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Tumor-derived interleukin-2-dependent lymphocytes in adoptive immunotherapy of lung cancer

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Summary. A trial of adoptive immunotherapy was performed in which long-term cultured, interleukin-2 (IL2)-dependent T-lymphocytes were administered to patients with metastatic adenocarcinoma of the lung. Lymphocytes were isolated from explants of cancer tissues that were cultured in medium with recombinant IL-2. These T-cells expressed surface markers of activation, and killed a broad panel of tumor targets. Intravenously injected ^{111}indium-labeled T-cell blasts distributed primarily to lungs, liver, and spleen. Despite a paucity of infused lymphocytes detected by external imaging at sites of tumor, five of seven patients showed reduction of their cancers. However, in no case was greater than 50% reduction of total tumor burden achieved. Evidence of increased delayed cutaneous hypersensitivity to protein antigens was observed in three patients following therapy. We conclude that long-term cultured tumor-derived T-cells can be transferred safely into humans and that these cells may be capable of enhancing immune responses and mediating tumor reduction in vivo.

Introduction

Tumor cells express surface antigens that serve as targets for cell-mediated immunological responses [22]. It has been repeatedly demonstrated that animals that have been immunized against a tumor can specifically reject subsequent challenges by that tumor [23]. Rejection in vivo is mediated by mononuclear cells and specificity for tumor is conferred by T-lymphocytes [3, 12]. Lymphocytes sensitized to syngeneic tumor in vitro can effectively produce regression and cure of certain advanced murine tumors [4, 18, 31, 38, 40]. In addition to antigen-specific T-lymphocytes, natural killer (NK) cells [21] and other nonspecific cytotoxic cells [27, 34] have been suggested to play a role in reducing tumor in vivo. Recently, Rosenberg et al. have reported clinical responses in some human cancers by the transfer of interleukin-2 (IL-2)-stimulated peripheral blood lymphocytes that display broad antitumor specificities [39].

T-lymphocytes that are isolated from human tumors are enriched for specific reactivities to their autologous tumor when examined in vitro [24, 44, 45]. The adoptive transfer of sensitized, tumor-specific T-lymphocytes, however, has not been applied to the treatment of human cancers due to difficulties in the isolation and propagation of large numbers of immune T-cells. T-lymphocytes can be isolated from autologous tumor tissues when fresh tumor explants are cultured in medium with IL-2 [24]. T-cells retrieved in this manner secrete lymphokines, bind to tumor cells, kill autologous and allogeneic cancer cells and the K562 erythroleukemia cell line. These T-lymphocytes also display enhanced killing of their autologous tumors beyond what is observed for peripheral blood lymphocytes from the homologous donors.

We report the results of an immunotherapeutic trial in metastatic adenocarcinoma of lung using the adoptive transfer of autologous, long-term cultured, tumor-derived T-cells. Five of seven patients who received adoptive transfers of autologous T-lymphocytes without adjuvant IL-2, showed a reduction in the size of their tumors. The antitumor effects of immunocompetent T-cells isolated from tumor tissues suggest that these cells may be of therapeutic value in human lung cancer.

Experimental methods

Patient population. Seven patients with metastatic adenocarcinoma of lung were studied. The criteria for entry into the trial included the presence of biopsy-proven metastatic adenocarcinoma of the lung that was not amenable to surgical extirpation. Patients were excluded from the trial if they were (1) >70 years old, (2) had arterial pO_2 <60 mm Hg, (3) a creatinine >3.0 mg/100 ml, (4) bilirubin >3.0 mg/100 ml, (5) active arthritis or autoimmune disease, or (6) were receiving steroids. All patients signed an approved informed consent form prior to entrance into the trial.

Four of the seven patients had completed courses of radiation to their primary lung tumor and mediastinum at least 1 month prior to treatment. One patient (D) completed a single course of chemotherapy, without achieving a clinical response, prior to beginning immunotherapy. Three (B, C, G) received palliative radiotherapy to painful boney lesions during their course of immunotherapy (Table 1). Irradiated sites in bone were excluded from posttreatment evaluation.

Lymphocyte harvest and culture. Recombinant IL-2 was a generous gift of the Genetics Institute (Boston, Mass.). In

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Table 1. Clinical details of patients involved in the study

Patient	Age	Tumor	Site of disease	Date of diagnosis	Prior treatment	Date of death
A	36	Adeno (PD)[a]	Lung, chest wall	11/84	Surgery, radiation	3/86 (5 days)[b]
B	60	Adeno (MD)	Lung, chest wall, bone	6/85	None	9/85 (2 months)
C	34	Adeno (PD)	Lung, bone	5/85	None	12/85 (3 months)
D	67	Adeno (MD)	Liver, bone, brain	10/85	Velban Mitomycin	4/86 (6 months)
E	53	Adeno (WD)	Lung, pleura	6/85	Radiation	Alive (3 months)
F	58	Adeno (WD)	Lung, pleura, pericardium	4/85	Radiation	3/86 (1 months)
G	61	Adeno (MD)	Lung, pleura, bone	11/85	Radiation	Alive (7 months)

[a] Histological grade of tumor, WD = well, MD = moderately, PD = poorly differentiated
[b] Numbers in parentheses represent survival following immunotherapy

all but one patient (D), lymphocytes were isolated and grown directly from biopsies of their tumor. In this patient long-term cultures (>4 weeks) of IL-2-dependent peripheral blood lymphocytes were used for therapy. Tumor was obtained from either primary or metastatic sites by various biopsy procedures (Table 2).

