Cancer Immunol Immunother (2003) 52: 28–32
DOI 10.1007/s00262-002-0333-z

ORIGINAL ARTICLE

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Interferon-γ secretion and perforin expression are impaired in CD8$^+$ T lymphocytes from patients with undifferentiated carcinoma of nasopharyngeal type

Received: 26 June 2002 / Accepted: 22 August 2002 / Published online: 26 November 2002

Abstract An efficent antitumor and antiviral cellular immune response requires optimal interferon-γ (IFN-γ) secretion and perforin expression in CD8$^+$ T cells. The aim of this study was to define whether CD4$^+$ and CD8$^+$ T cells from patients with undifferentiated carcinoma of nasopharyngeal type (UCNT), a tumor regularly associated with the Epstein-Barr virus (EBV), have abnormal phenotype profiles, cytokine production, perforin and CD3-zeta expressions. Our data showed that CD4 and CD8 subset distribution was not grossly altered in the peripheral blood of UCNT patients, while tumor biopsies contained an increased proportion of CD8$^+$ T cells. The analysis of the CD4$^+$ subset showed a defect in interleukin-2 (IL-2) production and a moderate increase of IL-10 production, a situation consistent with a Th1/Th2 imbalance. We have also demonstrated that CD8$^+$ lymphocytes from UCNT patients had a marked impairment of IFN-γ secretion and perforin expression. This impairment was not related to the presence of detectable EBV DNA in the plasma. In UCNT patients, the blockade of the perforin pathway and of IFN-γ production may constitute important mechanisms for immune escape by the tumor and for impaired control of EBV replication.

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Keywords CD8 lymphocyte · Interferon-γ · Perforin · Undifferentiated carcinoma of nasopharyngeal type

Introduction

Undifferentiated carcinoma of nasopharyngeal type (UCNT) is one of the most common malignancies in Southern China and Southeast Asia, but it is much rarer in western Europe [15, 25]. Since UCNT is a tumor that is regularly associated with the Epstein-Barr virus (EBV) [15], considerable attention has been paid to defining the immunological aspects of this tumor. Intact T-cell mediated immunity is, in fact, a key determinant for defense mechanisms against tumors and viral infections. Although the T cell-mediated immune response against tumors may include several mechanisms, many authors have focused their attention on the role of intracellular proteins contained in CD8 cells in the control of tumor progression and viral replication. It has been shown shown that two proteins, interferon-γ (IFN-γ) and perforin, provided optimal protection in separate tumor models [9, 13, 21, 24]. Furthermore, EBV-specific CD8 cells are capable of IFN-γ production and contain perforin, suggesting that a defect of these two molecules may also be related to the establishment of persistent viral infections [3, 10, 12, 26]. In addition, recently published reports have suggested that a reduced expression of the transduction molecule CD3-zeta may be involved in the impairment of the cell-mediated immune response against tumours and chronic viral infections [20, 22]. Besides the well-known dysregulation of antibody production, little information on the immunological status of UCNT patients is available at the moment. Immunological abnormalities have been found to occur in Asian UCNT patients, such as a suppressed cellular immune response, as measured in vitro by using recall antigens and polyclonal mitogens, and lower levels of EBV-specific cytotoxic T cells as compared to healthy virus carriers [1, 4, 5, 11, 23]. It has been suggested that

these immune defects are correlated to the burden of EBV [5], but the mechanisms underlying immune suppression present in these patients are still not well understood. In this study, we addressed the question of whether $CD8^+$ T cells from UCNT patients and healthy subjects differ in their IFN-γ production, perforin and CD3-zeta expression. Defining the expression of intracellular pathways involved in efficient antitumor and antiviral response may provide further insight into the pathophysiology of UCNT, and may help to design new immunotherapeutic strategies [14].

