SERA OF LUNG CANCER PATIENTS AFFECT THE RELEASE OF TH1, TH2 AND MONOCYTE-DERIVED CYTOKINES, AND THE EXPRESSION OF IL-2Rα BY NORMAL, STIMULATED MONONUCLEAR CELLS

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Abstract: We have shown that the sera of lung cancer patients affect the response of ConA-stimulated normal peripheral blood mononuclear cells by decreasing the expression of IL-2Rα and inhibiting the release of IL-1β and IL-2. A tendency to enhance the release of IL-6 was also observed. We conclude that an imbalance in the Th1/Th2 cytokine response, typical for cancer patients, may at least partly be related to soluble factors circulating in the patients’ blood. We discuss a putative role of serum IL-10, IL-1ra, and soluble IL-2Rα in the effects observed.

Key words: Cytokine Imbalance, Cancer Immunosuppression, Th1, Th2, IL-2R

INTRODUCTION

It has been recognised for many years that patients with both lymphoid and non-lymphoid malignancies frequently display impaired delayed-type hypersensitivity responses while retaining the normal capacity to generate antibody responses [1-5]. In addition, peripheral blood mononuclear cells from such patients exhibit impaired proliferative responses to plant mitogens and to allogeneic cells in vitro [4, 6-8].
Studies on cytokine profiles produced and released by T-helper cells disclosed a functional T-cell dichotomy and provided an explanation for the opposing cellular and humoral responses to various antigens [9]. In principle, two T-helper cell subsets, Th1 and Th2, have been distinguished by their cytokine profiles. Th1 lymphocytes, engaged mainly in generating delayed-type hypersensitivity responses, preferentially produce interleukin-2 (IL-2), interferon \( \gamma \) (IFN\( \gamma \)) and tumour necrosis factor \( \alpha \) (TNF\( \alpha \)), while Th2 lymphocytes, essential in the initiation of antibody responses, release IL-4, IL-5, IL-6 and IL-10. In addition, the activation of one of these subsets results in the suppression of the other. Several \textit{in vitro} studies revealed a decline in the production and/or release of the Th1 cytokines, IFN\( \gamma \) and IL-2, by mitogen-stimulated mononuclear cells from patients with various malignancies, i.e. colorectal, bladder, renal, prostate, breast, ovarian, cervical, endometrial and other carcinomas, as well as malignant melanoma [10-16]. In some of the malignancies, there is also an increase in the production and/or release of Th2 cytokines, typical for the development of immunological tolerance [11, 15, 17, 18]. The impaired proliferative responses of peripheral blood mononuclear cells from patients with malignancies have also been linked with the suppressive activity of monocytes/macrophages [4] and the decreased release of IL-1 [13]. In animal models, Th1 cells have been shown to be critically important for the induction of cellular immunity and the eradication of tumour mass \textit{in vivo} [19, 20].

So far, little is known about the nature of the factors responsible for the abnormal patterns of cytokine release and the poor proliferative response of peripheral mononuclear cells from cancer patients. We and others [21-23] have found increased amounts of cytokines and soluble receptors known to inhibit Th1 responses in ascitic fluids from patients with ovarian carcinoma. Ascitic fluids have also been shown to exert a suppressive effect on the proliferative responses of peripheral blood mononuclear cells from normal donors [24]. More recently, it has been demonstrated that the sera of patients with different non-lymphoid tumours contain elevated levels of various cytokines, including IL-10, IL-1 receptor antagonist (IL-1ra) and soluble IL-2 receptor \( \alpha \) (sIL-2R\( \alpha \)) [23, 25-29]. This may suggest that factors present in the sera of cancer patients may suppress Th1-like responses.

In this study, we report that the sera of patients with advanced lung carcinoma affect the proliferative response of ConA-stimulated mononuclear cells from normal subjects by simultaneously reducing the expression of IL-2 receptor \( \alpha \) (IL-2R\( \alpha \)) and inhibiting the release of IL-1\( \beta \) and IL-2. There was also a tendency towards increased IL-6 release in the presence of sera from cancer patients. These effects seem to relate to sIL-2R\( \alpha \), IL-1ra and/or IL-10 content in the cancer patients’ sera.
MATERIALS AND METHODS

Patients
Sera collected from healthy blood donors and untreated lung cancer patients were stored at -70°C. We previously assessed the concentrations of several cytokines in the sera of healthy volunteers (42 subjects aged 19 to 74, median age 42) and of lung cancer patients. For this study, we selected the sera of lung cancer patients with either elevated or normal levels of IL-1ra, IL-10 and sIL-2Rα (12 subjects aged 34 to 78, median age 55). The characteristics of the sera of the lung cancer patients included in this study are summarised in Tab. 1.