The tumors were cultured directly from biopsy explants as previously described [30]. Briefly, tumor was sectioned into 1–2 mm pieces with a sterile blade and placed into 24-well tissue culture plates that contained RPMI-1640 with 5% heat-inactivated human serum, 50 μg/ml, gentamicin (complete medium), and IL-2 (10 units/ml) or complete medium alone. The cultures were observed daily under an inverted light microscope. Cells were expanded in 24-well plates when cell density exceeded $\sim 5 \times 10^5$/ml. To attain large numbers of cells for infusion and testing, the cultures were periodically (every 2–4 weeks) restimulated with irradiated autologous peripheral blood mononuclear cells and phytohemagglutinin. (PHA, Wellcome HA 16/17, 1 μg/ml). The interval between the time of initiating cultures from the biopsy and the use of the cells for therapeutic infusions varied between 1 and 4 months. Immediately prior to each infusion, the cells were washed 6 times in endotoxin-free, acetate-buffered, normal saline, pH 7.3. Then 10 ml of the culture supernatants were planted in separate culture bottles containing thioglycollate medium and dextrose phosphate broth (GIBCO Life, Lawrence, Mass.), and these cultures were examined for bacterial growth. T-lymphocyte cultures were examined each day by direct microscopic observation to exclude bacterial and fungal contamination. Gram stains were prepared from culture supernatants prior to each infusion. An aliquot of the supernatant from the last saline wash of the cells was tested for the presence of bacterial endotoxin in a Limulus Amebocyte Lysate assay (MA Bioproducts, Walkersville, Md.) that is sensitive to >1.25 EU/ml of FDA reference endotoxin. Patient plasma was examined for the presence of preformed anti-lymphocyte antibodies by indirect immunofluorescent staining and flow cytometric analysis. Pooled sera containing anti-HLA antibodies from multiparous females served as the positive control for these studies. Control antibody binding was defined using a panel of sera from normal men and nonparous women.

Radiolabeling of T-cells. T-cells (2.5×10^8) were radiolabeled with 250 μCi of ^{111}indium-oxide using a method modified from previous reports [42]. The radiolabeled cells were infused over 5 min via a peripheral venous catheter. Sampling of peripheral blood was performed at regular intervals between 5 min and 48 h. Aliquots of 1 ml of blood were counted in a γ-counter, and the counts corrected for the radioactive decay of ^{111}indium ($t_{½} = 2.8$ days). Patients were scanned by γ-camera at 4, 24, 48, and 72 h to determine the distribution of the radiolabel. Images were re-

Table 2. Biopsy sites used and effects of immuno therapy procedures

Patient	Source of biopsy	Interval from biopsy to treatment	Number of infusions	Total number cells infused (x 10^{-9})	Tumor reduction	Clinical[a] response
A	Open lung	4 months	3	0.5	Subcutaneous mass	N.E.[b]
B	Excision of rib metastasis	1 months	9	4.0	Subcutaneous mass, lung	Progression
C	Fiberoptic bronchoscopy	3 months	6	3.0	No	Progression
D	Blood	2 months	14	4.6	Subcutaneous masses	No
E	Rigid bronchoscopy	2 months	6	4.8	Lung	No
F	Pleural biopsy	3 months	8	3.3	No	No
G	Rigid bronchoscopy	4 months	9	3.1	Lung, bone	Progression

[a] Based on evaluation at completion of therapy
[b] Therapy could not be evaluated due to death 5 days after starting therapy

corded with a medium energy, high resolution collimator. Data was recorded and stored in a dedicated computer with a 128×128 matrix.

Pretherapy evaluation. All patients were studied within 2 weeks of initiating treatment to define the location and extent of their tumor. Routine studies included chest X-rays, liver, spleen, and bone scans. Computerized tomography (CT) of the head, chest, or abdomen was obtained when indicated. Laboratory testing prior to therapy included complete blood count (CBC) with differential, liver function tests, serum creatinine, electrolytes, erythrocyte sedimentation rate (ESR) carcinoembryonic antigen, anti-nuclear factor, rheumatoid factor, hepatitis B surface antigen, electrocardiogram, urinalysis, pulmonary function studies. Values for CBC, ESR, electrolytes, and creatinine were obtained three times a week during treatment. All blood sampling was repeated at 1–3 weeks after the end of therapy.

Two-color flow cytometric analysis of the T-cell blasts used for infusion, and peripheral blood lymphocytes was performed immediately before, and 30 min after completion of the T-cell infusion, 1–3 times a week. Mouse anti-human monoclonal antibodies directed at a spectrum of lymphocyte antigens were used to stain the cells. These antibodies react with CD3 (Leu-4) that is present on peripheral blood T-lymphocytes, CD4 (Leu-3a) present on the helper/inducer subset of T-cells and monocytes, CD8 (Leu-2) present on the cytotoxic/supressor subset of T-cells, Leu-8 present on regulatory subsets of peripheral $CD4^+$ and $CD8^+$ T-cells, CD11 (Leu-15) present on the regulatory subset of $CD8^+$ cells and >90% of monocytes and granulocytes, HNK-1 (Leu-7) present on granular lymphocytes, CD16 (Leu-11) present on the majority of NK cells and granulocytes, CD25 (IL-2 receptor) present on activated T and B-lymphocytes, class II HLA-DR antigens expressed by activated human T-lymphocytes, B-cells, and monocytes.

Patients were skin tested with 0.1 ml of purified protein derivative (PPD, 5 units), mumps, or *Candida* specific antigens planted in the forearm before immunotherapy and again at the end of therapy. Tests were scored at 24, 48, 72 h. Induration at the skin test site >10 mm was scored as positive and the presence or absence of erythema was noted.

Treatment protocol. Each patient was skin tested with 5×10^6 autologous T-cells 48 h before receiving i.v. infusions of lymphocytes. T-cells for infusion were suspended and delivered in acetate or bicarbonate-buffered, normal saline, pH 7.3. In a single patient (A), intralesional injections (total of 4×10^8 cells) of a s. c. chest wall tumor were also performed. The numbers of cells delivered per i.v. infusion varied between 2×10^7 and 1.1×10^9 cells. In general, 2–3 i.v. infusions were performed per week for 3 weeks (Table 2). In two patients (D and E), infusions were continued on an outpatient basis at periodic intervals.

Evaluation of responses. All scans and X-rays were repeated at 1–3 weeks after the completion of therapy to assess changes in tumor size and distribution. Tumor size was determined by the product of the longest horizontal and vertical diameters of each lesion. The extent of measurable tumor was taken to be the summation of the size of all measurable lesions. Further testing was continued at periodic intervals when indicated. Antitumor responses were quantitated by the following criteria: complete response = total disappearance of all known malignant disease; partial response = 50% or greater decrease in the extent of malignant disease, no more than a 25% increase in the size of any measurable lesion and no new malignant lesions. No response = <50% decrease in the extent of a measurable lesion with <25% increase in the size of any malignant lesion and no new lesions. Progressive disease = >25% increase in the size of any measurable lesion or the development of a new malignant lesion.

