Report of a Clinical Trial in 12 Patients With Head and Neck Cancer Treated Intratumorally and Peritumorally With Multikine

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Background: There is cumulative evidence suggesting that cells of the immune system recognize and may participate in eradicating neoplastic cells. As a result, immune modulation, first with interleukin 2 and later with other cytokines, has been tried in the clinical setting as part of antitumor therapy.

Objective: To examine the effectiveness and toxicity of a combination of natural interleukins in patients with squamous cell head and neck cancer.

Methods: Twelve previously untreated patients with various head and neck cancers were treated by peritumoral injection of a combination of cytokines (Multikine), in addition to zinc sulfate, indomethacin, and a single dose of cyclophosphamide, which were administered systemically. Response was evaluated clinically and histopathologically. T-lymphocyte determinants were studied by fluorescence-activated cell sorter analysis (against controls).

Results: Two patients showed complete regression and another 2 showed partial regression. There were no serious adverse effects of treatment. Pathological study results showed tumor fragmentation and the appearance of multinucleated macrophages. Fluorescence-activated cell sorter analysis showed lymphocyte activation, reflected by an unusually high number of cytotoxic T-lymphocyte activation 4 cells and natural killer cells.

Conclusion: Multikine warrants further investigation for inclusion in the pharmacotherapeutic armamentarium of head and neck cancer.

recombinant IL-2. Hadden5,6 reported tumor regression from one to three cycles of 800 U peritumoral injections of Multikine, to complete regression of 2 lip cancers after local injections of recombinant IL-2 at the perilymphatic approach.22 Saito et al, in the Japanese literature,22 reported on the complete regression of recurrent head and neck tumors with local injections of a mixture of natural ILs, zinc, and cyclophosphamide, and asked about the degree of tongue mobility and pain.23-25 In clinical situations, patients with carcinoma of the colon and breast who were treated with intratumoral and peritumoral IL injections showed an immediate elevation in neutrophil count and an increase in NK cell activity on long-term follow-up.15 Intratumoral injection has also been associated with cellular infiltration of the tumor, consisting mainly of polymorphs and macrophages that form centers of necrosis.7,12 The necrosis may be confluent, producing a ballooning effect.15,19 In 2 studies,20,21 endolymphatic administration of ILs caused an activation of the regional nodes. There were also indications of widespread activation of lymphoid tissue, which may result in an increase in host resistance against tumor cells and the metastatic process.20,21 Regarding head and neck cancer, the effect of immune-enhancing treatment has been investigated in only a few patients. Cortesina et al22 suggested that temporary regression of recurrent head and neck tumors may be achieved with low doses of recombinant IL-2, and Saito et al, in the Japanese literature,22 reported on the complete regression of 2 lip cancers after local injections of recombinant IL-2. Hadden5,6 reported tumor regression during the past 10 years with intravenous and perilymphatic administration of a mixture of natural ILs, zinc, cyclophosphamide, and indomethacin.

The present study examines the toxicity and effectiveness of the peritumoral and perilymphatic administration of a pharmaceutical preparation of mixed natural ILs (Multikine) in patients with squamous cell head and neck cancer.

### METHODS

#### PATIENTS

Twelve patients with previously untreated head and neck carcinomas were included in this clinical trial. The primary site of the lesions is presented in Table 1. Inclusion criteria were as follows: older than 18 years, histological diagnosis of squamous cell carcinoma on surgical or fine-needle aspiration biopsy specimen, and an expected survival of at least 6 months.

Patients who were pregnant, had received radiation to the same site, or had a gastric or duodenal ulcer were excluded. Patients proved anergic by a skin test result were also excluded because we assumed they lacked the potential to respond to immune stimulation by Multikine, and patients allergic to ciprofloxacin were excluded because the Multikine solution contains ciprofloxacin as a protection against bacterial contamination. All participants signed an informed consent form in accordance with the recommendations of the Declaration of Helsinki (1985). The study was approved by the Rabin Medical Center Review Board.