Tab. 1. IL-1ra, IL-10 and sIL-2Rα (pg/ml) in the sera of patients with lung cancer.

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Sex</th>
<th>Histopathology of lung cancer</th>
<th>Stage</th>
<th>Serum cytokines (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>51</td>
<td>M</td>
<td>Squamous cell carcinoma</td>
<td>3B</td>
<td>IL-1ra (473) IL-10 (7.3) sIL2Rα (1753)</td>
</tr>
<tr>
<td>2</td>
<td>49</td>
<td>M</td>
<td>Squamous cell carcinoma</td>
<td>1</td>
<td>359  4.8  1374</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>F</td>
<td>Adenocarcinoma</td>
<td>3B</td>
<td>1032 52.5 596</td>
</tr>
<tr>
<td>4</td>
<td>66</td>
<td>M</td>
<td>Squamous and large cell carcinoma</td>
<td>3A</td>
<td>151 46.7 2094</td>
</tr>
<tr>
<td>5</td>
<td>34</td>
<td>M</td>
<td>Squamous cell carcinoma</td>
<td>2</td>
<td>982  2.0  1384</td>
</tr>
<tr>
<td>6</td>
<td>75</td>
<td>F</td>
<td>Adenocarcinoma</td>
<td>4</td>
<td>132  2.0  1876</td>
</tr>
<tr>
<td>7</td>
<td>70</td>
<td>M</td>
<td>Adenocarcinoma</td>
<td>2</td>
<td>183  8.1  2321</td>
</tr>
<tr>
<td>8</td>
<td>59</td>
<td>M</td>
<td>Adenocarcinoma</td>
<td>4</td>
<td>189  4.5  1524</td>
</tr>
<tr>
<td>9</td>
<td>66</td>
<td>M</td>
<td>Squamous cell carcinoma</td>
<td>3B</td>
<td>471 15.0 1704</td>
</tr>
<tr>
<td>10</td>
<td>78</td>
<td>F</td>
<td>Squamous cell carcinoma</td>
<td>4</td>
<td>515  6.0  2488</td>
</tr>
<tr>
<td>11</td>
<td>40</td>
<td>M</td>
<td>Non-small-cell carcinoma</td>
<td>3B</td>
<td>624 24.3 5504</td>
</tr>
<tr>
<td>12</td>
<td>38</td>
<td>M</td>
<td>Squamous cell carcinoma</td>
<td>3A</td>
<td>450  2.7  1020</td>
</tr>
</tbody>
</table>

1Median age, 2TNM classification, 3in brackets, upper level in normal sera, evaluated in a group of 42 healthy donors (30 women and 12 men, aged 19 to 74, median age: 42).

Cell cultures
Peripheral blood mononuclear cells from healthy donors were separated by Ficoll-Uropoline centrifugation, washed, and cryopreserved in liquid nitrogen. Thawed and washed cells were suspended 5x10⁶/ml in RPMI 1640 supplemented with kanamycine, glutamine and 6% FCS. For mitogenic stimulation, concanavalin A (ConA) at a final concentration of 5 µg/ml was added (standard cultures). Except for standard cultures, lung cancer patients’ sera (CP) or pooled normal human AB serum (Sigma, collected from healthy male donors, min. age 17, max. age 69) (AB) were added to cultures at the arbitrarily selected concentrations of 5 and 25%. Pooled commercial serum was chosen as a control, to avoid random differences between the sera of individual healthy donors. Cells were incubated in glass culture tubes at 37°C, in a
humidified atmosphere of 5% CO₂. After 1, 2 and 3 days, the cultures were centrifuged and the supernatants were collected, aliquoted and stored at -70°C until assayed for cytokine contents. The cells were washed in PBS, collected by cytopinning and stained for IL-2Rα.

**Cytokine assays**

Commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis) were used for the quantitative determination of cytokines in the sera and in culture media. For IL-4 determination, high sensitivity tests were used. The tests were performed according to the manufacturer’s instructions.

