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Immunology

Selective expression of interleukin 10, interferon γ, and granulocyte–macrophage colony-stimulating factor in ovarian cancer biopsies

(mRNA/polymerase chain reaction/T-cell receptor)

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ABSTRACT **The variable clinical response seen with most cancer immunotherapy suggests that there is a large interindividual variation in immunologic response to tumors. One of the key functional parameters of an immune response is the local production of cytokines. As a method to survey the immune status of tumor-infiltrating cells, we have investigated the constitutive expression of cytokine mRNA in biopsies from epithelial ovarian carcinomas by using a PCR-assisted mRNA amplification assay. Using a set of cytokine-specific primers for 10 different cytokines, we have found selective expression of interleukin 10 (IL-10), granulocyte–macrophage colony-stimulating factor, and interferon γ mRNA in ovarian tumor tissue as compared to normal ovaries and ovarian tumor cell lines. Such differences could not be explained by the extent of T-cell infiltration, since comparing samples with the same intensity of T-cell receptor (TCR) constant region α-chain product from the tumor and normal biopsies demonstrated different cytokine patterns. No IL-2 gene expression was detected in the tumor biopsies. IL-2 mRNA, however, became expressed after stimulation of the tumor-derived cells via the CD3 molecule but not after growth in recombinant IL-2 alone. Using the same methodology, we also analyzed the TCR variable region β-chain gene repertoire. No restriction or biased expression of these genes was observed.**

The variable clinical response seen with most cancer immunotherapy suggests that individual patients have very different immunological mechanisms involved in the pathophysiology of their specific malignancy (1, 2). These can include important differences in the existence, nature, and distribution of tumor antigens on the malignant cells, as well as the specific immune response to the tumor. Analysis of tumor-infiltrating inflammatory cells revealed various numbers of cytotoxic and helper T cells, natural killer (NK) cells, macrophages, and neutrophils (3–5). Freshly isolated tumor-infiltrating lymphocytes (TILs) are functionally deficient as measured by their proliferation and cytotoxicity (6, 7). Addition of recombinant interleukin 2 (rIL-2), however, renders these cells lytic *in vitro* (8) and capable of antitumor activity in adoptive transfer studies (1). These data suggest, therefore, that the failure of the immune system to recognize and destroy cancer cells may be in part a result of insufficient immunological activation. The mechanism underlying decreased immunological reactivity is not known and could involve suppression effectuated by immunologically active cells, anergy due to aberrant presentation of antigen, and secretion of suppressor molecules from the tumor cells.

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One of the key functional parameters of an immune response is the local production of cytokines. Under physiological conditions, these are transiently produced by more than one cell type and provide short-range signaling within the tissue. Depending on the sequence of their action, they may act either synergistically or antagonistically on multiple types of target cells (9). Recognizing these intricate interactions, attention is shifting from cytokine action on individual cells to cytokine action in tissues.

As a method to survey the immune status of tumor-infiltrating cells, we have investigated the constitutive expression of cytokine mRNA in biopsies from epithelial ovarian carcinomas by using a PCR-assisted mRNA amplification assay. Using a set of cytokine-specific primers for 10 different cytokines, we have found a selective expression of IL-10, granulocyte–macrophage colony-stimulating factor (GM-CSF), and interferon γ (IFN-γ) mRNA in the ovarian tumor tissue as compared to normal ovaries and ovarian tumor cell lines. By the same methodology, experiments were also performed to correlate the cytokine mRNA pattern of each tumor biopsy with its T-cell receptor variable region β-chain (TCR V_β) gene repertoire.

MATERIALS AND METHODS

Biopsy Samples. Ovarian tumor biopsy specimens were obtained from 11 patients with advanced epithelial ovarian carcinomas, histologically classified according to World Health Organization criteria (10). All patients underwent primary extensive debulking surgery.

Biopsy specimens from normal ovaries were obtained in patients laparotomized for nonmalignant gynecological disorders. Three of these women were postmenopausal. Informed consent according to the Helsinki agreement was obtained from the patients before surgery.