#### PATIENT EXAMINATION

General assessment before treatment included obtaining a medical history, performing a physical examination and a hematological and blood chemistry workup, electrocardiography, chest cardiography, and obtaining a computed tomographic scan of the head and neck. A picture of the tumor was also taken, when possible. Tumor size was determined by measuring the 2 major perpendicular diameters. The patients were interviewed daily and asked about the degree of tongue mobility and pain.

#### TREATMENT

A Multikine solution (Cel Sci Corporation) was injected peritumorally (patients 1-11) or perilymphatically (patient 12) for 10 days. The manufacturer lists the following components in the Multikine solution: IL-2, IL-1α, IL-1β, granulocyte-macrophage colony-stimulating factor, interferon α, tumor necrosis factor α, tumor necrosis factor β, IL-3, IL-4, IL-6, IL-8, IL-10, macrophage inflammatory protein 1α, and granulocyte colony-stimulating factor. For the peritumoral approach, the total quantity of 2 or 4 mL was divided into 4 equal parts and injected at 4 equidistant points along the periphery of the tumor to a depth of 5 mm. For the perilymphatic approach, the total quantity of 4 mL was divided and injected at 4 arbitrary points at the periphery of the mastoid tip adjacent to the jugulodigastric nodes. Ten patients (patients 1-10) received 800 U, and 2 (patients 11-12) received 1600 U, at a concentration of

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<table>
<thead>
<tr>
<th>Patient No./Sex/Age, y</th>
<th>Site of the Primary Tumor</th>
<th>Grade</th>
<th>Dose, mL</th>
<th>Clinical Response†</th>
<th>Outcome</th>
<th>Follow-up, mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/F/61</td>
<td>Hard palate</td>
<td>Mod.</td>
<td>2</td>
<td>None</td>
<td>AWD</td>
<td>28</td>
</tr>
<tr>
<td>2/M/73</td>
<td>Tongue</td>
<td>Mod.</td>
<td>2</td>
<td>Partial</td>
<td>AWD</td>
<td>16</td>
</tr>
<tr>
<td>3/M/82</td>
<td>Hard palate</td>
<td>Well</td>
<td>2</td>
<td>None</td>
<td>AND</td>
<td>20</td>
</tr>
<tr>
<td>4/M/69</td>
<td>Retromolar triangle</td>
<td>Mod.</td>
<td>2</td>
<td>Complete§</td>
<td>Dead</td>
<td>20</td>
</tr>
<tr>
<td>5/F/77</td>
<td>Tongue</td>
<td>Mod.</td>
<td>2</td>
<td>None</td>
<td>Dead</td>
<td>7</td>
</tr>
<tr>
<td>6/M/73</td>
<td>Lip</td>
<td>Well</td>
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<tr>
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<td>Mod.</td>
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<td>Partial</td>
<td>AND</td>
<td>20</td>
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<td>AND</td>
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<tr>
<td>9/F/89</td>
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<td>None</td>
<td>AND</td>
<td>18</td>
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<td>Retromolar triangle</td>
<td>Mod.</td>
<td>2</td>
<td>None§</td>
<td>AND</td>
<td>18</td>
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<tr>
<td>11/M/79</td>
<td>Tongue</td>
<td>Well</td>
<td>4</td>
<td>Partial</td>
<td>AND</td>
<td>16</td>
</tr>
<tr>
<td>12/M/82</td>
<td>Unknown</td>
<td>NA</td>
<td>4</td>
<td>Complete</td>
<td>AND</td>
<td>12</td>
</tr>
</tbody>
</table>

Abbreviations: AND, alive with no evidence of disease; AWD, alive with disease; Mod, moderately differentiated squamous cell carcinoma; NA, data not applicable; Well, well-differentiated squamous cell carcinoma.

*All patients had a T2 N0 M0 tumor, except for patient 5 (who had a T2 N0 M1 tumor).

†This patient had a second primary tumor in the oral cavity.

‡This patient developed Wegener granulomatosis.

§This patient had developed Wegener granulomatosis.

