IMMUNOTHERAPY WITH THE USE OF TUMOR- INFILTRATING LYMPHOCYTES AND INTERLEUKIN-2 AS ADJUVANT TREATMENT IN STAGE III NON-SMALL-CELL LUNG CANCER

A pilot study

This study assesses the feasibility and toxicity of adoptive immunotherapy with tumor infiltrating lymphocytes and recombinant interleukin-2 in 29 patients who underwent resection for stage III non-small-cell lung cancer. In five patients cultures yielded no growth of tumor infiltrating lymphocytes. In the remaining 24 patients (stage IIIa, 14 cases; stage IIIb, 10 cases) tumor infiltrating lymphocytes were in vitro expanded from surgically obtained tissue samples, including samples from both the tumor and surrounding lung. A number of tumor infiltrating lymphocytes, ranging from 4 to 70 billion cells, were reinfused intravenously 4 to 6 weeks after operation. Interleukin-2 was administered subcutaneously at escalating doses for 2 weeks and then at reduced doses for 2 to 3 months. Median survival was 14 months, and the 2-year survival was 40%. Three patients remain alive and disease-free at more than 2 years after operation. Two of these patients did not have complete resection at thoracotomy. Multivariate analysis showed no correlation between the factor of incomplete resection and survival. Intrathoracic recurrence without concomitant distant failure was documented in two patients only and none of the patients with incomplete resection (12 cases) had relapse within the thorax. The present experience demonstrates that adoptive immunotherapy may be applied with safety in patients operated on for stage III non-small-cell lung cancer and suggests that it can be useful, notably in patients with locally advanced disease. (J THORAC CARDIOVASC SURG 1995;109:1212-7)

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Adoptive immunotherapy with tumor-infiltrating lymphocytes (TIL) and recombinant interleukin-2 (IL-2) has been shown to be capable of mediating the regression of cancer.^{1,2} Rosenberg² demonstrated the efficacy of in vitro-expanded TIL cells in metastatic melanoma, and other groups obtained similar results in ovarian carcinoma.³ Pre-clinical studies demonstrated that expanded TIL, derived from non-small-cell lung cancers (NSCLCs), are able to recognize and efficiently lyse autologous cancer cells.^{4,5} No clinical study investigated the

role of adoptive immunotherapy in the treatment of NSCLC. Anecdotal reports in patients with unresectable tumors basically showed no benefit from this approach.⁶⁻⁸ The reasons accounting for such lack of efficacy of adoptive immunotherapy could be (1) the small number of infused TIL cells with respect to the large number of tumor cells or (2) TIL activity inhibition by tumor-derived suppressive factors. On the basis of these assumptions, we planned a pilot study to assess the feasibility and toxicity of adoptive immunotherapy in patients who underwent resection for stage III NSCLC.

Patients and methods

Preoperative assessment included (1) computed tomographic (CT) scans of the chest, upper abdomen, and brain, (2) bronchoscopy, (3) ultrasonographic scans of the liver and adrenal glands, (4) radionuclide bone scan, and (5) cervical or left anterior mediastinoscopy, or both, as indicated. For ethical concerns, patients with T3 NO-1 NSCLC or with minimal N2 disease (that is, involvement of one single mediastinal nodal site) were not offered adoptive immunotherapy, because their disease could be

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cured by surgical treatment. Inclusion criteria were (1) histologically confirmed NSCLC, (2) mediastinoscopy results that demonstrated multiple nodal site metastases (and excluded contralateral mediastinal involvement), (3) cardiopulmonary function adequate for the planned operation, (4) performance status from 0 to 1 (ECOG/WHO: 0 to 5 classification), (5) normal hematologic, renal, and hepatic function, (6) no prior therapy with biologic response modifiers (such as interferon or interleukins), (7) negative serologic test results for human immunodeficiency virus and hepatitis B virus antibodies, (8) no previous treatment with antineoplastic therapy, and (9) no steroid therapy.