Materials and methods

Patients

Twenty-five patients with histologically confirmed UCNT were enrolled in this study. UCNT was classified on the basis of the simplified World Health Organization (WHO) classification for nasopharyngeal carcinomas [19]. The median age was 56 years (range: 36–72 years). Five patients were included in WHO stage I, 5 in stage II, 7 in stage III and 8 in stage IV. The biological samples were obtained at diagnosis before any antineoplastic treatment was initiated. Ten subjects attending the Nose and Throat Clinic for non-neoplastic problems were enrolled in the study as controls.

Lymphocyte subpopulations in peripheral blood and in tumor biopsies

Peripheral blood samples were obtained in ethylene diamine tetraacetic acid (EDTA), and the percentages and absolute counts of lymphocyte subsets were evaluated by a single platform whole blood lysing technique [2]. Briefly, 100 µl of blood was added to the appropriate monoclonal antibody (mAb) combination and fluorescent microbeads, and incubated for 15 min: after this, the samples were lysed and fixed by a commercial preparation (Immunoprep, Beckmann-Coulter, Milan). The CD3/CD4/CD8/CD45 mAb combination (Beckmann-Coulter, Milan) was used to stain peripheral blood lymphocytes (PBL). The percentages and absolute counts were then obtained via an EPICS XL flow cytometer (Beckman-Coulter, Milan). Mononuclear cells were also isolated from tumor biopsies, as previously described [27]. Each biopsy was minced and filtered through a 40-µm nylon mesh; cells were then separated by density gradient centrifugation, and stained as described for peripheral blood.

Lymphocyte subpopulations were studied in detail by the following mAb combinations: CD4/CD45R0 (Dako, Milan; memory cells), CD4/CD45RA/CD62L (Dako, Milan; naive cells), CD4/HLA-DR (activated cells), CD8/CD28, CD8/CD38 (activated cells; all the mAb were obtained from Becton Dickinson, Milan), CD8/C1.7 (Beckmann-Coulter, Milan; activated/cytotoxic cells) [17].

Flow cytometric determination of cytokine production and perforin content in CD4 and CD8 lymphocytes

Cytokine production was measured in Ficoll-Hypaque purified peripheral blood mononuclear cells (PMBC); briefly, 2×10^6 cells/ml were mixed with 10 ng/ml PMA (phorbol 12-myristate 13 acetate; Sigma, Milan), 0.75 µg/ml ionomycin (Sigma, Milan) and 1 µg/ml brefeldin A; cells were then aliquoted into 12×75-mm plastic tubes, and incubated for 5 h or overnight in 5% CO_2 at 37ºC; after washing and centrifugation, the cellular pellet was resuspended in 100 µl of staining buffer containing an optimal concentration of fluorochrome-labelled mAb against surface antigens. Cells were then fixed by adding 500 µl of CytoFix/CytoPerm solution (Pharmingen, San Diego, Calif.), permeabilized by adding Perm/Wash reagent, and stained with 2 ml Perm/Wash solution containing an optimal concentration of labelled anti intracellular protein mAb [6]. The phenotypically different $CD4^+$ subsets were studied using the following mAb combinations: CD3/CD8 (gated on $CD3^+/CD8^-$ cells to overcome CD4 down-regulation) plus anti IL-2 or anti IL-10 (BD-Pharmingen, Milan). A CD3/CD8/CD28/IFN-γ mAb combination was used to study the $CD8^+$ subset. CD3/CD8/C1.7/perforin (anti-perforin antibody obtained from BD-Pharmingen, Milan) and CD3/CD8/CD3 zeta chain (anti CD3 zeta chain antibody was purchased from Santa Cruz Biotechnology) intracellular expression was measured in unstimulated permeabilized PBMC.

As negative controls, we used stimulated fixed/permeabilized cells stained with non-conjugated isotype-matched mouse immunoglobulin or unstimulated fixed/permeabilized cells stained with the above-mentioned mAb against intracellular cytokines or intracellular proteins. The percentage of cells expressing the various combinations of surface and intracellular antigens was determined after live gating on $CD3^+$/CD8 negative or $CD3^+/CD8^+$ populations.