*This patient had a second primary tumor in the oral cavity.
Patients were advised to undergo either surgery or radiation as definitive treatment at 21 days after Multikine treatment. Blood count and levels of electrolytes and enzymes were recorded. All adverse reactions during the study were also documented. Patient response was categorized as follows: complete regression, complete tumor regression documented clinically and histologically; partial regression, tumor shrinkage of at least 50% of its original size according to the physician’s examination; and no response, tumor shrinkage of less than 50% of its original size. Tumor regression was recorded as absent or present. We also attempted to evaluate the degree of regression based on the presence of keratin remnants.

### POSTOPERATIVE CLINICAL ASSESSMENT

Patient response was categorized as follows: complete regression, complete tumor regression documented clinically and histologically; partial regression, tumor shrinkage of at least 50% of its original size according to the physician’s examination; and no response, tumor shrinkage of less than 50% of its original size (Table 1). Tongue mobility and patient well-being were also documented. All adverse reactions during the study were recorded. Blood count and levels of electrolytes and enzymes were evaluated before and after Multikine treatment.

### HISTOPATHOLOGICAL ASSESSMENT

In 11 patients, the diagnosis was based on the results of a surgical biopsy of the primary tumor, and in 1 patient (with a neck node metastasis of unknown origin), on the results of fine-needle aspiration biopsy and quadroscopy. Posttreatment samples were available for histopathological analysis in 10 patients. Sections (4-µm thick) from the paraffin-embedded specimens were stained with hematoxylin-eosin. The tumors were graded according to the degree of differentiation (well differentiated, moderately differentiated, and poorly differentiated). Lymphocytes, macrophages (mononucleated and multinucleated), plasma cells, granulocytes, and eosinophils were graded as follows: 0, no infiltration; 1, low infiltration; 2, moderate infiltration; and 3, heavy infiltration. Special note was taken of the extension of inflammatory cells into the tumor. Tumor necrosis and fragmentation, stromal edema, and stromal fibrosis were graded from 0 to 3 (0, absent; 1, mild; 2, moderate; 3, prominent). Tumor regression was recorded as absent or present. We also attempted to evaluate the degree of regression based on the presence of keratin remnants (Table 2).

### FLOW CYTOMETRY ANALYSIS

Of the 12 patients, 8 were studied for various T-lymphocyte determinants by fluorescence-activated cell sorter (FACS) analysis. Human peripheral blood mononuclear cells were obtained by density centrifugation (Ficoll-Hypaque method) and washed and suspended in phosphate-buffered saline. Aliquots of 0.5 × 10⁶ cells per test tube were used. The cells were incubated with the following monoclonal antibodies: CD3, CD4, CD8, CD56, CD28, cytokotoxic T-lymphocyte activation 4 (CTLA-4), CD25, and Burkitt lymphoma cell membrane activated T cells (BAT: Pharmingen, San Diego, Calif) for 30 minutes at 4°C. After washing and centrifugation for 5 minutes at 1200 rpm with phosphate-buffered saline, paraformaldehyde was added for fixation. Cell labeling was performed by incubation with anti–mouse fluorescein isothiocyanate (Jackson, Immunoresearch Labs, Palo Alto, Calif). Mouse anti–human IgG or anti–mouse fluorescein isothiocyanate was used as an isotope control for the monoclonal antibodies. Cells were analyzed by FACS analysis (FACScan; Becton Dickinson Microbiology Systems, Cockeysville, Md). The results of FACS analysis of 6 patients with head and neck cancer who had not received Mul-

### Table 2. Histopathological Analysis of Pretreatment and Posttreatment Specimens

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Period</th>
<th>Diff</th>
<th>Edema*</th>
<th>Necrosis*</th>
<th>Lymphocytic Infiltrates*</th>
<th>Plasma Cells*</th>
<th>Granulocytes*</th>
<th>Eosinophils*</th>
<th>Macrophages*</th>
<th>TR, %</th>
<th>Fibrosis*</th>
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<td>2</td>
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<td>0</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>Mod</td>
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<td>1</td>
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<td>2</td>
<td>0</td>
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<td>0</td>
<td>0</td>
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</tr>
<tr>
<td></td>
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<td>2</td>
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<tr>
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<td>1</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
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<td>1</td>
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</tr>
<tr>
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<td>Post</td>
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<tr>
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<td>NA</td>
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<td>Mod</td>
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<td>Mod</td>
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<td>NA</td>
<td>NA</td>
<td>CR</td>
<td>1</td>
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</tr>
</tbody>
</table>

*See the “Histopathological Assessment” subsection of the “Methods” section for a description of the grading for these categories.