**Determination of IL-2Rα positive cells**

Immunocytochemical staining was performed using monoclonal mouse anti-human interleukin-2 receptor α (anti-CD25, DAKO) as a primary antibody, and the Universal LSAB+ detection system (DAKO), employing the avidin-biotin technique based on biotinylated secondary antibody reacting with several phosphatase conjugated streptavidin molecules.

The examined MNC and, for the positive control, IL-2 dependent human T-cell line “S1 line 5”, kindly provided by Dr. J.K. Siwicki from the Maria Skłodowska Memorial Cancer Centre and Institute of Oncology in Warsaw [30], were collected as cytocentrifuge preparations.

Prior to incubation with the first antibody, the slides were fixed in cold concentrated acetone for 5 minutes and air-dried. After being washed in Tris buffer (pH 7.6), the slides were incubated for 1 hour at room temperature with the primary antibody diluted 1:50 in DAKO Antibody Diluent with Background Reducing Components. For the negative control, mouse IgG1 (DAKO) was used as a primary antibody. After washing with Tris buffer, the slides were developed with the use of a LSAB Kit, according to the manufacturer’s instructions. Briefly, the slides were incubated with biotinilated anti-mouse immunoglobulins for 15 minutes, rinsed and incubated with streptavidin conjugated to alkaline phosphatase. The specimens were rinsed and a substrate-chromogen solution with New Fuchsin was used to visualise the immunoreaction product. Positive immunostaining resulted in a red precipitate on the cell membrane and within the cytoplasm. The slides were counterstained with hematoxylin, mounted and examined in a light microscope.

**Statistical analysis**

Statistica version 6.0 (StatSoft) was used for all the statistical analyses. The distribution of cytokine levels in the culture supernatants was found normal by both the Kolgomorov-Smirnov and the Shapiro-Wilk tests. Therefore, variations in cytokine release and receptor expression in the presence of human sera were analysed using Student’s t-test for matched pairs (against the respective value obtained in standard culture); correlations were examined using Pearson
coefficient. The effects of lung cancer patients’ sera were compared with those of pooled normal human AB serum by Student’s t-test for independent data. A \( p \) of <0.05 was considered significant.

RESULTS

Fig. 1 shows the effects of sera of lung cancer patients and of pooled normal human AB serum on the release of IL-1\( \beta \), IL-2, IL-4 and IL-6 and on the expression of IL-2R\( \alpha \) by normal ConA-stimulated mononuclear blood cells. Maximum IL-1\( \beta \) concentration was observed 1 day after stimulation. Compared to standard cultures containing no human serum, upon the addition of 25\% of

![Graph](image)

Fig.1. The influence of 5\% and 25\% of sera of lung cancer patients (CP – grey checked boxes) and of normal pooled AB serum (Sigma) (AB – grey boxes) on IL-1\( \beta \), IL-2, IL-4 and IL-6 release and on IL-2R\( \alpha \) expression by normal human ConA-stimulated (5 \( \mu \)g/ml) mononuclear cells cultured for 1, 2 and 3 days. Cytokine levels in the culture supernatants were measured by ELISA. IL-2R\( \alpha \) expression was determined by immunocytochemistry. The results are shown as the percentages of standard values obtained in cultures with no human serum (standard cultures). The 10\(^{th}\), 25\(^{th}\), 50\(^{th}\) (median), 75\(^{th}\) and 90\(^{th}\) percentiles are shown. An asterix indicates a significant (\( p < 0.05 \)) difference from standard cultures; \( p \) values indicate significant differences between the effects of AB and CP sera.
AB serum, a significant increase of IL-1β-level was found in 1-, 2- and 3-day cultures. The 25% addition of CP sera resulted in a significant decrease in IL-1β concentration on days 1 and 2. The decrease observed on day 3 was not significant (p=0.05) compared to the standard cultures. The effects of CP sera on IL-1β release were found to be significantly different from those of AB serum on all the examined days of culture. There was no correlation between the secretion of IL-1β and the expression of IL-2Rα by normal mononuclear cells cultured in the presence of human sera (data not shown).