Immediately after surgical removal of the first tumor specimen, biopsies were trimmed, frozen in CO_2-chilled isopentane, and kept at −70°C until analysis.

Tumor Cell Lines. Tumor tissue was minced with a scalpel and digested with collagenase type S (1 mg/ml), DNase (200 μg/ml), and hyaluronidase (100 units/ml) (all Sigma). Plastic adherent cells were cultured in Dulbecco's modified Eagle's medium (GIBCO), supplemented with L-glutamine (300 μg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal calf serum (GIBCO). Temporary selection medium

Abbreviations: IL, interleukin; rIL, recombinant IL; IFN, interferon; TNF, tumor necrosis factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; TCR V and C genes, T-cell receptor variable and constant region genes; TIL, tumor-infiltrating lymphocyte; DIG, dUTP-11-digoxigenin.

(MEM C-VAL; GIBCO) was used to prevent fibroblast growth.

PCR-Assisted mRNA Amplification. *RNA preparation.* Total RNA preparation was performed essentially as described (11). In brief, 50–150 mg of frozen tissue was lysed in a proportionate volume (0.5–1.5 ml) of solution, consisting of 4 M guanidine thiocyanate (Fluka), 25 mM sodium citrate (pH 7), 0.5% sodium *N*-lauroylsarcosinate (Fluka), and 100 mM 2-mercaptoethanol (Sigma). To each 1 ml of lysate was sequentially added 0.1 ml of 2 M sodium acetate, 1.0 ml of water-saturated phenol (Fluka), and 0.4 ml of chloroform/isoamyl alcohol (49:1); ingredients were thoroughly mixed by inversion after the addition of each reagent. The final suspension was shaken for 10 sec and chilled on ice for 15 min. Samples were spun at 10,000 × *g* for 20 min at +4°C, and the aqueous phase was transferred to a clean Eppendorf tube; RNA was precipitated in an equal volume of 2-propanol at −20°C for 60 min. Precipitates were pelleted at 10,000 × *g* at +4°C, redissolved in lysis solution, and ethanol precipitated overnight at −20°C; they were subsequently washed twice in 75% ethanol. Air-dried pellets were resuspended in 0.1–0.3 ml of RNase-free water.

First-strand cDNA synthesis. RNA was denatured for 5 min at 70°C and then chilled on ice. First-strand cDNA synthesis was performed from 5–10 μg of RNA at 40°C for 45 min in a final vol of 0.1 ml: 50 μl of denatured RNA, 20 μl of 5× buffer (BRL), 7.5 μl of 100 mM dithiothreitol (BRL), 10 μl of dNTP [dATP, dCTP, dGTP, and dTTP (5 mM each); Pharmacia], 2.5 μl of RNasin (40 units/μl; Promega), 5 μl of 1 mM random hexamer primers (Pharmacia), 5 μl of Moloney murine leukemia virus reverse transcriptase (200 units/μl; BRL).

Tubes were afterwards heated for 5 min at 95°C. cDNA from each sample was synthesized in one tube and then divided into separate tubes for the PCR.