Detection of EBV plasma viremia

EBV DNA status was initially determined by qualitative PCR. Amplifiability was evaluated by co-amplifying plasma samples with an EBV DNA competitor containing a fragment of the LMP2A unique region of the EBV genome with a 28-bp internal deletion. Quantitative competitive PCR was then performed on plasma samples with the addition of increasing amounts of the EBV competitive template. After amplification, the PCR products were resolved on 8% non-denaturing polyacrylamide mini-gels and quantified by densitometric scanning of the relative intensities of the ethidium bromide stained bands.

Statistical analysis

The Mann-Whitney test was used to compare the distributions of variables between two groups [2, 27].

Results

CD4 and CD8 counts in peripheral blood and biopsies and EBV plasma viremia in UCNT patients

Compared with healthy controls, a statistically significant increase of $CD8^+$ cells was observed in tumor biopsies but not in the peripheral blood, from UCNT patients (Table 1).The percentages and absolute numbers of CD4 lymphocytes in UCNT patients were not significantly different from healthy controls. Plasma EBV DNA was detectable in 58.3% of the UCNT patients and in 0% of the healthy controls.

CD4 and CD8 subpopulations in peripheral blood and biopsies of UCNT patients

The percentages and absolute numbers of CD4 memory ($CD45R0^+$), CD4 naive ($CD45RA/CD62L^+$) and activated ($HLA\text{-}DR^+$) CD4 lymphocytes are shown in Table 2. No significant differences were present in the peripheral blood and tumor biopsies from UCNT patients as compared to healthy controls. When considering the CD8 subset, a significant proportion of

Table 1 Percentages and absolute counts (cells/mmc) of $CD4^+$ and $CD8^+$ T cells in the peripheral blood (*PB*) and tumor biopsies (*BIO*), and percentage of plasma EBV-positive UCNT patients and controls

	CD4 % PB	CD4 PB counts	CD4 % BIO	CD8 % PB	CD8 PB counts	CD8 % BIO	EBV % viremia$^+$
UCNT	39.6 ± 11	606 ± 417	48.4 ± 12	32.1 ± 13	439 ± 231	25.9 ± 11*	58.3
Controls	42.4 ± 7	732 ± 100	54.6 ± 8	27.5 ± 4	487 ± 133	12.2 ± 8	0

*$P < 0.05$ as compared to controls

Table 2 Percentages and absolute counts (cells/mmc) of gated $CD4^+$ T cell subpopulations in the peripheral blood and tumor biopsies from UCNT patients and controls

	% PB memory	PB memory counts	% BIO memory	% PB naive	PB naive counts	% BIO naive	%PB DR^+	PB DR^+ counts	% BIO DR^+
UCNT	23.9 ± 9	352 ± 230	41.7 ± 13	13.5 ± 8*	254 ± 238	6.8 ± 7	6.7 ± 6	88 ± 60	20.6 ± 10
Controls	21.4 ± 3	358 ± 110	42 ± 10	18.9 ± 4	230 ± 95	9.8 ± 5	4.7 ± 2	59 ± 10	21 ± 7

*$P < 0.05$ as compared to controls

lymphocytes bearing the phenotype of activated/cytotoxic cells ($CD38^+$ and $C1.7^+$) was found in the peripheral blood of UCNT subjects (Table 3).

Intracellular cytokine production, perforin and CD3 zeta expression in peripheral blood lymphocyte subsets of UCNT patients

UCNT patients had a statistically significant decrease in peripheral blood CD4 cells secreting IL-2, while IL-10 production was increased (Table 4). A severe depletion of IFN-γ-producing cells and of perforin-expressing CD8 lymphocytes was characteristic of UCNT patients. The reduction in IFN-γ secretion and perforin expression was similar in UCNT patients with detectable plasma EBV-DNA levels as compared to patients who were EBV-DNA negative (data not shown). The expression of the CD3-zeta signal transduction molecule was evaluated in the $CD8^+$ T cell compartment. The results obtained in UCNT patients were similar to those found in healthy controls (data not shown).

