identified in the healthy subjects and melanoma patients.

Table II. Expansion index of γδ T cells and Vδ2 T cells in healthy subjects and melanoma patients.

<table>
<thead>
<tr>
<th>Donors</th>
<th>γδ T cells</th>
<th>Vδ2 T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy subjects</td>
<td>86±6.7*</td>
<td>58±4.4</td>
</tr>
<tr>
<td>Melanoma patients</td>
<td>4.4±3.8**</td>
<td>3.3±3.6**</td>
</tr>
</tbody>
</table>

*nThe expansion index was calculated by dividing the absolute number of γδ T cells in stimulated cultures by the absolute number of γδ T cells before culture. *Data are expressed as mean±SD.
*p<0.02 and **p<0.03 vs healthy subjects.

Figure 3. Percentage of γδ T cells producing cytokines in melanoma patients and healthy donors. γδ+ T cells from patients with cutaneous primary melanomas or healthy subjects were activated as reported in Fig 2 and analyzed for cytokine production after dual staining with cell surface anti-pan γδ (FITC) MoAb and intracellular anti-IFN-γ or anti-TNF-α (PE) MoAb. The mean percentage of γδ T cells producing either IFN-γ or TNF-α was significantly lower in melanoma patients in comparison with healthy subjects (p<0.01 and p<0.02 for IFN-γ or TNF-α, respectively).

Figure 4. Evaluation of γδ T cell expansion in melanoma patients and healthy donors. PBMC from patients with cutaneous primary melanomas or healthy subjects were stimulated for 10 d in the presence of IPP (30 μg per ml) and IL-2 (100 U per ml). The expansion index (left panel) and the percentage (right panel) of in vitro expanded γδ+ T cells among total CD3+ T cells were significantly lower in melanoma patients in comparison with healthy subjects (p<0.02 and p<0.01).

patients. Instead, the lytic activity of γδ T cells, evidenced by their cytotoxicity toward Daudi and K562 tumor cells and their production of perforins, seems to be well preserved in melanoma patients.

On the basis of the data reported in this paper, we suggest that the impairment of γδ T cell number and function may contribute
same geographical area (Ancona area in Italy), and melanoma pa-
tients did not have family histories of melanoma. In conclusion, the demonstration of a numerical and functional derangement of γδ T cells, which we have found in patients with cutaneous primary melanomas, make them a potentially useful tool for the planning of new approaches of immune therapy in malignant melanoma.

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