Maximum IL-2 concentrations in standard cultures were found after the first day following ConA stimulation. Those concentrations decreased during the subsequent days of culture. The addition of AB serum seemed to increase IL-2 concentration in the culture supernatants, but no statistical significance characterised this effect. In the presence of CP sera, IL-2 release was significantly diminished in 1-day cultures as compared both to standard cultures (p<0.0001 and p<0.00001, for 5% and 25% addition of serum, respectively) as well as to the cultures with AB serum supplement (p<0.05). A negative correlation between the release of IL-2 and the expression of IL-2Rα was found, but only in 1-day cultures with a 5% addition of patients’ sera (p<0.01; data not shown).

IL-4 levels in standard culture supernatants were found to be very low, with the maximum in 2- or 3-day cultures. Upon the addition of 25% of AB serum, a significant decrease in IL-4 levels in 2-day cultures was observed. The addition of 25% of most CP sera resulted in an increase of IL-4 release, but it was found not significant. There was no significant difference between the effects of CP and AB sera on IL-4 release.

The concentrations of IL-6 in standard culture media usually tended to increase over three consecutive days. Pooled AB serum significantly affected IL-6 levels only in 1-day cultures, at a concentration of 25%. In the presence of 5% and 25% of CP sera, the levels of IL-6 were significantly elevated in 2- and 3-day cultures and in 2-day cultures, respectively (p<0.01). In 2-day cultures, the stimulatory effect of CP sera was stronger than the effect of AB serum, but this proved to be significant only in cultures with a 5% addition of human sera.

The highest IL-2Rα expression in standard cultures was observed after 1 day of incubation. The presence of human sera, both AB and CP, significantly diminished the percentages of normal mononuclear cells expressing IL-2Rα. In 2- and 3-day cultures, the effects of CP sera on IL-2Rα expression were significantly more pronounced than those of AB serum.

The effects of lung cancer patients’ sera on cytokine release and IL-2Rα expression varied considerably in intensity with individual sera tested. As shown in Fig. 2, this might at least in part correspond to concentrations of cytokine
Fig. 2. Cytokine release and IL-2Rα expression and inhibitory factors in patients’ sera. IL-1β, IL-2, IL-4, IL-6 release and IL-2Rα expression by normal, ConA-stimulated peripheral blood mononuclear cells was assessed in 2-day cultures with a 25% addition of lung cancer patients’ sera containing normal or elevated levels of IL-10 (A) [see Tab. 1. sera Nos. 1, 2, 5, 6, 8, 10, and 12 containing normal levels of IL-10, and sera Nos. 3, 4, 7, 9, and 11 containing elevated levels of IL-10], IL-1ra (B) [see Tab. 1. sera Nos. 1, 2, 4, 6-9, and 12 containing normal IL-1ra levels and Nos. 3, 5, 10, and 11 containing elevated IL-1ra levels] and sIL2Rα (C) [see Tab. 1. sera Nos. 1-3, 5, 8, 9, and 12, containing normal sIL-2Rα levels and sera Nos. 4, 6, 7, 10, and 11 containing elevated sIL-2Rα levels]. The results are expressed as indexes relating cytokine release/receptor expression in cultures with patients’ sera to the values obtained in standard cultures, i.e. without the addition of human sera (set at 1). The 10th, 25th, 50th (median), 75th and 90th percentiles of the index values are shown.

Inhibitors in the patients’ sera. Strong inhibition of IL-1β release appeared to correspond particularly to increased IL-10 serum levels. The sera with elevated IL-10 as well as sIL-2Rα seemed also to exert inhibitory effects on the levels of
IL-2; those sera induced a similar decrease in IL-2 release in 2-day and 1-day cultures (the results for 1-day cultures not shown). No clear-cut pattern emerged concerning the relationship between the IL-4 and IL-6 concentration in culture supernatants and the content of individual inhibitory cytokines in sera. The inhibition of IL-2Rα expression seemed more pronounced in the presence of patients’ sera containing elevated concentrations of IL-10, IL-1ra and/or sIL-2Rα. Due to the limited number of sera characterised by similar concentrations of inhibitory cytokines, the relationship between the effects of CP sera and their inhibitory cytokine content could not be verified statistically.

DISCUSSION

We have shown that, compared to the effects of normal human AB serum, sera of patients with lung cancer inhibited, in a concentration dependent manner, the expression of IL-2Rα and the synthesis and/or release of IL-1β and IL-2 in cultures of ConA-stimulated mononuclear blood cells from normal donors. The degree of inhibition appeared to correspond to the concentrations of IL-10, IL-1ra and sIL-2Rα in the patients’ sera. The synthesis and/or release of IL-6 tended to increase in the presence of patients’ sera. These results suggest that the functional impairment of lymphocytes and changes in cytokine profiles observed in cancer patients may relate to the presence of soluble regulatory factors released into the body fluids of cancer patients.