Results

Phenotype of cells used for therapy

The cell surface phenotype of the T-cells that were used for therapy is shown in Table 3. Five of the seven patients showed a dominance of $CD4^+$ cells in their cultures; $CD8^+$ cells dominated in the cultures of two patients. Differences in the percentage of T-cells that expressed IL-2 receptors, and to a lesser degree class II HLA-DR antigens, varied inversely with the time interval between PHA stimulation and infusion. The expression of IL-2 receptors on cells peaked at 2–5 days after stimulation, then decayed rapidly and was present on less than 10% of cells by day 12. The decrease in IL-2 receptor expression was accompanied by diminished proliferation of the cells in culture but did not correlate with their cytotoxic activities (data not shown). HLA-DR antigen diminished slowly on the surface of activated T-cells and was present on more than 70% of T-cells at day 12 (Fig. 1). Cultured T-cells were routinely used for infusion at 7–14 days after restimulation.

All lymphocyte cultures were tested for cytotoxicity against allogeneic and NK tumor targets in 4-h ^{51}Cr release assays [24]. The amount of allogeneic and NK target killing varied among long-term T-cell cultures (data not shown). In two cases, the lymphocytes were examined for killing of autologous tumor cells that had been isolated in

Table 3. Phenotype of T-cell blasts used in therapy

Antigen	Patient						
	A	B	C	D	E	F	G
CD4	61	72	55	1	5	91	58
CD8	20	12	35	98	91	5	22
CD4/IL-R[a, b]	11	51	13	0	1	85	12
CD8/IL-R	8	8	5	1	1	1	2
CD4/HLA-DR[b]	23	nd	57	1	5	93	14
CD8/HLA-DR	52	nd	38	80	86	3	9
CD4/Leu-8	8	40	10	0	1	41	56
CD8/CD11	36	3	3	1	22	2	8
HNK-1/CD8	20	2	8	0	14	1	8
HNK-1/CD16	8	1	2	0	0	1	2

Long-term cultured T-cells were stained with monoclonal antibodies tagged with FITC and phycoerythrin, fixed in 1% paraformaldehyde, and analyzed on a Becton-Dickinson FACS 440

[a] data represents the percentage of T-cell blasts that stained positively for both antigens and is representative of the phenotype used for infusions in each patient

[b] the percentage of T-cells bearing IL-2R, and to a lesser extent HLA-DR, varied based on the interval between PHA stimulation and staining

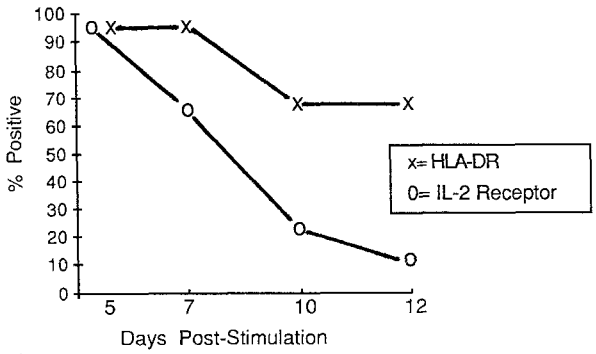

Fig. 1. Expression of cell surface IL-2 receptors and class II HLA-DR antigens on T-cells following restimulation with PHA
T-cells cultured from tissues in medium with IL-2 were periodically (2–4 weeks) restimulated in the presence of PHA (1 μg/ml) and irradiated peripheral blood mononuclear cells. T-cells were stained with monoclonal antibodies reactive with the IL-2 receptor and class II HLA-DR antigens at 2–3 day intervals, and analyzed on a Spectrum III flow cytometer (Ortho Diagnostics, Westwood Mass.). Nearly all T-cells (>95%) expressed IL-2 receptors and HLA-DR antigens 5 days after stimulation. IL-2 receptor expression decreased rapidly after day 5, with less than 10% of T-cells staining positively by day 12. Cellular proliferation paralleled the expression of the IL-2 receptor (data not shown). HLA-DR expression was maximal at day 5, and then gradually diminished with time so that ~70% of T-cells were HLA-DR$^+$ at day 12. Data represent the average values from three different tumor-derived T-cell cultures stimulated in PHA, and analyzed in parallel

stationary cultures from tumor tissue explants. In one case (A), T-cells showed enhanced killing of autologous tumor above what was observed for IL-2-stimulated, homologous peripheral blood (Fig. 2). In the other case, minimal cytotoxic activity was observed against syngeneic, allogeneic, and NK tumor targets (data not shown).

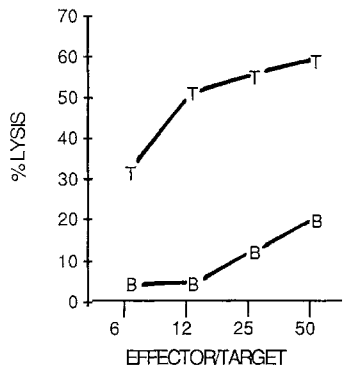

Fig. 2. Lysis of autologous tumor by IL-2-dependent, tumor-derived and long-term cultured peripheral blood T-cells
Tumor-derived lymphocytes and peripheral blood lymphocytes were cultured in parallel in IL-2, and tested for their ability to lyse cultured autologous adenocarcinoma cells in a 4-h ^{51}Cr release assay. Note that tumor-derived T-cells were more effective at lysing autologous tumor at all effector: target ratios than peripheral blood lymphocytes. Tumor-derived and blood T-cells also killed allogeneic tumor targets and NK targets (data not shown). Data is representative of one of five seperate assays performed periodically over a 6-month period using these cultures

Localization of radiolabeled cells

Whole-body imaging of the distribution of ^{111}indium-labeled T-lymphocytes, performed within 4 h after infusion, showed concentration of radiolabel in lungs, liver, and spleen, with minimal uptake in bone. With time, the amount of radiolabel in the lungs decreased, while activity in liver and spleen increased. In two cases (B and C), the rate of lung clearance of radiolabel was dramatically faster than for the other five patients (Fig. 3). Minimal activity was present in areas that corresponded to sites of primary lung tumor. A patient with liver metastases (D) also showed less uptake of label in regions of hepatic metastases that had been previously detected by ^{99}technetium colloid imaging (Fig. 4). In no case were labeled cells concentrated above tissue background within known pulmonary, hepatic, subcutaneous, or brain tumors as judged by γ-imaging. γ-Counting of peripheral blood ($n=3$) showed the circulating radiolabel to be reduced to ~8%–10% of the injected dose by 5 min following infusion, suggesting