Cytokine cDNA amplification. Forty microliters of PCR mixture was added to 10 μl of first-strand cDNA. PCR mixture contained 5 μl of 10× buffer [100 mM Tris·HCl/500 mM KCl/0.1% (wt/vol) gelatin, pH 8.3], 5 μl of 20 mM $MgCl_2$, 8 μl of dNTP (1.25 mM each; Pharmacia), 11.75 μl of sterile water, 5 μl of each primer (10 μM), and 0.25 μl of *Taq* polymerase (Cetus). The reaction mixture was amplified with a Perkin–Elmer thermal cycler for 30 cycles. The temperature profile used was 94°C for 1 min for denaturation, 58°C for 1 min for annealing, and 72°C for 1 min for primer extension. PCR products were separated on ethidium bromide-stained 1.6% agarose gel (Pharmacia). Lymphokine-specific primers were synthesized on a DNA synthesizer (Applied Biosystems). All primers were RNA specific and nonreactive with DNA. The following oligonucleotide 5′ and 3′ primer sequences were used: IL-1α, GCCAATGACTCAGAGGAAGA and TCTCAGGCATCTCCTTCAGC; IL-2, TGTACAGGATGCAACTCCTG and CAATGGTTGCTGTCTCATCAG; IL-3, CTCCTGCTCCAACTCCTGGT and AGGCTCAAAGTCGTCTGTTG; IL-4, CCTCTGTTCTTCCTGCTAGC and CCGTTTCAGGAATCGGATCA; IL-6, TGAACTCCTTCTCCACAAGC and ATCCAGATTGGAAGCATCCA; IL-10, CTGAGAACCAAGACCCAGACATCAAGG and CAATAAGGTTTCTCAAGGGGCTGGGTC (12); tumor necrosis factor α (TNF-α), TGAGCACTGAAAGCATGATC and TTATCTCTCAGCTCCACGCC; IFN-γ, TCTGCATCGTTTTGGGTTCT and CAGCTTTTCGAAGTCATCTC; GM-CSF, TGCAGAGCCTGCTGCTCTTG and CAAGCAGAAAGTCCTTCAGG; G-CSF, CAGAGCCCCATGAAGCTGAT and TATGGAGTTGGCTCAAGCAG; β-actin, ATGGATGATGATATCGCCGCG and CTAGAAGCATTTGCGGTGGAC.

Amplification of TCR V_β and constant region α-chain (C_α) cDNA. One microliter of cDNA was mixed with 19 μl of PCR mixture containing 0.5 μM (final concentration) TCR C_α- or V_β-specific oligonucleotide. Primer sequences (13) were verified by analysis of RNA extracted from phytohemagglutinin-stimulated lymphocytes, which resulted in successful amplification of all TCR V_β genes (data not shown). The PCR profile used was denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min for 35 cycles. Five microliters of the TCR C_α amplified product was separated on an ethidium bromide-stained 1.6% agarose gel and visualized in UV light. The V_β PCR amplified product was separated on a 1% agarose gel and blotted by alkali transfer to a nylon filter (Hybond-N; Amersham). A C_β-specific probe (14) was prepared as follows: 10 ng of plasmid cDNA was amplified (total vol, 50 μl) using primers 5′C_β-AGGACCTGAAC and 3′C_β-GGGAGATCTCTGCTTCTGATGG (final concentration, 0.5 μM each) and dNTP with a 2:1 ratio between dTTP and dUTP-11-digoxigenin (DIG) (Boehringer Mannheim). The PCR profile used was 94°C for 1 min, 35°C for 1 min, and 72°C for 1 min for 30 cycles. Filters were hybridized overnight at 42°C with DIG-labeled probe and incubated with alkaline phosphatase-labeled antibody. Finally, membranes were developed using the chemiluminescent substrate 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane (AMPPD) by autoradiography for 5–45 min according to the manufacturer's instructions (Boehringer Mannheim).

Statistical Analysis. Fisher's exact probability test was used for statistical analysis of cytokine gene expression.

RESULTS

Expression of cytokine and TCR V_β genes was analyzed in biopsies taken from 11 ovarian carcinomas by the PCR-assisted mRNA amplification assay. Biopsies from 8 healthy ovaries and 2 *in vitro* established ovarian carcinoma cell lines were included as controls.

T-Cell Infiltration. To estimate the degree of T-cell infiltration in the tumors and normal ovaries, the TCR C_α gene was analyzed by PCR. C_α was successfully amplified in all 11 tumor biopsies, with bands of high intensity, as detected on ethidium bromide-stained gels (Fig. 1*A*). In contrast, 3 of the 8 normal ovaries tested failed to express mRNA for TCR C_α (Fig. 1*B*). The intensity of the amplified product in these 5 biopsies varied. These results demonstrate T-cell infiltration in all tumor biopsies and in most, but not all, normal ovaries.

