Cancer Immunol Immunother (1984) 18: 5–12

Cancer Immunology Immunotherapy

Original articles

Intrapleural administration of OK432 in cancer patients: Augmentation of autologous tumor killing activity of tumor-associated large granular lymphocytes

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Summary. *Ten patients with carcinomatous pleural effusions were treated with single intrapleural (i.pl.) injections of the streptococcal preparation OK432 on day 0 and the effects of i.pl. OK432 on the lysis of fresh or cryopreserved autologous tumor cells isolated from the pleural effusions were observed on day 7. In eight patients tumor cells in the effusions had decreased or disappeared by day 7. The other two patients, however, had no clinical evidence of therapeutic benefit from i.pl. OK432. Effusion tumor cells were relatively resistant to lysis by autologous lymphocytes when tested in a 4-h ^{51}Cr-release assay. Positive reactions were recorded for blood and effusion lymphocytes in two of ten untreated patients. Injection of OK432 i.pl. resulted in an induction or augmentation of cytotoxicity against autologous tumor cells and K562 in the effusions of seven of ten subjects by day 7. In contrast, autologous tumor killing activity of blood lymphocytes was not always modified by i.pl. OK432. Purification of large granular lymphocytes (LGL) by discontinuous Percoll gradient centrifugation enriched autologous tumor killing activity, with no reactivity in LGL-depleted, small T lymphocytes. Significant lysis of autologous tumor cells was observed with effusion LGL from seven of ten untreated patients. Seven days after i.pl. OK432 injection, effusion LGL expressed enhanced cytotoxicity against autologous effusion tumor cells, whereas T cells were still not cytotoxic to autologous tumor cells on day 7. The frequency of LGL among effusion lymphocytes was not altered by i. pl. OK432. Adherent effusion cells were not involved in lysis of autologous effusion tumor cells in either untreated or OK432-treated patients. In vitro treatment of blood and effusion lymphocytes with OK432 induced an enhancement of autologous tumor-killing activity in patients who subsequently responded to i.pl. OK432 treatment. OK432 augmented in vitro autologous tumor killing activity of LGL, whereas T cells failed to lyse autologous tumor cells even after in vitro activation with OK432. These results indicate that i.pl. administration of OK432 to cancer patients will result in an augmentation of autologous tumor killing activity of LGL in the pleural effusions, and that this could be responsible for the antitumor activity of i.pl. OK432 therapy.*

Introduction

In vitro cell-mediated cytotoxicity has been considered an expression of the host immune defense mechanisms. In the majority of studies on cytotoxicity of spontaneous and activated killer cells in cancer patients, cultured human tumor cell lines have been used as targets. It is difficult to interpret the data on cytotoxicity against cell line targets, however, since tumor cells aquire susceptibility to natural killer (NK) cells through growth in vitro [2, 4]. For the better evaluation of cytotoxicity of lymphocytes in cancer patients studies on autologous combinations of fresh effector and fresh target cells have been performed. Peripheral blood lymphocytes from approximately 25% of cancer patients have been demonstrated to be cytotoxic to autologous, freshly isolated tumor cells from solid neoplasms [9, 33–35, 37, 38] and from malignant effusions [1, 20, 28–30]. We have recently demonstrated that cytotoxic potential for autologous tumor cells is present in the peripheral blood and pleural effusions of cancer patients, and that it is strongly associated with a minor proportion of large granular lymphocytes (LGL) and restricted to the cell population that can lyse NK-sensitive K562 cells [20, 28, 30]. Similar observations have been made with tumor biopsy cells from solid tumors, although reactivity is also present in T lymphocytes [35]. Since fresh human tumor cells are relatively resistant to lysis by autologous unstimulated lymphocytes, several attempts have been made to induce or augment autologous tumor killing activity. The lysis of fresh human tumor cells has been observed with autologous blood lymphocytes activated in vitro by allosensitization [5, 18], by lectins [13], and by interleukin 2 (IL 2) [6, 7, 37].

OK432, a heat- and penicillin-treated lyophilized powder of the Su substrain of *Streptococcus pyogenes* A3, has been used as an immunomodulating agent and shown to have antitumor activity in cancer patients [22, 26]. Although the therapeutic usefulness of OK432 has been demonstrated, the mechanism responsible for its antitumor activity is not fully understood. Experimental animal studies have revealed that OK432 stimulates the cytostatic or cytotoxic activity of marcophages [14, 17], induces immune interferon (IFN) [12], and enhances NK cell activity [16]. In cancer patients administration of OK432 has been shown to result in an increase in lymphoproliferative response to mitogens and antigens [21, 22] and in NK cell activity of blood lymphocytes [26, 31]. Furthermore, in vitro treatment of blood lymphocytes and tumor-associated lymphocytes with OK432 has enhanced NK cell activity [3, 23, 31, 39] and autologous tumor killing activity [3, 29, 31] independently of IFN induction. Our previous studies have demonstrated that intrapleural (i.pl.) administration of OK432 to patients with carcinomatous pleural effusions induces an augmentation of effusion NK cell

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activity and a reduction of tumor cells in the effusions [26, 27]. We have suggested on the basis of these findings that OK432-activated NK cells may interact in vivo with tumor cells in the pleural effusions of patients who have received i.pl. OK432 treatment. For a better evaluation of the biologic role of NK cells and the antitumor activity of OK432, it seems of importance to ascertain whether OK432 administration augments the autologous tumor killing activity of LGL in cancer patients. The present study was designed to investigate the effects of i.pl. administration of OK432 on cytotoxicity against autologous effusion tumor cells in patients with carcinomatous pleural effusions.