Fig. 1 shows the cytofluorimetric analysis of IFN-γ and perforin expression in the peripheral blood $CD8^+$ T cells of a UCNT patient as compared to a healthy control.

Discussion

In the present investigation, we undertook a multiparametric cytofluorimetric analysis of CD4 and CD8 T cell subsets in the peripheral blood and tumour biopsies of UCNT patients. Our data showed that the CD4 and CD8 subset distribution was not grossly altered in the peripheral blood of UCNT patients while tumor biopsies contained an increased proportion of $CD8^+$ T cells.

Analysis of the $CD4^+$ subset showed a defect in IL-2 production and a moderate increase in IL-10 production, which was consistent with a Th1/Th2 dysregulation. Dysregulated synthesis of Th1 and Th2 cytokines has previously been shown to occur in solid tumors; it has been hypothesized that initial tumor development is facilitated because of the Th2 switch in the immune

Table 3 Percentages and absolute counts (cells/mmc) of gated $CD8^+$ T cell subpopulations in the peripheral blood and tumor biopsies from UCNT patients and controls

	% PB $CD28^+$	PB $CD28^+$ counts	% BIO $CD28^+$	% PB $CD38^+$	PB $CD38^+$ counts	% PB $C1.7^+$	PB $C1.7^+$ counts
UCNT	16 ± 7	253 ± 183	16.5 ± 11	5.1 ± 3*	69 ± 40	25.1 ± 10*	356 ± 166
Controls	15.1 ± 4	267 ± 110	8.6 ± 2	3.0 ± 1	15 ± 3	12.9 ± 3	227 ± 88

*$P < 0.05$ as compared to controls

Table 4 Percentages of intracellular protein expression in $CD4^+$ and $CD8^+$ T cell subpopulations in the peripheral blood of UCNT patients and controls

	$CD4/IL2^+$	$CD4/IL10^+$	$CD8/C1.7^+$ IFN-$\gamma+$	$CD8/CD28^+$ IFN-γ^+	$CD8/CD28^-$ IFN-γ^+	$CD8/C1.7^+$ perforin$^+$	$CD8/CD28^-$ perforin$^+$
UCNT	10.3 ± 5*	3.9 ± 1*	7.0 ± 4*	4.0 ± 1*	3.5 ± 3*	3.6 ± 2*	2.6 ± 1*
Controls	20.6 ± 5	1.0 ± 1	23.3 ± 9	13.5 ± 5	7.7 ± 2	14.3 ± 3	14.0 ± 4