Cytokine mRNA Expression. Cytokine gene expression was detectable in 10 of the 11 tumor biopsies, in 5 of the 8 normal ovaries, and in both tumor cell lines (Table 1). To rule out the possibility that a negative result in some samples was due to

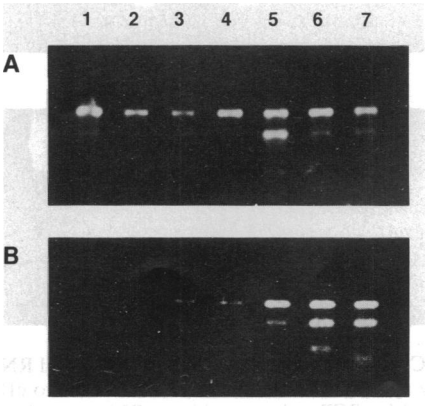

FIG. 1. TCR C_α gene expression in tumor biopsies and normal ovaries. cDNA from ovarian tumor biopsies (*A*) and normal ovaries (*B*) was amplified by using oligonucleotide primers specific for TCR C_α. The size of the amplification product is 600 base pairs (bp). (*A*) Lanes 1–7 correspond to patients 1–7 in Table 2. (*B*) Lanes 5–7 correspond to normal ovaries 1–3 from the same table.

Table 1. Cytokine mRNA expression in ovarian biopsies from cancer patients, healthy donors, and ovarian tumor cell lines

	IL-1α	IL-2	IL-3	IL-4	IL-6	IL-10	TNF-α	IFN-γ	GM-CSF	G-CSF
Healthy donors (n = 8)	0	0	0	0	3	0	5	2	0	0
Cancer patients (n = 11)	8	0	1	0	7	10*	10	8*	8*	1
Tumor cell lines (n = 2)	2	0	0	0	2	0	1	0	0	1

*P values for IL-10, IFN-γ, and GM-CSF in the tumor biopsies compared to normal ovaries are 0.0001, 0.0500, and 0.0021, respectively.

inefficient transcription and/or failure of the PCR, mRNA for the β-actin gene was successfully amplified in all cases. Ten of 11 tumor biopsies expressed mRNA for IL-10 and TNF-α; in 8 tumors, IL-1α, IFN-γ, and GM-CSF were observed; 7 exhibited IL-6; and 1 biopsy also expressed mRNA for G-CSF (Fig. 2; see also Fig. 4*A*). In contrast to the tumor biopsies, none of the 8 normal ovaries expressed IL-10 or GM-CSF mRNA (Fig. 3*A*). Three of them expressed IL-6 mRNA, 5 expressed TNF-α, and 2 expressed IFN-γ mRNA. Of the 2 ovarian tumor lines (passages 2 and 20), cytokine mRNA for IL-1α and IL-6 was detected in both, while TNF-α and G-CSF were expressed in 1 (Fig. 3*B*).

Cytokine expression analysis (Table 1) showed that the presence of IL-10 (P = 0.0001) and GM-CSF (P = 0.0218) mRNA was unique for the tumor biopsies. IFN-γ (P = 0.0500) gene expression was more frequent in the tumor biopsies but it was also present in healthy ovaries. Genes coding for IL-1α, IL-6, TNF-α, and G-CSF were expressed in tumor biopsies, tumor lines, and/or healthy ovaries, while IL-2, -3, and -4 mRNA were not detectable in any of the biopsies tested. No consistent pattern was seen when comparing the pathological classification of tumors (7 endometrioid, 3 seropapillary, and 1 mucinous) with their cytokine profile (data not shown).

To analyze the potential of the T cells infiltrating the tumor biopsies to express IL-2, -3, and -4 mRNA, we polyclonally activated one tumor sample, available as an ascites, by an anti-CD3 monoclonal antibody. This sample expressed a typical cytokine profile observed among the tumor biopsies (Fig. 4*A*). A 7-hr culture with the OKT3 antibody resulted in expression of all cytokines except IL-3 (Fig. 4*B*). However, cells cultured for 10 days with rIL-2 alone exhibited their original cytokine profile, without the expression of IL-2, -3, or -4, demonstrating that TCR stimulation was necessary for such expression (data not shown).