Abbreviations: CR, complete remission; Diff, degree of differentiation; Mod, moderately differentiated squamous cell carcinoma; NA, data not available; Post, after treatment; Pre, before treatment; TR, tumor regression; Well, well-differentiated squamous cell carcinoma.
CLINICAL RESULTS

Of the 12 patients, 9 had a primary lesion in the oral cavity and 2 had skin cancers (squamous cell carcinoma) of the head and neck area. One patient was treated for a neck metastasis of unknown origin. Ten patients underwent surgical treatment after Multikine injection, and 2 (patients 1 and 5) refused surgery and underwent a biopsy only.

Of the latter 2 patients, patient 1, who underwent partial reduction of a palatal tumor by laser, is alive at 30 months after the Multikine regimen, with persistent disease confined to the hard palate. Patient 5, who had tongue cancer, showed a reduction in tumor size in response to Multikine, but was unable to be located for follow-up and died 7 months later of unknown causes.

The other 7 patients with oral cavity tumors showed various degrees of response to treatment. One patient (patient 4) had complete tumor regression clinically and histologically, and 2 patients (patients 2 and 11) had a partial response clinically and pathologically. Additional responses noted by the treating physician (R.S.) and reported by another 4 patients after Multikine treatment included flattening of the tumor, increased mobility of the tongue, and a reduction in pain. Patient 4, whose primary tumor completely regressed, also had a neck node metastasis that did not respond to peritumoral Multikine injections; this was treated with neck dissection followed by radiation. The patient died 12 months later of sepsis associated with marked cervical and bilateral parotid enlargement. Biopsy specimens obtained from one of the parotid glands and from a lymph node diagnosed the patient as having a granulomatous disease. There was no evidence of recurrence of the primary tumor or of neck metastases at death. Patient 6 (Figure 1) underwent surgical removal of a lip tumor, and although 2 margins were microscopically involved, he refused additional surgery or radiation. At present, 24 months postoperatively, there is no evidence of tumor recurrence or persistence. The 2 patients with skin cancer (patients 8 and 9) failed to show a significant response to treatment. Patient 12, with a neck metastasis of unknown origin, was treated by a perinodal injection of Multikine. He showed complete tumor regression clinically and histologically, and major regression on a computed tomographic scan (Figure 2). The patient underwent a neck dissection. A histopathological analysis showed replacement of the metastasis by fibrosis. One patient (patient 10) had a second primary tumor in the palate 1 year after initial treatment. He received radiotherapy as definitive treatment, and is healthy, with no evidence of disease in either site.

Overall, of the 12 patients in the study group, 10 are alive, 1 with persistent disease and 1 after treatment for a second primary tumor (this patient is healthy). One of the patients who died had no evidence of tumor at death (but had cervical lymphadenopathy, consistent with a granulomatous disease). There are no details available concerning the state of disease in the second patient participating in the study who died, because he was unable to be located for follow-up.

LABORATORY RESULTS

There were no obvious effects of the protocol on routine laboratory variables (electrolyte levels, liver function, and hemoglobin level) either during or after treatment. There was, however, an elevation in total white blood cell count, from a mean of 245 to 100/µL after treatment. This difference was statistically significant ($P<.05, \chi^2$ test). One patient with type 2 diabetes mellitus showed a decrease in fasting blood glucose levels, from a mean of 245 to 100 mg/dL (13.6-5.5 mmol/L).