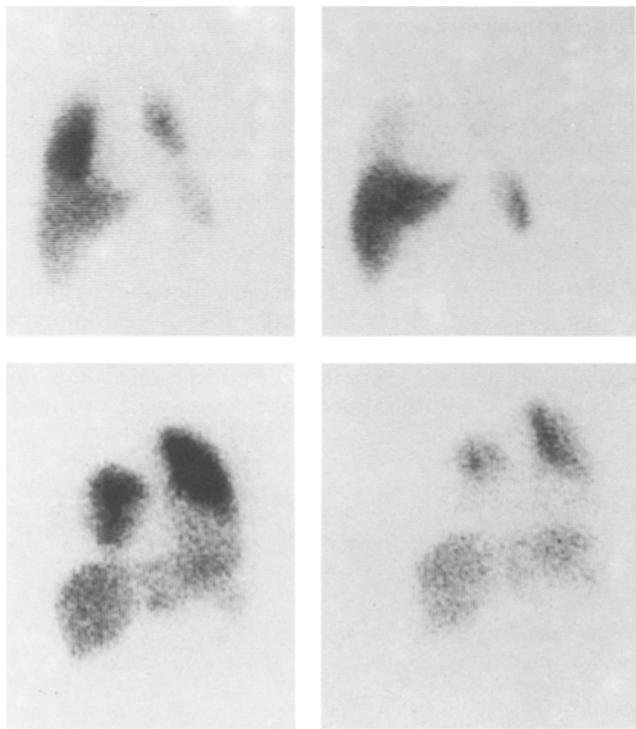

Fig. 3. γ-Imaging of patients following infusion of ^{111}indium-labeled T-cells
T-lymphocytes (2.5×10^8) were labeled with 250 μCi of ^{111}indium. The cells were washed, and infused via a peripheral vein. Whole-body images were obtained by γ-scanning at 4 h and 24 h. The upper panel (left) shows the 4 h upper torso scan of a patient (B) who had a tumor in the left lower lobe. Note the concentration of label in lungs, liver, and spleen. Minimal activity in other organs, and blood pool is seen. Less activity is present in the left lower lung zone than in other pulmonary zones. At 24 h (upper panel, right) there had been a marked decrease in the amount of activity in the lungs and increased label is present in liver and spleen. The lower panel (left) shows the appearance in another patient (E) at 4 h, who had a tumor in the right upper lobe that had involved ipsilateral pleura. Note the concentration of label in lungs, liver, and spleen, and the presence of focally diminished activity in the right lung. At 24 h, label persisted in the lungs, but activity was diminished compared to the earlier time point

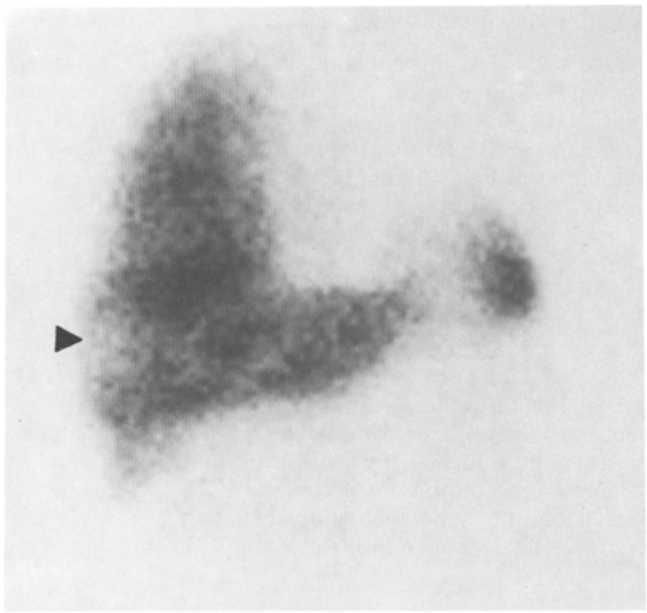

Fig. 4. γ-Imaging of a patient with liver metastases
γ-Imaging at 4 h following the infusion of radiolabeled cells in a patient (HK) with liver metastases showed a discrete zone of diminished activity in the right lobe of the liver (arrow), as well as several other inhomogeneous zones in that organ. These areas of diminished activity corresponded to the location of metastases as judged by ^{99}technetium colloid scanning (not shown). Note the absence of the left lung, which had been previously resected

that most cells had been sequestered following the initial circulatory passes. A gradual, further loss of the radiolabel from the blood occurred over hours, and only ~1%–2% of the injected label was present in the circulation at 12 h. For two patients, blooid disappearance curves were constructed on initiation of therapy and at the end of the 3-week course of T-cell infusions. No significant difference in the kinetics of disappearance of the radiolabel was detected between these time points (data not shown).

Histology of the lungs following infusions

Transbronchial biopsies of nontumor-bearing lung (right lower lobe) were performed in two patients 24 h after they received their first i.v. infusion of radiolabeled T-cells. The lungs in both cases showed focal alveolar septal injury as judged by the presence of episeptal and intraalveolar fibrin. A lymphocytic infiltrate was present around small septal vessels, within alveolar septa, and in air spaces (Fig. 5).

Analysis of the cells in the bronchoalveolar lavage (BAL) revealed that up to 80% of the leukocytes in the BAL were lymphocytes (normal 10%–20%). More than 90% of these lymphocytes were CD3$^+$ (Fig. 6). In one patient (AR), the BAL fluid was examined for the presence radioactivity. All of the radioactivity (614 cpm/ml lavage fluid) was associated with the cell pellet confirming that T-cells that had been infused had been retrieved in the BAL.