Normal human serum is known to exert immunosuppressive activity. For example, it inhibits the proliferative response of stimulated human lymphocytes [31, 32]. However, the changes in IL-2Rα expression and cytokine responses of normal mononuclear cells in the presence of sera from lung cancer patients that we demonstrated here were, in general, different from those observed in the presence of normal human serum, and similar to those seen by others in cultures of mononuclear cells from cancer patients [16]. The decline in the level of the Th1 derived cytokines IFNγ and IL-2 as well as in IL-2Rα expression was found in mitogen-stimulated peripheral blood lymphocytes (PBL) from patients with different carcinomas, including lung, colorectal, bladder, renal, prostate and gynaecological carcinomas, with malignant melanoma and non-Hodgkin’s lymphoma [10-13, 15, 16, 33]. In addition, increased levels of Th2 cytokines – IL-4, IL-6 and IL-10 – were found in cultures of PBL from cancer patients [11, 14, 15].

Such alterations in Th1/Th2 cytokine profiles were also described in tumour-infiltrating lymphocytes from, for example, non-small cell lung carcinoma, glioblastomas and renal cell carcinoma [34-36]. In addition, Onishi et al. [37] found that the elevated intratumoral production of Th2-like cytokines in renal cell carcinoma correlated with the stage and grade of malignancy. Tatsumi et al. [38] have recently shown a Th2-dominated response of peripheral blood T-lymphocytes from patients with renal cell carcinoma or melanoma to the tumour-associated antigen MAGE-6. Most of the available clinical data and the
results obtained in animal models [39] demonstrate a shift from a Th1- to Th2-type response with the progression of cancer. The suppression of IL-1β release in the presence of lung cancer patients’ sera reported in the current study suggests the impact of the patients’ sera on antigen-presenting cells, what could provide an explanation for the delayed release of IL-2. This is in accordance with our earlier unpublished observation that the suppressive effects of ovarian cancer ascitic fluids on the proliferation of normal mononuclear cells are dependent on adherent cells. The mechanism of cytokine release inhibition induced by cancer patients’ body fluids requires further studies.

A variety of factors can be responsible for the effects of cancer patients’ sera on cytokine release and receptor expression by mononuclear cells. Soluble regulatory factors initially thought to be released by immune cells in response to a tumour may also originate from neoplastic and/or stromal tumour cells, known to produce and release various cytokines as well as cytokine inhibitors [40]. In our preliminary attempts to relate the effects of cancer patients’ sera to soluble regulatory factors, we analysed the concentrations of IL-10, IL-1ra and sIL-2Rα because of their well-characterised immunosuppressive activity [41-43], their increased levels found in sera from patients with a variety of malignancies [25-29], and their production demonstrated in malignant and/or stromal tumour cells [5, 16, 34, 37, 44-47]. Our results suggest that elevated concentrations of IL-10 in patients’ sera may relate to the impairment of IL-1β and IL-2 release and IL-2Rα expression, while high concentrations of sIL-2Rα may relate to the inhibition of IL-2 release and IL-2Rα expression. At least part of these results might be explained by the known regulatory functions of IL-10, which inhibits the synthesis of several monocyte/macrophage-derived cytokines, including IL-1 and Th1 cytokines [40]. Both IL-10 and soluble IL-2Rα receptors are thought to switch Th1 to Th2 response [25]. IL-1ra is known to down-regulate IL-1 induced responses, but it requires a hundred-fold surplus to abolish IL-1 activity [42]. It should be realised that increased IL-10, IL-1ra and sIL-2Rα levels in sera from cancer patients may be accompanied by elevated levels of other regulatory factors affecting lymphocyte functions.

Our preliminary data suggest that IL-10, IL-1ra and sIL-2Rα found in the sera of cancer patients are likely to be involved in a shift towards Th2 response; further studies are required to confirm these observations. Whether these factors mainly originate from immune cells or rather from neoplastic cells or other components of tumour microenvironment remains to be elucidated.

In conclusion, our results demonstrate that factors present in the sera from cancer patients may influence Th1/Th2 balance in the response of normal mononuclear cells, in a similar direction to that observed in the response of mononuclear cells from cancer patients.
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