TCR V_β mRNA Expression in Biopsies from Ovarian Carcinomas. Since the observed expression of cytokine mRNA indicated the presence of immunologically active cells, we also analyzed the T-cell repertoire in tumor and normal ovarian tissues. A representative Southern blot analysis of the PCR-amplified TCR V_β gene is shown in Fig. 5. Results from the analysis of all 11 ovarian cancer biopsies and from 3 normal ovaries are summarized in Table 2. As can be seen for most tumors, there was a heterogeneity in the TCR V_β repertoire. V_β gene usage varied from very limited (biopsies 2, 3, 6, and 7) to a broad repertoire of TCR V_β expression. The observed differences in the intensity of the signals indicated quantitative variation between different V_β genes, as shown in Table 2 by + for a strong signal and by ± for a weak signal. There was no biased usage of individual V_β genes in the tumor biopsies, although V_β1 to -9 seemed to be more frequently expressed and V_β11, -15, and -20 were only detected in a minority of samples. The normal ovaries that demonstrated T-cell infiltration also had heterogeneous expression of TCR V_β genes (Table 2). No correlation between cytokine expression and V_β gene usage was observed (data not shown).

DISCUSSION

A major coordinating function in an immune response is executed by cytokines. Recent published data provide evidence that the pattern of cytokine expression in skin biopsies from patients with leprosy correlated with resistance or

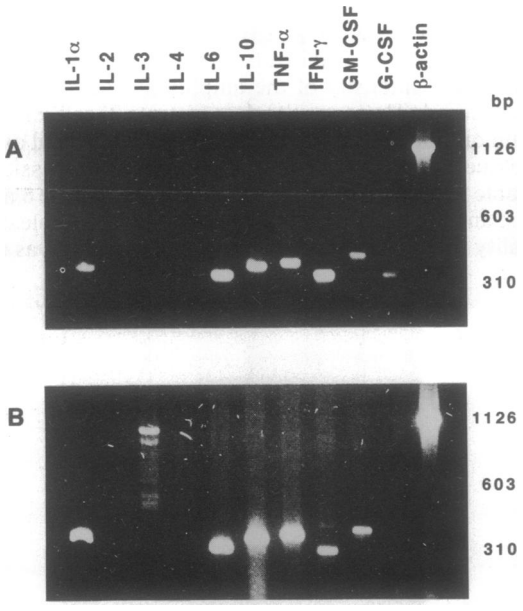

FIG. 2. Cytokine profile in tumor biopsies. Total RNA extracted from biopsy specimens was reverse-transcribed into cDNA. cDNA was amplified by PCR using cytokine mRNA-specific oligonucleotide primers. As an internal control of reverse transcription and PCR, β-actin mRNA was successfully amplified in all samples. (*A* and *B*) Cytokine gene expression in tumor biopsies 1 and 10 in Table 2, respectively. Bands >500 bp in lane IL-3 in *B* are larger than the expected IL-3 PCR product (425 bp) and are due to DNA contamination.

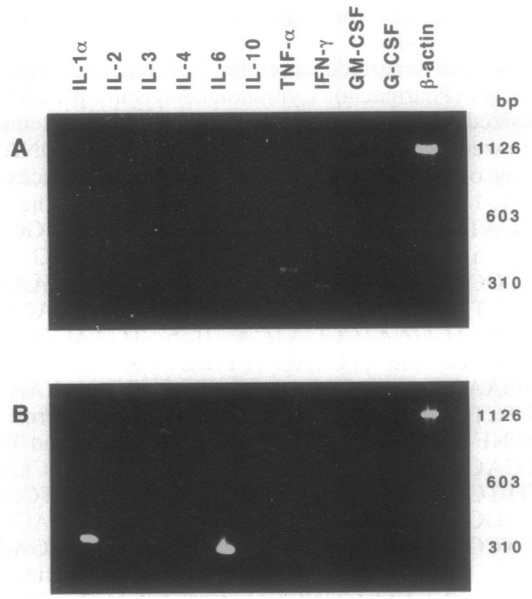

FIG. 3. Cytokine profile in normal ovaries and ovarian tumor cell lines. A representative cytokine profile from a normal ovary (ovary 3 in Table 2) is shown in *A* and one from an *in vitro* established ovarian tumor cell line (passage 20) is shown in *B*.