Skin testing for antigen

Five patients displayed cutaneous anergy to all of the protein antigens used for skin testing at the start of therapy. In one patient (D), cutaneous anergy to *Candida* specific antigens was replaced by an erythematous and indurated (>20 mm) response, when he was retested at the conclusion of therapy. In two nonanergic patients (G and E),

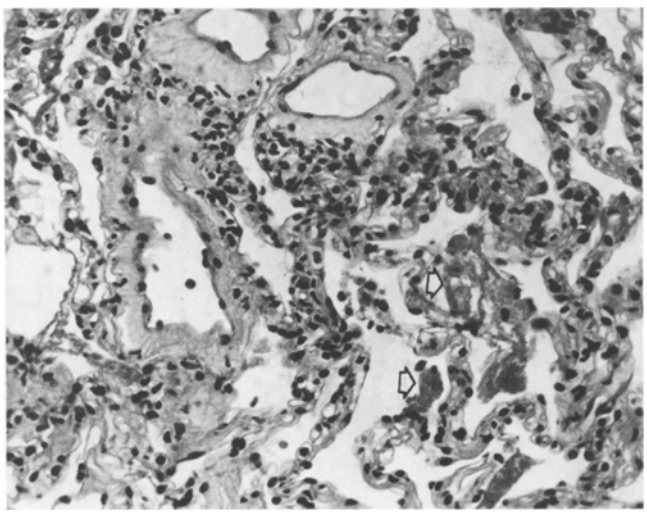

Fig. 5. Histology of the lung after T-cell infusion
Two patients underwent transbronchial biopsy of nontumor-bearing right lower lobe 24 h after receiving an infusion of T-cells. The lung tissue showed a lymphocytic infiltrate that involved the alveolar septa and surrounding small parenchymal vessels. Several foci of septal and intraalveolar fibrin are present (white arrows) indicating the presence of microvascular injury. Hematoxylin and eosin. Magnification ×313

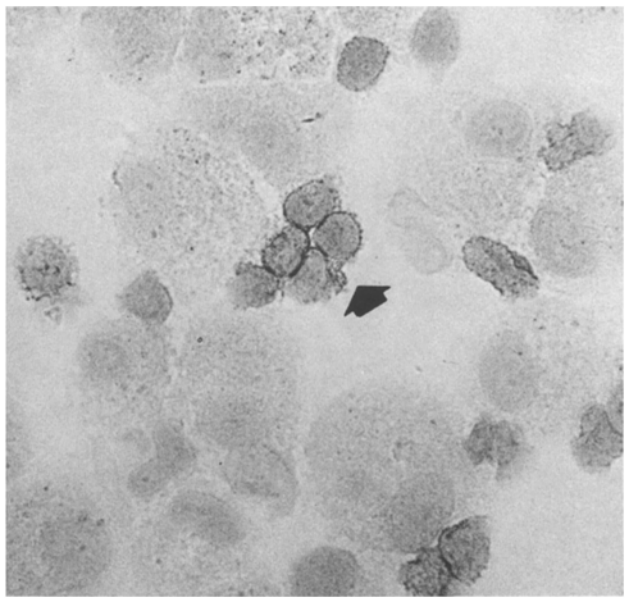

Fig. 6. Phenotype of lymphocytes retrieved in the BAL fluid
Prior to transbronchial biopsy, patients underwent a 300 cc saline lavage of their right middle lobe. Most of the white blood cells in the fluid that was retrieved were lymphocytes. Cytocentrifuge preparations of the cell pellet were prepared, and stained using an avidin-biotin immunoperoxidase technique for CD3. More than 90% of the lymphocytes stained for the CD3 antigen. Clusters of positively stained T-cells (black arrow), are seen amidst larger, nonstaining alveolar macrophages. Immunoperoxidase. Magnification ×450

positive responses with induration (> 10–20 mm), and erythema at the initial skin test sites were observed at 24 h, and persisted for more than 1 week during the T-cell infusions. Both of these patients showed a greater than 10 mm increase in their cutaneous skin test responses to *Candida* antigens after therapy. Skin test conversion to PPD responsiveness did not occur for any patient.

Toxicity

Infusions of long-term cultured T-cells were generally well tolerated. Fever was detected in one patient (A) who had been febrile, apparently due to his extensive tumor burden, prior to the initiation of immunotherapy. Nausea, without vomiting, was reported by three patients, all of whom were receiving narcotic analgesics, and was controlled with antiemetics. A mild decrease in pulmonary function parameters (up to 21% of vital capacity) was measured for all patients at the end of therapy (Table 4) without any signs of respiratory distress. Local reactions at the site of infusion were not observed. Serious hypercalcemia (> 12 mg/100 ml) occurred in two patients who had widespread boney disease and pathological fractures. These patients required treatment with vigorous saline diuresis and mithramycin. In one patient (C), calcium levels that had become elevated during T-cell infusions normalized within 3 weeks after the completion of therapy.

Tumor response to T-cell therapy

All patients had metastatic adenocarcinoma that was clinically and histologically consistent with a primary site in the lung. The histological grade of the tumors varied between well, moderately, and poorly differentiated cancer (Table 1). Tumor reduction following therapy was seen in five of seven patients, however, no patient achieved a complete or partial (> 50% reduction of total measurable tumor) response. Two of the seven patients (G and E) are alive at 3 and 9 months after treatment respectively.

In our first patient (A), two intralesional injections of T-cells into a large (11 × 10 cm) left-sided chest wall tumor were followed within 72 h by progressive softening at the injected sites and a ~20% decrease in the size of the tumor. No decrease in size was seen at sites within this tumor that had been injected directly with saline. No change was seen in the size of the underlying lung tumor, which completely filled the left hemithorax as judged by CT scanning. The patient died on the 5th day following the initiation of therapy. Biopsy of the subcutaneous tumor at limited autopsy revealed tumor cells undergoing necrosis and a lymphocytic infiltrate (Fig. 7). γ-Counting of biopsied tumor, and tissues of the contralateral chest wall that did not contain tumor, was performed 48 h after i.v. infusion of radiolabeled T-cells. These counts showed that radioactivity was concentrated within the tumor at a ratio of 3.2/1 above normal control tissue (1932 vs 601 cpm/g wet weight of tissue). This accumulation of radiolabel within tumors was not detected by external imaging.

B had an elevated, 7 × 5 cm, chest wall tumor that was contiguous with a metastatic lesion within an underlying rib. During the 2nd week of therapy, the mass became erythematous, warm, and painful, and then progressively diminished to less than 10% of its original size over the next week. During this time, the patient was afebrile and did not have an elevated white blood cell count. An opacified region in the left lower lobe on chest film, which was believed to contain the primary tumor, was reduced after therapy. Tumor progressed in bone, as judged by CT scanning, and he eventually succumbed to complications of pathological fractures, hypercalcemia, and renal failure.