Informed consent was required before patients entered the study. Beginning in January 1991, 29 patients with stage III NSCLC were scheduled for adjuvant immunotherapy with the use of TIL cells and rIL-2. In 5 patients cultures yielded no growth of TIL. The remaining 24 patients underwent surgical treatment and subsequent immunotherapy. Clinical and pathologic data are reported in Table I. The resection was classified as complete (R0) or microscopically (R1) or macroscopically incomplete (R2), according to previous definitions.⁹

TIL preparation and characterization. Tissue samples, including samples from both the tumor and surrounding lung, were obtained with a sterile technique from the surgically removed specimens (median sample weight, 24 gm). They were immediately processed, according to a previously described method.⁴ In brief, after removal of necrotic tissue and apparently normal lung, neoplastic and paraneoplastic tissues were dissociated mechanically, incubated (for 4 hours at 37° C with use of a magnetic stirrer) in RPMI 1640 medium (Microbiological Associates, Pool Biological Italiana, Milan, Italy) in the presence of deoxyribonuclease (Sigma Chemical Co., St. Louis, Mo., 0.002%, 100 IU/mg), collagenase type IV (Sigma, final dilution 200 IU/ml), hyaluronidase (Sigma, 1500 U/ml), and gentamicin (16 mg/100 ml). Erythrocytes, debris, and dead cells were eliminated with use of a Ficoll-Paque discontinuous gradient (Pharmacia, Inc., Piscataway, N.J.). The cells found in the gradient interface (such as lymphocytes, cancer cells, and alveolar macrophages) were washed and placed at 5×10^5 cells/ml in complete medium, containing recombinant IL-2 (Proleukin, Eurocetus, Amsterdam, The Netherlands, 500 IU/ml) and phytohemagglutinin (1% vol/vol; Difco Laboratories, Inc., Detroit, Mich.), to induce the activation and proliferation of any viable TIL.¹⁰ Living mononuclear cells, including both lymphocytes and cancer cells, were then incubated in RPMI 1640 medium, with 10% human AB serum and 500 IU/ml of recombinant IL-2.

When cells reached the concentration of 3 to 4×10^6 cells/ml, cultures were divided 1:2 in complete medium and their expansion allowed to continue. Cancer cells and other "nonlymphoid" cells disappeared from positive cultures in 2 to 3 weeks and a pure population of proliferating lymphocytes was obtained. Cultures yielded growth of adequate numbers (1×10^9) of TIL cells in more than 80% of cases. Single cancer cell suspensions were obtained as described.⁴ Neoplastic cells were frozen in liquid nitrogen for further studies or labeled with ⁵¹Cr for cytotoxicity tests.

Table I. Clinical and pathologic data

Age (yr $\pm$ SD)	60 $\pm$ 9
Male/female	21/3
Histologic type	
Squamous carcinoma	14
Adenocarcinoma	8
Large-cell carcinoma	2
Stage IIIa	
T2 N2	7
T3 N2	7
Stage IIIb	
T4 N2	10
Operation	
Lobectomy	13
Sleeve lobectomy	2
Pneumonectomy	9
Resection group*	
0	12
1	8
2	4

SD, Standard deviation.

*See text for description of groups.

Indirect immunofluorescence and flow cytometry were used for culture immunophenotype definition. The following monoclonal antibodies were used: CD3 (leu-4, Becton Dickinson Inc., Oxnard, Calif.), CD4, CD8, CD19, CD25, HLA-DR (T4, T8, and B4, Coulter Corporation, Hialeah, Fla.), CD16 (KD1, produced by our laboratory, and fluorescein isothiocyanate-labeled antihuman immunoglobulin G, Southern Biological Associated, Birmingham, Ala.). Lymphocyte cultures (0.05 ml) were incubated with a pretitered dilution of monoclonal antibodies for 30 minutes at 4° C, washed, and then incubated with 0.05 ml of fluorescein isothiocyanate-labeled antimouse immunoglobulin G (Southern Biological Associated) for 30 minutes. Cells were extensively washed, analyzed on an EPICS Elite flow cytometer, and the percentages of positive cells were calculated.

Cytolytic activities were studied on different targets. Natural killer (NK) activity was evaluated on K562, a human erythroleukemic cell line. Lymphokine-activated killer (LAK) cell activity was tested on the NK-resistant Daudi cell line. Finally, specific lysis was studied on autologous and allogenic cells from primary cultures of NSCLC. Cells were labeled with Na₂⁵¹CrO₄ as described.¹¹ Different effector/target dilutions (ranging from 50:1 to 1.5:1) were incubated for 4 hours at 37° C and specific lysis was calculated.¹¹

TIL infusion. Four to 6 weeks from the date of operation, in vitro-expanded TIL were infused intravenously (day 0). Recombinant IL-2 (Proleukin, Eurocetus) was administered subcutaneously, as shown in Fig. 1. Antipyretic agents (paracetamol or indomethacin), ranitidine, and antiemetics (ondansetron) were given to control fever, gastritis, and vomiting. No corticosteroids or anti-neoplastic drugs were administered during treatment. Toxicity of recombinant IL-2 administration was scored according to World Health Organization criteria. All patients were treated in general medical wards and none

Fig. 1. Recombinant IL-2 (rIL-2) scheduling after infusion of in vitro-expanded TIL.