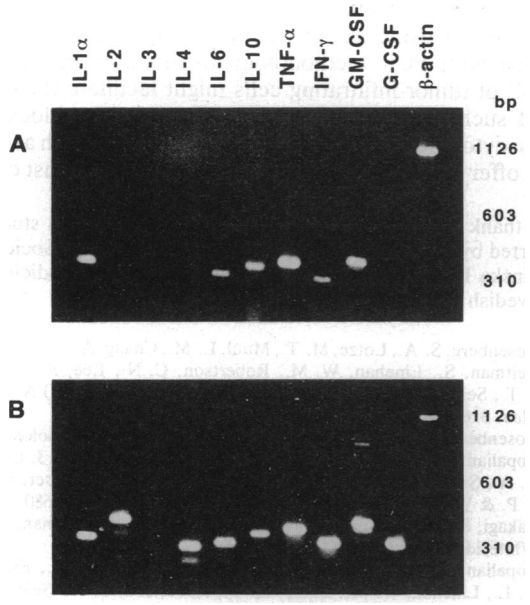

FIG. 4. Cytokine gene expression in ovarian tumor cells before and after OKT3 stimulation. An ovarian tumor from patient 4 in Table 2 available as an ascites and exhibiting a typical tumor cytokine profile was cultured (2×10^6 cells per ml) in RPMI medium with purified OKT3 (25 ng/ml) murine monoclonal antibody (American Type Culture Collection) for 7 hr. To reduce nonspecific stimulation, the serum source was excluded from the culture. Total RNA representing 5×10^4 cells from unstimulated (*A*) and stimulated (*B*) culture was used for amplification of each cytokine gene.

susceptibility to the infection (15). Furthermore, it was reported that the different functions of human $CD4^+$ and $CD8^+$ T-cell subsets can be discriminated and predicted by analysis of their cytokine profile (16). We now show that cytokine mRNA profiles of ovarian cancer biopsies differ from healthy ovaries. Comparison of an ovarian tumor cell line with cancer biopsy cytokine profiles revealed that the observed difference could not be accounted for by cytokine production of cancer cells only. Neither could the discrepancies be explained by the extent of T-cell infiltration, since comparing samples with the same intensity of TCR C_α product from the tumor (Fig. 1*A*, lane 1) and normal (Fig. 1*B*, lane 5) biopsies demonstrated different cytokine patterns (Figs. 2*A* and 3*A*, respectively). Our results demonstrate that

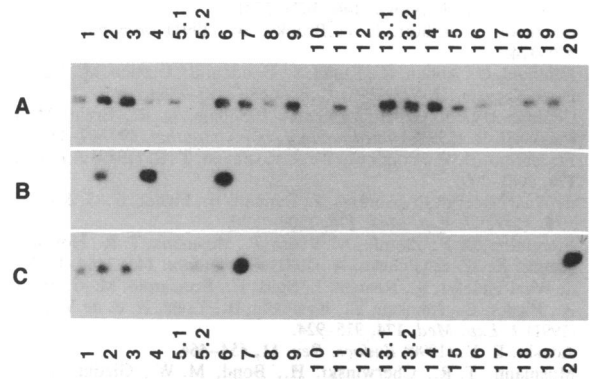

FIG. 5. Southern blot analysis of PCR-amplified TCR V_β genes in ovarian tumor biopsies. Total RNA extracted from biopsy specimens was reverse-transcribed into cDNA by a random hexamer priming method. Oligonucleotide primers specific for 22 different TCR V_β genes were used to amplify the cDNA in 35 cycles. Products were separated on 1% agarose gel, blotted, probed with DIG-labeled C_β probe, and detected with alkaline phosphatase-labeled antibody and chemiluminescence AMPPD substrate. (*A–C*) Analysis of tumor biopsies from patients 1–3 in Table 2, respectively.