D achieved complete regression of two (3 × 2 cm and 2 × 2 cm) rapidly enlarging, subcutaneous tumor nodules during therapy but no change in the size of several large liver metastases as judged by ^{99}technetium colloid liver-spleen imaging. He received monthly infusions of T-cells with stabilization of his tumor for 4 months, but then developed rapidly progressive disease in lymph nodes and lung.

G had a dramatic decrease in the radiographic appearance of a right mid lung metastatic pulmonary nodule (Fig. 8), and an apparent decrease in the size of a sternal lesion as judged by bone scanning. Her tumor progressed in other bones, brain, and breast 1 month following treatment. A breast biopsy showed infiltrating mucinous adenocarcinoma that was histologically consistent with her original tumor. The breast tumor was estrogen receptor negative. It was unclear from the histology whether the breast tumor represented the primary or a metastatic site.

The size of a large right upper lobe primary that had been irradiated 2 months prior to starting therapy continued to decrease after immunotherapy in patient E. Her tumor status was stable for 5 months, however, she then developed synchronous brain and bone metastases. These have been treated with local irradiation.

C had extensive boney and intrapulmonary metastatic disease. Her immunotherapy was complicated by a pathological hip fracture that occurred in hospital just prior to starting treatment. Her disease continued to progress in several boney sites during immunotherapy and she died from complications of her widespread disease.

Table 4. Pulmonary function before and after T-cell therapy

Patient	Before therapy		After therapy	% Change
B	FEV_1 (L)	1.62 (50)	1.43 (40)	−12
	VC (L)	2.11 (51)	1.84 (45)	−21
	TLC (L)	3.15 (55)	2.99 (53)	− 5
	D_LCO (ml/min/mm HG)	12.80 (56)	10.40 (39)	−19
	pO_2	79	75	
	A−a	14	27	
C	FEV1	0.73 (24)	0.80 (26)	+10
	VC	0.92 (25)	1.04 (29)	+13
	pO_2	72	63	
	A−a	23	39	
E	FEV1	1.36 (50)	1.10 (40)	−19
	VC	1.78 (53)	1.40 (42)	−21
	TLC	3.17 (62)	2.62 (51)	−17
F	FEV1	1.83 (72)	1.71 (60)	−17
	VC	2.16 (67)	1.86 (59)	−14
	TLC	4.41 (88)	3.71 (76)	−16

Numbers in parentheses represent percent predicted for age, height, and weight. All blood gas determinations were obtained breathing room air. The A−a gradient for oxygen was calculated by the following formula: FiO_2 (760) − (47) − pCO_2/0.8. Three patients were unable to complete testing because of inability to perform test maneuvers

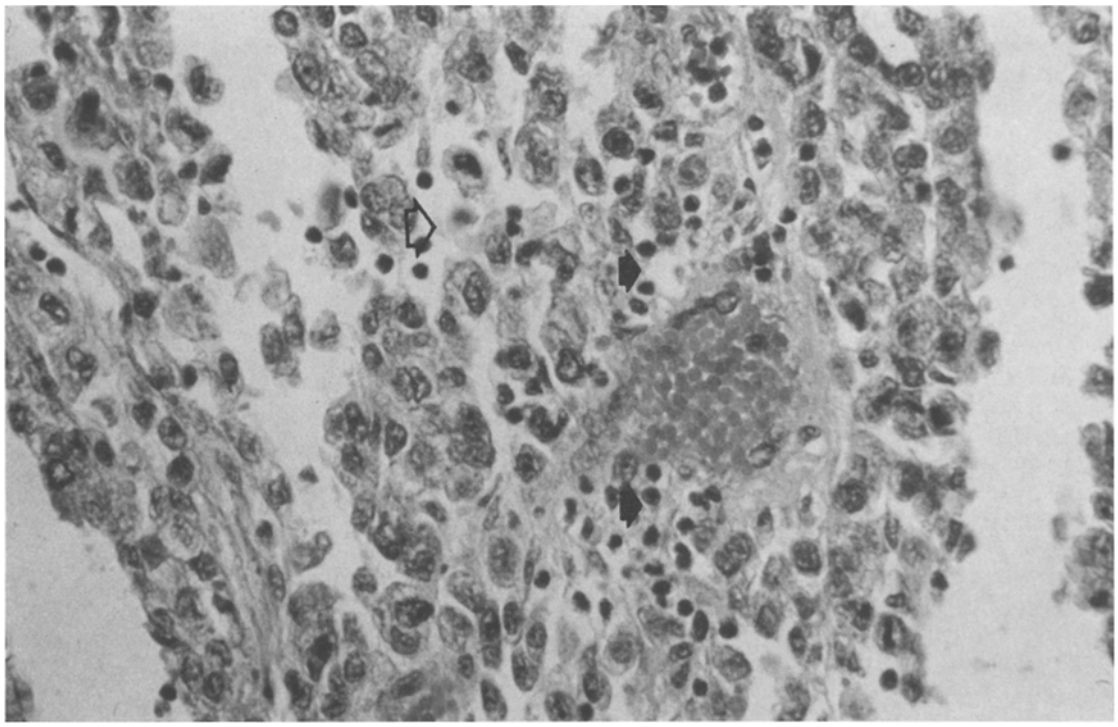

Fig. 7. Biopsy of patient tumor following intralesional and i.v. infusions of tumor-derived T cells
Patient A received intralesional injections of T-cells into a large subcutaneous chest wall mass as well as several i.v. infusions of T-cells. His tumor decreased in size, and was biopsied 5 days after the first injection. The histology of the biopsy shows poorly differentiated tumor infiltrated by lymphocytes (black arrows). Multiple foci of tumor cell necrosis (white arrow) were present within the biopsy. Increased numbers of radioactive counts were detected in this biopsy after the infusion of radiolabeled cells. Hematoxylin and eosin. Magnification $\times 400$

F died from a rapidly progressing, idiopathic pneumonia that began in the portal of previous pulmonary irradiation completed 8 months prior to her death, and 3 weeks after her course of immunotherapy had ended. An open lung biopsy showed nonspecific changes including the presence of interstitial and intraalveolar fibrin and fibrosis, consistent with the proliferative stage of diffuse alveolar damage. Bacterial, viral, and mycoplasma cultures failed to reveal an infectious agent. Tumor cells were not present within the lung biopsy. A small pericardial effusion that had been noted prior to beginning immunotherapy was sampled and found to contain adenocarcinoma cells.