Table II. Characteristics of infused TIL in 24 patients with NSCLC (mean and range)

	Percent
Surface phenotype	
CD3+ lymphocytes	95 (91-100)
CD4+ lymphocytes	39 (5-88)
CD8+ lymphocytes	49 (18-82)
CD16+ lymphocytes	0 (0-2)
CD19+ lymphocytes	0 (0-2)
CD25+ lymphocytes	52 (39-86)
HLA-DR+ lymphocytes	80 (59-85)
Cytolytic properties*	
K562 (NK cell target)	56 (28-100)
Daudi (LAK cell target)	35 (12-98)
Autologous NSCLC cells	41 (0-75)
Allogeneic NSCLC cells	12 (0-31)

*Percent of lysis was calculated at the effector/target ratio of 50:1.

required intensive care unit monitoring. The follow-up ranged from 6 to 32 months (median follow-up, 14 months) and included (1) CT scan of the chest and ultrasonographic scan of the upper abdomen every 6 months and (2) bronchoscopy, bone scan, and CT scan of the brain every 12 months.

Survival was calculated from the date of operation by the Kaplan-Meier method. Although the feasibility of the overall approach was the study end point, the influence of major prognostic factors (listed in Table I) on survival was also evaluated (by the Cox test).

Results

In eight patients the resection was classified as R1 because of (1) metastases in the last-removed node in the paratracheal or subcarinal region (4 cases), (2) extracapsular mediastinal node disease (3 cases), and (3) neoplastic infiltration of the aortic resection line (1 case). In four patients the resection was classified as R2 because of (1) neoplastic infiltration

Fig. 2. Survival of patients with stage III NSCLC who underwent surgical treatment and adoptive immunotherapy, according to completeness of resection. Number of patients at risk for each time interval is given in parentheses.

of the myocardial resection line (2 cases), (2) multiple parietal pleural metastases (1 case), and (3) tracheal wall infiltration by extracapsular node disease (1 case). One subject, in whom supraclavicular node metastases developed 8 months after operation, underwent lymphadenectomy followed by a second adoptive immunotherapy treatment (TIL and recombinant IL-2). This patient died 4 months after the second TIL infusion. A number of TIL cells ranging from 4 to 70 billion cells was obtained. Surface phenotype and cytolytic activity of TIL cells are shown in Table II. A correlation between the cytolytic CD8+ phenotype and the actual cytolytic activity of expanded TIL could not be documented. There was no correlation between the number of infused TIL and the clinical outcome of patients.

Median survival was 14 months. Three patients remain alive and disease free at more than 2 years after operation. Two of these long-term survivors did not receive complete resection at thoracotomy (R2). Fig. 2 illustrates survival curves of the complete resection and incomplete resection groups. There was no significant difference between the two curves. Multivariate analysis confirmed that in patients treated with adoptive immunotherapy, the factor of incomplete resection had no influence on prognosis (coefficient beta, -0.20). Survival was not significantly correlated with any of the investigated prognostic factors.

The sites of disease relapse are depicted in Table III. Despite the locally advanced disease stage, intrathoracic recurrence without concomitant distant failure was documented in two patients only. In particular, none of the patients with incomplete resection (R1 and R2) had a relapse within the thorax.

The toxic effects of adoptive immunotherapy were due to recombinant IL-2 administration. Toxicities were encountered in the course of the first 14 days of therapy and gradually resolved with continued administration at reduced doses. There were no grade IV (World Health Organization) toxic events. All patients had fever (higher than 38° C), chills, malaise, and nausea, which were easily managed with appropriate drugs. A rise in levels of creatinine (2 cases) or transaminases (1 case), a cutaneous rash (1 case), and oliguria and weight increase greater than 10% (2 cases) returned to normal after reduction of recombinant IL-2 doses. No modification of circulating white blood cells was observed after the infusion of TIL, whereas after recombinant IL-2 administration an expansion of eosinophils, associated with a rise in the percentage of CD16+ and CD25+ cells, was found.