HISTOPATHOLOGICAL RESULTS

Biopsy samples of 9 patients were analyzed before and after treatment (the 2 skin lesions were excluded). The patient with a neck metastasis of unknown primary origin was only partially evaluated. Four had well-differentiated carcinoma, and 6 had moderately differentiated carcinoma. Before treatment, fibrosis, necrosis, and stromal edema were not prominent, but lymphocytic infiltrates to various extents were present in all patients (Table 2 and Figure 3A). The infiltrates were found mainly in the stroma, and only focal infiltration into the tumor itself was noted.

After treatment, 2 patients (patients 4 and 12) showed complete regression, 2 (patients 2 and 11) showed...
partial regression, and 1 (patient 3) had a clear focus of regression. (In patient 4, the primary tumor regressed, whereas in patient 12, the neck metastasis disappeared.) An increase in the degree of lymphocytic infiltration was noted in 5 patients (Table 2 and Figure 3). The infiltrate also contained plasma cells, neutrophils, and eosinophils, as in the pretreatment specimens. Of special interest was the new posttreatment appearance of up to 5 multinucleated macrophages per high-power field in the patients with significant tumor regression (Figure 3B). The multinucleated macrophages were detected in 2 specific locations, namely, around the keratin debris and in the tumor-stroma interface (Figure 4), and they seemed to be actively engulfing the tumor cells. In 1 patient (patient 1), up to 3 multinucleated cells per high-power field were found in the pretreatment and post-treatment biopsy specimens. Although there was no evidence of major clinical tumor regression, this patient, who refused definitive surgery, remained healthy, without significant tumor growth throughout 30 months of follow-up. Stromal fibrosis, which was not present in any of the pretreatment biopsy specimens, was noted mainly in tumors showing regression, seeming to replace the tumors.
FACS ANALYSIS OF T-LYMPHOCYTE DETERMINANTS

A comparison of FACS analysis results of peripheral blood mononuclear cell samples from 6 patients with head and neck tumors who had not received Multikine treatment with patients after Multikine treatment reveals a dramatic change in the general appearance of the cell population (Figure 5). The post–Multikine treatment blood samples show a new distinctive population composed of highly scattered and granular cells, representing monocytes, macrophages, and neutrophils (Figure 5A). This population is nearly missing in the pretreatment group (Figure 5B).

Figure 6 summarizes the results obtained by FACS analysis of specific subpopulations in peripheral blood mononuclear cells from 8 of the patients treated by our protocol and compared with 6 patients with head and neck cancer who were not participating in our study (Figure 6). The T-lymphocyte (CD3-positive) count was maintained at a normal average rate of 65%, in contrast to the high count of NK cells (79% were CD56 positive) in the posttreatment blood samples, compared with controls (50%). The CD4/CD8 ratio was 1:1 (30% and 39%, respectively). CD28 and CTLA-4 are T-lymphocyte activation determinants. While a normal percentage of CD28-positive cells was found in the peripheral blood of all 8 patients, 6 of them exhibited an exceptionally high expression of CTLA-4, compared with controls (none had any positive CTLA-4 reading). Lymphocytes from 4 of the 8 patients had more than 90% IL-2 receptor-positive T lymphocytes (CD25).

TOXICITY

No significant toxic effect of the treatment was registered in any of the patients participating in the study during or after treatment. Two patients complained of minor adverse effects, namely, headaches and palpitations, but it was unclear if they were related to the Multikine treatment. It was also unclear whether the development of sepsis and Wegener granulomatosis in patient 4 was connected to the Multikine treatment.

COMMENT

Recent findings of a depressed lymphoproliferative response to mitogens,23-27 depressed IL-2 production,28 and a depressed cutaneous reaction to recall antigens24,25,27 in patients with head and neck cancer all point to the presence of a depressed immune status. Studies29,30 using mouse patients with head and neck cancer all point to the presence of otherwise lethal tumor challenges. Whether this is related to Multikine treatment through stimulation of lymphocytes by ILs to an abnormal degree of proliferation cannot be completely ruled out. In another patient (patient 9), a second tumor developed in the oral cavity (alveolar ridge) 1 year after Multikine treatment. This cannot be directly related to Multikine, because second primary tumors are known to occur in 15% of patients with head and neck cancer.3