No consistent changes in CBC, serum enzymes, ESR, or carcinoembryonic levels were seen following therapy in these patients.

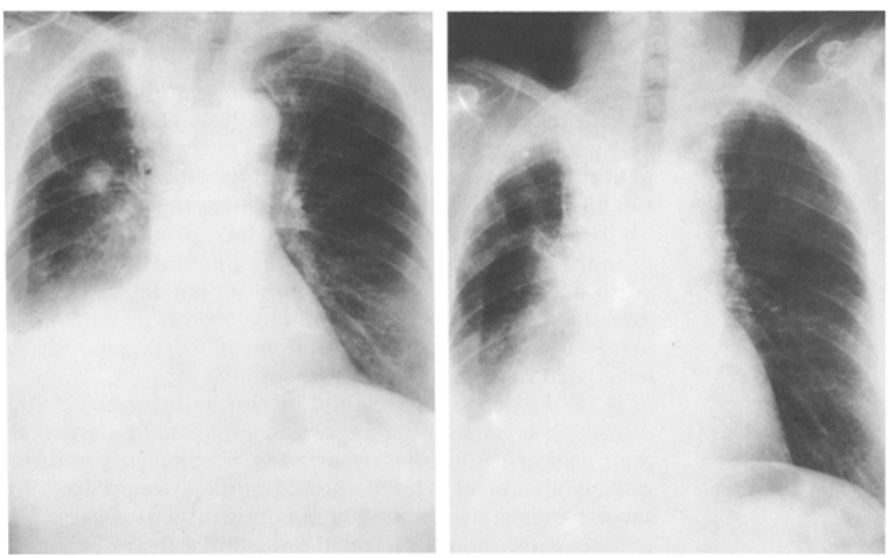

Fig. 8. Tumor response in patient G following infusions of tumor-derived IL-2-dependent T-cells
Posterior-anterior X-ray views of the chest in a patient with metastatic lung cancer shows the presence of a right apical abnormality and a right mid-lung nodule prior to the initiation of immunotherapy (left). After completion of immunotherapy, the mid-lung nodule was greatly diminished in size. Tumor progressed, however, at other sites

Discussion

We have previously reported that immunocompetent T-lymphocytes can be isolated and propagated directly from cancer tissues by culturing tumor explants in the presence of IL-2. In the present study, we have shown that autologous, long-term cultured T-cells can be safely transferred into cancer patients, and may mediate the reduction of advanced tumors. The adoptive transfer of autologous T-lymphocytes alone, however, did not produce clinically significant responses (> 50% reduction in total disease) in any patient. It is possible that combination of T-cells and systemic IL-2 may prove to be more effective in this regard [39].

The long-term cultured lymphoid cells administered in these studies were T-cells as judged by their expression of CD3, CD4, or CD8 antigens on the cell surface. Long-term IL-2-dependent cultures from tumor and peripheral blood showed much smaller numbers of HNK-1$^+$ and CD16$^+$ cells, which are associated with the presence of large granular lymphocytes and NK cell activity [26]. T-cells isolated from the tumor explants expressed IL-2 receptors, class II MHC antigens, and proliferated in the presence of exogenous IL-2. These findings indicate that these lymphoid cells represent the expanded progeny of T-cells which had been activated in vivo, possibly in response to autologous tumor-associated antigens. This is supported by the finding that T-cells cultured from tumors in IL-2 were specifically enriched for cytotoxic specificities against autologous tumors in one of two cases examined. Vanky et al. have suggested that the presence of autologous tumor-reactive lymphocytes in situ may have positive prognostic implications [44].

In contrast to the dominance of CD8$^+$ cells that we had previously observed in lung cancer-derived T-cells [24], five of the present seven cases displayed larger numbers of CD4$^+$ cells in culture. In our earlier report, tumor was obtained by lobectomy and malignant tissue was harvested directly from the resected specimen and cultured in IL-2. It is possible that tissues sampled via bronchoscopy, or from sites of metastatic tumor, may preferentially yield growth of CD4$^+$ cells. Most of the patients in the current series had larger cumulative tumor burdens than the subjects in our earlier report. It remains to be determined whether factors such as tumor burden and duration of disease can influence the phenotype of activated T-cells within tumors. Examination of larger numbers of cancer specimens will be required to answer this question.

The lymphoid cells that were infused contained few, if any, contaminating monocytes, B-cells or null cells as judged by flow cytometric surface phenotype analysis of the cells. The absence of contaminating monocytes, which are a source of interleukin-1, from the infusions, may explain our failure to observe fever in this series of patients. Fever was encountered in all patients that received infusions of lectin-activated peripheral blood mononuclear cells in another adoptive therapy trial of cancer [32].

Radiolabeled T-cells were demonstrated to distribute primarily to lung, liver, and spleen. Most radiolabeled cells were present in the lungs within 4 h of infusion. Thereafter, radioactivity in the lungs decreased, but simultaneously increased in liver and spleen. These results are in agreement with studies by Lotze et al. who examined the distribution of radiolalabeled long-term cultured T-cells [28] but contrast with reports of adoptive transfer of PHA-activated (PAK) lymphocytes in patients with cancer [32]. In the latter study, it was noted that little activity was present within the lungs at 24 h after i.v. infusion of labeled cells, with virtually all of the activity present in liver and spleen. Intrapulmonary activity was noted to increase progressively for patients, after they had received multiple, nonlabeled, PAK cell infusions.

The factors which determine the characteristic distribution of long-term cultured T-cells are not known. The early distribution of radiolabeled T-cells primarily in lungs, with lesser amounts in liver and spleen does not correspond directly to the distribution of blood flow, nor to the distribution of other radiolabeled leukocytes [33]. The number of transferred radiolabeled cells in the circulation is markedly reduced by as early as 5 min after infusion, indicating that most of the T-cells are sequestered in tissues after the first passes through the circulation. It is unlikely that T-cells are selectively removed from the circulation as a result of membrane injury that might occur as a result of radiolabeling. T-cells remain viable after labeling and continue to exclude trypan blue and proliferate in response to IL-2 (data not shown).