Table 2. Southern blot analysis of PCR-amplified TCR V_β gene expression in ovarian tumor biopsies and normal ovaries

	Tumor biopsies												Normal ovaries			
V_β gene	1	2	3	4	5	6	7	8	9	10	11	n = 11	1	2	3	n = 3
1	+	−	+	+	+	+	−	±	+	+	±	9	+	−	+	2
2	+	+	+	±	+	+	−	±	+	+	±	10	+	−	−	1
3	+	−	+	+	+	+	−	±	±	±	±	9	+	−	+	2
4	+	+	−	+	+	+	−	±	±	+	±	9	±	−	+	2
5.1	+	−	±	+	+	+	−	±	+	±	±	9	+	−	+	2
5.2	±	−	±	±	+	−	−	+	+	+	+	8	+	−	−	1
6	+	+	+	+	+	−	−	±	+	+	±	9	+	−	−	1
7	+	−	+	−	+	−	+	+	+	+	±	8	+	−	−	1
8	+	−	±	+	+	−	+	+	+	+	±	9	+	−	+	2
9	+	−	−	+	+	−	−	+	−	+	+	6	+	−	−	1
10	−	−	−	+	+	−	−	±	+	+	±	6	±	−	−	1
11	+	−	−	+	+	−	−	+	−	−	−	4	±	−	−	1
12	±	−	−	+	+	−	−	+	+	+	±	7	+	−	−	1
13.1	+	−	−	+	+	−	−	+	±	+	±	7	+	±	−	2
13.2	+	−	−	+	+	−	−	+	+	+	+	7	+	+	−	2
14	+	−	−	+	+	−	−	+	−	+	+	6	+	−	+	2
15	+	−	−	±	−	−	−	−	+	+	−	4	−	−	−	0
16	+	−	−	+	±	−	−	−	+	−	+	6	−	+	−	1
17	+	−	−	+	+	−	−	+	+	±	+	7	+	+	−	2
18	+	−	−	+	+	−	−	+	+	−	±	7	±	+	−	2
19	+	−	−	±	+	−	−	−	+	−	+	6	−	+	−	1
20	±	−	+	−	−	−	−	−	+	−	+	3	+	−	−	1
V_β usage	21	3	9	20	20	5	2	18	19	17	20		19	6	6	

+, Strong signal; −, no signal; ±, intermediate/weak signal.

some cytokines predominantly but not exclusively produced by T cells, such as IL-10, IFN-γ, and GM-CSF, are selectively expressed in the cancer lesions. Interestingly, mRNAs for other cytokines also believed to be of T-cell origin (IL-2, -3, and -4) were absent.

Previous *in vitro* studies also describe TNF-α, IFN-γ, and GM-CSF production in TILs expanded in rIL-2 and stimulated with autologous tumors (17–19). These and other studies (20) report a specific defect in IL-2 and IL-4 secretion by TILs, which might be overcome by phytohemagglutinin stimulation (21). Recently published data also demonstrate that generation of lymphokine-activated killer cells from peripheral blood lymphocytes of renal cancer patients may be down-regulated by coculture with autologous TILs or a cell-free supernatant from such cells (22). It was also suggested that defective IL-2 production might be one of the reasons for impaired immunity in cancer patients. Our results support this notion and further indicate that a defect in IL-2 and IL-4 gene expression is probably not at the transcriptional level, since stimulation of the TCR via the CD3 molecule induces expression of IL-2 and IL-4 mRNA. The data further indicate that the IL-2 deficiency cannot be overcome by mere rIL-2 stimulation, as the 10-day TIL culture exhibited the same cytokine profile as the original tumor and was also deficient in IL-2 mRNA. The observed lack of IL-2 expression therefore could result from a lack of TCR-mediated stimulation *in vivo*—e.g., resulting from an immunosuppression at the antigen presentation level. Such a defect could be in part explained by the observed expression of IL-10 in the cancer lesions, a cytokine that was first described as a cytokine synthesis inhibitory factor (23, 24). Recently, it was also demonstrated that IL-10 strongly inhibits antigen-specific T-cell proliferation through down-regulation of the major histocompatibility complex class II expression and in that way inhibits the antigen presenting capacity of monocytes (25, 26). However, the true role of

IL-10 in this context remains to be analyzed with the help of, e.g., blocking antibodies or antisense RNA constructs.