*$P < 0.05$; **$P < 0.01$ as compared to controls

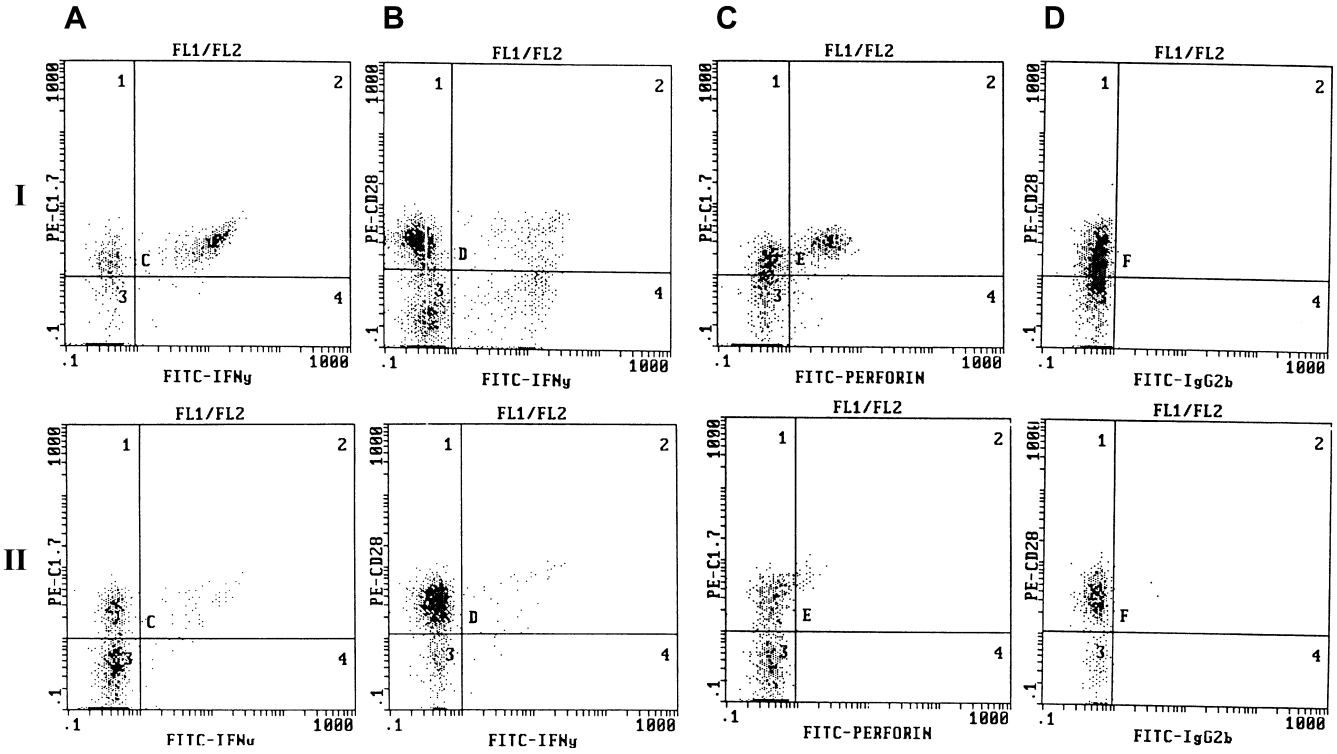

Fig. 1 Flow cytometric expression of C1.7/IFN-γ (*panel A*), CD28/IFN-γ (*panel B*), C1.7/ perforin (*panel C*) and CD28/isotypic control (*panel D*) in gated $CD8^+$ T lymphocytes from a healthy control (*panel I*) and from a representative UCNT patient (*panel II*)

response, which is not able to generate specific reactive antitumor cytotoxic cells [7, 8, 16]. Whether this hypothesis may be relevant for the pathogenesis of UCNT or other head and neck cancers remains to be established.

Among $CD8^+$ T lymphocytes, $CD38/C1.7^+$ activated cells predominated in peripheral blood; IFN-γ secretion and perforin expression were markedly impaired in these cells. On the contrary, the data presented here do not support the down-regulation of the CD3-zeta molecule as a mechanism contributing to the immune defect in UCNT.

It has previously been demonstrated that UCNT patients had lower levels of cytotoxic T cell activity, but the mechanisms involved have not been fully investigated [5]. We therefore focused our analysis on the intracellular pathways that are essential for an efficient antiviral and antitumor immune response to occur. Perforins "perforate" targeted tumor cells and virally infected cells by forming transmembrane pores which ultimately cause cell lysis. The antitumoral and antiviral activities of IFN-γ are more complex, including stimulation of antigen presentation by upregulation of major histocompatibility complex (MHC) antigens, induction of protein kinase and reduction of cell proliferation.

Our data suggest that the mechanism(s) of immune impairment in UCNT subjects may depend on the inability of $CD8^+$ lymphocytes to secrete IFN-γ and to express perforin. This impairment did not depend on the presence of detectable EBV-DNA in the plasma of UCNT patients, and therefore is probably not correlated with the extent of EBV replication.

These results are particularly relevant because the blockade of the perforin pathway and IFN-γ production constitute mechanisms for immune escape by UCNT neoplastic cells and for impaired control of EBV replication [13, 18, 21]. Our data may help to establish the rationale to appropriately plan immunotherapeutic protocols in UCNT patients.

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