Activated T-cell blasts express a sequence of antigens that are not observed on resting T-lymphocytes. These include the c-myc antigen [20], IL-2 receptors [15], transferrin receptors [43], and class II MHC antigens [10]. Activated T-cells fail to display the MEL-14 glycoprotein [13], or its human analogue the Hermes-1 antigen [19]. Loss of the MEL-14 antigen in the mouse, or Hermes-1 in man, is associated with diminished binding by T-cells to high endothelial venules and failure to achieve hematolymphatic circulation. Diminished circulation of long-term cultured T-cells has been suggested to be a potential obstacle to the therapeutic success of adoptive transfers in metastatic cancers [8]. We observed that the intrapulmonary distribution of radiolabeled cells to sites of tumor may be specifically diminished. In the lung, localized hypoxic pulmonary vasoconstriction could help to account for decreased perfusion to the tumor. Neoplasms in the lung are nourished by the systemic rather than the pulmonary circulation, a fact that could interfere with the distribution of large numbers of i.v. transfered T-cells to tumor. The observation that liver metastases may also receive diminished numbers of infused lymphocytes raises questions as to whether the abnormal vascular microenvironment of tumors may also limit migration of T-cells.

The presence of transferred long-term cultured lymphocytes in a variety of organs has been reported by Cheever et al. [7]. They detected cultured cells in lymph nodes and peritoneal exudates of mice as late as 120 days after adoptive transfer. Some of these cells displayed specific immunological memory for tumor antigens in vitro. Cheever et al. [6] and Eberlein et al. [9] have shown that long-term cultured lymphocytes are effective at reducing the size of tumors present at a distance from the sites of inoculation. Thus, failure of large numbers of cultured T-cells to migrate to sites of tumor does not mean that adoptive therapy will be ineffective. Indeed, it is noteworthy that we were unable to correlate the localization of radiolabeled lymphocytes on scans with sites of tumor reduction. This finding favors a mechanism of tumor reduction in vivo by which antitumor effector cells in the host are recruited by smaller numbers of transfered T-lymphocytes.

The presence of increased numbers of T-lymphocytes

within the BAL fluid after infusion indicates that the infused T-cells can exit through the pulmonary microvasculature. Lung biopsies showed lymphocytic infiltrates and alveolar septal injury as judged by the presence of episeptal and intraalveolar fibrin. These findings were accompanied in all cases by a modest decrease in pulmonary function, as reflected by diminished lung volume, diffusing capacity for carbon monoxide, and arterial pO_2. Microvascular injury, extravascular fluid accumulation, and respiratory distress have been regularly observed after infusions of high doses of IL-2 in the absence of transfered T-cells [29]. It is uncertain as to whether a toxic effect of exogenous IL-2 alone, or a secondary effect of cells activated by IL-2 in vivo, produces microvascular injury and subsequent increase in vascular permeability. Based on our preliminary observations it appears that activated T-lymphoblasts alone may be capable of mediating microvascular injury directly. The mechanism of T-cell mediated vascular injury is unknown.

A variety of lymphoid populations have been reported to lyse tumor in vitro. Human T-lymphocytes, as judged by their expression of the CD3 antigen and genomic rearrangement of DNA encoding the antigen receptor, appear to be capable of mediating antitumor effects via two distinct mechanisms. Classical cytotoxic T-cells recognize tumor-antigen restricted by class I or class II HLA antigens that are expressed on the surface of some tumor cells [2, 36]. T-cells cultured in IL-2, however, can be activated to lyse tumors via mechanisms that are not MHC-restricted. T-cells activated by IL-2 kill a wide panel of fresh and long-term cultured syngeneic and allogeneic tumor targets [16]. The distinct increase in non-MHC restricted aggression by lymphocytes following activation in IL-2 has not yet been explained. Finally, a subset of granular $CD3^-$ lymphocytes that do not exhibit rearrangements of the genes encoding for the T-cell receptor can lyse certain tumor targets in a non-MHC restricted manner. Clones of these so-called "NK" cells also proliferate in IL-2 [1] and display augmented nonspecific tumor killing in response to γ-interferon [46].

The phenotype and mechanism of tumor cell recognition by the lymphoid cells which mediate the phenomena of lymphokine-activated killing (LAK) remains to be fully defined. LAK cells have been reported to derive from $CD3^-$ $CD2^-$ precursors that are $CD16^+$ and $CD11^-$ [17] indicating that they may not be of thymic lineage. Following stimulation by IL-2, LAK cells may remain $CD3^-$, although reports concerning the surface phenotype of the LAK effector cell have varied [37]. We and others [24, 25] have shown that allogeneic killing of NK-resistant tumor targets, as well as killing of NK targets, can be mediated by populations of IL-2-activated $CD3^+$ $CD2^+$ lymphocytes. This indicates that cytotoxic activities may be widely shared by phenotypically diverse lymphoid cells.

The mechanism of tumor reduction following adoptive transfer of T-cell blasts in vivo is uncertain. Enhanced skin test responses to protein antigens following adoptive immunotherapy appear to reflect systemic augmentation of delayed-type hypersensitivity responses. The principal mode of tumor reduction after adoptive transfers of lymphocytes may be via enhanced delayed-type immune responses, rather than direct cytotoxic activities of the transferred cells [25]. Murine studies have demonstrated a requirement for the presence of a $Lyt1^+2^-$ population in the reduction of tumor in vivo [11]. If noncytotoxic, $CD4^+$ cells are needed to mediate tumor reduction in the human, this may reflect requirements for T-cell help in immunoglobulin production, amplification of cytotoxic lymphocyte responses, or initiation of delayed-type hypersensitivity responses. In each of these instances, interaction with other competent effector cells of the host is required. The immunosuppressive effects of combination chemotherapies, or steroids, would be expected to adversely influence antitumor responses to adoptive immunotherapy. A potential role may exist, however, for combining adoptive immunotherapy with selective chemotherapeutic or biological response modifiers [5].

We conclude that long-term cultured T-cells can effect systemic immunomodulation and mediate tumor reduction in vivo. Sensitized, tumor-derived T-cells may prove more effective than peripheral blood lymphocytes at reducing tumor in vivo in humans as they have in certain murine systems [41]. Although no clinical responses were achieved in this trial, it is possible that the combination of systemic IL-2 with tumor-derived T-cell infusions may promote further increases in antitumor responses in vivo. Based on these hypotheses, we have initiated a trial of tumor-derived T-cells and systemic IL-2 in patients with non-small cell cancers of the lung.

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