Certain cytokine expression in normal ovaries is not surprising, since, e.g., IL-1 and TNF-α were implicated as putative intraovarian regulators (27). Normal ovaries are known to be infiltrated by T lymphocytes (27), as confirmed by our data, where the majority of normal ovaries expressed C_α mRNA. They exhibited, however, a different cytokine profile as compared to tumor biopsies, with a lack of IL-10 and GM-CSF expression and seldom expressing IFN-γ. We therefore conclude that the difference in cytokine expression between tumor and normal ovarian tissue cannot simply depend on a quantitative difference in T-cell infiltration.

Since the majority of TILs are believed to be of the $CD3^+$ $CD4^+/CD8^+$ phenotype (3, 28) the question that naturally arises is which T-cell subset predominantly produces these cytokines. Murine $CD4^+$ T cells have been divided according to their patterns of cytokine production (29, 30). There is now evidence that even human type 1 $CD4^+$ cells produce IL-2, IFN-γ, and GM-CSF (16, 31). The human T-cell subset with helper activity for B cells, corresponding to the murine T_H2 cells, produces IL-4, IL-5, and GM-CSF. The human $CD8^+$ population can also be divided into subsets: $CD8^+$ cytotoxic cells (type 1) that produce mainly IFN-γ and IL-10 and $CD8^+$ suppressor cells (type 2) that produce IL-4 (16). In the present study, the cytokine profile of individual T-cell clones derived from the tumor biopsies has not been addressed. Therefore, it is unclear to what extent the same subdivision of T-cell subsets in infectious diseases is also valid for the TILs. If so, however, then the predominant T-cell subset in the tumor biopsies would correspond to a type 1 $CD8^+$ T cell, with no production of IL-4 but with significant amounts of IL-10, GM-CSF, and IFN-γ.

Regarding the possibility that γ/δ T cells are responsible for the observed cytokine expression, the phenotype of fresh noncultured tumor-infiltrating cells was analyzed by cytofluorometry. Only 1–3% of the $CD3^+$ cells expressed the γ/δ TCR (data not shown), not exceeding what is usually observed in peripheral blood. Taking into account the sensitivity of PCR methodology, this does not entirely rule out the fact that γ/δ T cells could be responsible for the observed cytokine expression, particularly as they are known to produce several cytokines, including IL-2, IL-4, IFN-γ, and GM-CSF (32). Even the contribution of $CD3^-$ NK cells as well as activated macrophages to the observed cytokine profile should also be considered, since these were shown to produce IL-10 (24); however, their presence in ovarian malignancies is rare (5).

Little information exists on the T-cell repertoire of TILs in humans, although limited expression of TCR V_α genes was reported in human melanomas (33). The majority of tumors in our study demonstrated heterogeneous expression of a broad TCR V_β gene repertoire, but four of the tumor biopsies exhibited restricted TCR V_β usage, with only two to five V_β genes expressed (Table 2). The finding of relatively restricted V_β expression in two of the normal ovaries may indicate, however, that this selectivity is tissue specific rather than characteristic of ovarian tumors.

Immunotherapy offers medical treatment of great specificity. However, the unique nature of the host–tumor relationship and the unfavorable risk/benefit ratio of the treatment are arguing against its indiscriminate application. Precise diagnostic assays able to describe the specific pathophysiology in individual patients could identify the "likely to respond" group of patients. Clinical efficacy in immunotherapy also requires determination of the optimal dose for individual patients. Our results demonstrate specific cytokine mRNA profiles in ovarian tumor biopsies, which might be of prognostic significance for immunotherapy. The possibility of using a fine needle biopsy technique in conjunction with PCR methodology to monitor the "immune status" of tumor-infiltrating cells might facilitate the clinical use of such treatments. Furthermore, selective blocking of certain cytokines locally produced in the tumor, such as IL-10, might offer new immunotherapeutic principles against cancer.

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