Discussion

Very few reports deal with the use of adoptive immunotherapy in the treatment of NSCLC. Eight patients with advanced, unresectable NSCLC were treated with in vitro-expanded TIL cells and continuous infusion of recombinant IL-2 by Kradin and associates.⁶ None of the patients in that report responded to the treatment. Recombinant IL-2 with LAK cells or continuous infusion of recombinant IL-2 alone has been used in 10 patients with disseminated disease and failed to obtain major responses.^{7,8} Different explanations could be provided for the lack of efficacy of adoptive immunotherapy in patients with unresectable NSCLC. TIL cells that exhibit in vitro a specific response against autologous tumor cells might suffer in vivo from inhibition by tumor cell-derived immunosuppressive factors.⁵ Alternatively, the ratio of tumor cells to TIL cells might be so high as to make adoptive immunotherapy ineffective. This assumption is supported by a study that reported that cytolytic effects can be documented only when a number of TIL cells is used that is equal to or superior to the number of tumor cells.² Thus we tested the feasibility and toxicity of adoptive immunotherapy in patients who

Table III. Sites of failure in 24 patients with stage III NSCLC who underwent operation followed by adoptive immunotherapy

	No. of failures
Intrathoracic	
Lung	2
Distant	
Supraclavicular nodes	1
Brain	5
Bone	2
Liver	1
Adrenals	1
Intrathoracic and distant	
Lung and bone	1
Lung and brain	1

had undergone resection of the primary neoplasm. Our experience demonstrated that such an approach can be applied without severe toxic effects in more than 80% of subjects with stage III NSCLC. Prolonged administration of recombinant IL-2 may be done with safety, in the outpatient setting, so that the potential long-term maintenance of TIL activity is ensured. Several studies have shown that such recombinant IL-2 scheduling has a clinical efficacy comparable with that of the more toxic regimens.¹²⁻¹⁴ Besides its clinical acceptability, continued administration of recombinant IL-2 at nontoxic doses has been suggested to induce profound and prolonged effects on the patient's immune system.⁷

The 2-year survival in patients undergoing operation followed by adoptive immunotherapy was 40% and median survival was 14 months. According to current data in the literature, the median survival is about 12 months for subjects with stage IIIa and 8 months for subjects with stage IIIb NSCLC treated by intensive means.^{15,16} It should be noted that, in our study, patients with T3 N0-1 lesions (stage IIIa), whose disease stage has been associated with a better prognosis, were excluded.

Unexpectedly, in the present series, the subset of patients with incomplete resections (R1 and R2) had an outcome similar to that of patients with complete resection. This finding is at variance with the results of a previous study from our institution that demonstrated that patients with stage III NSCLC who underwent incomplete resection have a prognosis significantly worse than that of patients with complete resection.¹⁷ In the incomplete resection group, median survival was 9.1 months and the 2-year survival was 14%. These data would suggest that reinfused TIL cells are able to recognize and

lyse the intrathoracic component of the disease. Such assumption is supported by the analysis of the patterns of NSCLC relapse, revealing a very low intrathoracic failure rate after adoptive immunotherapy. In particular, immunotherapy succeeded in achieving disease control in patients with residual cancer. The effectiveness of immunotherapy in destroying intrathoracic cancer cells could be explained by a previous observation from our laboratory. One hundred million ^{99m}Tc labeled TIL cells were injected intravenously and monitored on the routine clinical gamma-camera. Washout analysis of the lungs showed that the majority of labeled TIL cells were trapped in the lung during the first 24 to 36 hours after infusion (manuscript in preparation). Thus a prolonged contact, within the chest, between the large number of infused TIL cells and residual cancer cells could be allowed. The reduced TIL concentration, once passed into the systemic circulation, could explain the minor impact of adoptive immunotherapy on distant metastases. Further, we have observed that, *in vitro*, TIL cells have a prominent LAK activity. Therefore we could suggest that the large number of effector cells concentrated in the lung may destroy cancer cells, independently from the presence of the wide spectrum of adhesion molecules, essential for the recognition of tumor cells in organs different from the lung.

The mechanisms by which TIL and recombinant IL-2 act remain uncertain. The results we have obtained, which indicate a lack of correlation between the percentage of cytolytic CD8+ cells infused and clinical response, seem to support the activation of local immunologic mechanisms. These mechanisms might include the production of lymphokines and the potentiation of antigen presentation mediated by specific helper T cells. This hypothesis is supported by a recent study that investigated radiolabeled TIL distribution and revealed that the majority of TIL cells do not specifically move to tumor sites.⁶

In conclusion, the present study shows that adoptive immunotherapy may be applied with safety in patients operated on for stage III NSCLC and suggests that it can be useful, notably in patients with locally advanced disease. However, the presented results should be considered with caution, because chance findings as a result of the study power may not be excluded. A randomized study has been planned to assess the effectiveness of adoptive immunotherapy as adjuvant treatment.

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