TOXICITY

Previous studies50 have shown that, compared with the intravenous route, locoregional administration of IL-2 results in minimal or no adverse effects. This finding is supported herein by the absence of major toxic effects of treatment in any of the 12 patients. The minor adverse effects noted, mainly headaches and palpitations, were transient and could not be directly related to the administration of Multikine. One of our patients, who was included in the study because of a retromolar carcinoma, died of Wegener granulomatosis, which developed 10 months after surgery. Whether this is related to Multikine treatment through stimulation of lymphocytes by ILs to an abnormal degree of proliferation cannot be completely ruled out. In another patient (patient 9), a second tumor developed in the oral cavity (alveolar ridge) 1 year after Multikine treatment. This cannot be directly related to Multikine, because second primary tumors are known to occur in 15% of patients with head and neck cancer.3

EFFECT ON THE TUMOR

The tumors in our sample were not homogeneous, making it difficult for us to draw statistically sound conclusions. One patient with retromolar cavity cancer (pa-
Of the 12 patients treated with Multikine, 2 patients (patients 2 and 11) with oral cavity tongue cancers had a partial response by clinical and pathological criteria (50% of the tumor was replaced by fibrosis). The 2 patients with skin cancers showed no real response to treatment. The single patient with a neck metastasis of unknown origin (patient 12), treated by perilymphatic injection, also showed complete regression (ie, resolution of the metastatic neck node). Although the node was not palpable after Multikine treatment and before surgery, the patient underwent supraomohyoid neck dissection, and a subsequent histological analysis revealed a 1.5 × 1.5-cm node with multinucleated macrophages engulfing and eating keratin debris—evidence of the past presence of epithelial tumor cells.

To better understand the effect of Multikine on tumor behavior, the tumors were carefully inspected by an experienced pathologist (M.F.). Traditionally, head and neck tumors are not referred to as immunogenic, because they lack the heavy lymphocytic infiltration that is present in melanoma and renal cell carcinoma, the prototypes of the so-called immunogenic tumors. Nevertheless, most of the pretreatment biopsy specimens in our study showed evidence of lymphocytic infiltration (Table 2 and Figure 3A). Because alcohol consumption is not common in Israel, it...
is possible that our population with head and neck cancer is not subjected to the depressing effect alcohol has on the immune system. This may be the explanation for the lymphocytic infiltration in the pretreatment biopsy specimens. Based on our observation of accelerated tumor regression after Multikine treatment, we suggest that Multikine has the potential to activate peritumoral macrophages already present before treatment and, thereby, to facilitate tumor killing. The presence of multinucleated macrophages in most of the posttreatment, but not the pretreatment, biopsy samples supports this assumption. Further support is provided by the complete tumor regression in 2 patients and the regression of varying degrees in several other patients, all associated with the keratin debris being eaten by macrophages (Figure 5).

We propose that peritumoral injections of Multikine, besides affecting the primary tumor, may be absorbed by the abundant submucosal lymphatic system and transmitted into the circulation, possibly leading to a general systemic effect. This theory is in line with our FACS and peripheral blood analysis findings of an increase in white blood cells in the peripheral blood, a marked increase in NK cell count (79% were CD56 positive vs 50% of the controls) (Figure 6), and an unusually high number of CTLA-4 cells (which express a T-lymphocyte activation determinant) compared with the control group (Figure 6). While the increase in peripheral white blood cells may be attributable to inflammamatory response secondary to the injection rather than to a direct Multikine effect, this is probably not enough to explain the dramatic effect on CTLA-4. The elevation of CTLA-4 is probably the result of Multikine treatment, suggesting that Multikine may possess antitumor potential.

In conclusion, the present study, using histological and peripheral blood analyses, indicates that the peritumoral and perilymphatic administration of Multikine, in combination with zinc, indomethin, and cyclophosphamide, is relatively safe and has an effect on tumor regression, probably through the enlacement of macrophages and the activation of lymphocytes. Further studies are needed to substantiate these findings and to define the best dosage and mode of administration of Multikine in patients with head and neck cancer.

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