Materials and methods

OK432. OK432 was supplied by Chugai Pharmaceutical Co., Tokyo, Japan. The 'KE' unit is used to express the strength of the preparation, 1 KE corresponding to 0.1 mg dried streptococci [21, 22].

Patients and treatment. Ten patients with carcinomatous pleural effusions were entered into this study. Histologic diagnosis revealed that eight patients had adenocarcinoma and two had squamous cell carcinoma of the lung. The patients, ranging in age from 33 to 72 years, had not received any anticancer agents at the time of the study. OK432 was administered i.pl. at a dose of 10 KE in 10 ml physiological saline on day 0, and clinical and immunological parameters were evaluated on day 7. No systemic treatment was given during the entire treatment period. Pleural effusions (500–1,000 ml) and peripheral blood (20–50 ml) were obtained from the patients on day 0 and 7 just before i.pl. injection of OK432. Specimens of peripheral blood from 20 healthy normal individuals were used as roughly age- and sex-matched controls.

Blood and effusion effector cells. Effector cells were prepared as described in detail elsewhere [24, 25, 27–29]. Lymphocyte-rich mononuclear cells were isolated from heparinized peripheral blood by centrifugation on Ficoll-Hypaque gradients. The mononuclear cells in the interface were collected, washed, and suspended in RPMI-1640 medium supplemented with 2 m*M* L-glutamine, 25 m*M* Hepes, 100 units penicillin/ml, 100 μg streptomycin/ml, and 10% heat-inactivated fetal calf serum (Gibco Bio-Cult, Glasgow, Scotland) (complete medium).

Specimens of pleural effusions were obtained from the patients by thoracentesis. Specimens were immediately centrifuged at 400 *g* for 5 min. The cells were washed, suspended at a concentration of 1×10^6/ml in complete medium, and layered on discontinuous gradients of 75% and 100% Ficoll-Hypaque. After centrifugation at 400 *g* for 30 min, lymphocyte-rich mononuclear cells were collected from the 100% interface, tumor cells and mesothelial cells from the 75% interface, and erythrocytes, polymorphonuclear cells, and aggregated tumor cells from the bottom. The procedure was repeated if the separation was not successful as judged by morphologic examination. Mononuclear cells having less than 5% tumor cells, as judged by morphologic examination of Wright-Giemsa-stained smears, were accepted for use.

The mononuclear cells were then incubated for 1 h at 37° C in plastic dishes precoated with fetal calf serum. After incubation, nonadherent cells were removed, and the dish was washed with cold medium. Adherent cells were harvested from the dish after 15 min incubation with Versene (1/5,000; Gibco) and by vigorous washing with a pipette, then washed and suspended in complete medium. The adherent cells contained more than 93% monocyte/macrophages, as judged by morphologic examination and nonspecific esterase staining. The nonadherent cells were passed through Sephadex G10 columns at 37° C to remove further contaminating monocyte/macrophages and tumor cells. The G10-passed lymphocytes were incubated in nylon-wool columns for 1 h at 37° C and eluted with warm complete medium.

The nonadherent lymphocytes were then fractionated by centrifugation on discontinuous Percoll density gradients, as previously described [19, 23, 28, 30]. Briefly, complete medium and Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) were adjusted to 285 mosmol/kg H_2O with distilled water and 10-fold concentrated phosphate-buffered saline, respectively. Seven different concentrations of Percoll in medium (40%–55%) were prepared by 2.5% increments. After layering of the gradients into 15-ml plastic tubes, 5×10^7 nonadherent lymphocytes were placed on the top of the gradients, and the tube was centrifuged at 550 *g* for 30 min. Cells from the seven layers were collected, washed, and suspended in complete medium. The cells from low-density fractions 2 and 3 usually contained more than 65% LGL, as judged by morphologic examination of Giemsa-stained cytocentrifuged smears. The cells from high-density fractions 6 and 7 usually contained less than 2% LGL. Every fraction was more than 96% viable according to the trypan blue dye exclusion test.

Effusion tumor cells. Cell suspension enriched for tumor cells obtained as described above were contaminated by mesothelial cells, monocyte/macrophages, and lymphoid cells. To eliminate these contaminating host nonmalignant cells, the cell suspension was layered on discontinuous gradients containing 4 ml each of 25%, 15%, and 10% Percoll in complete medium in 15-ml plastic tubes, then centrifugated at 25 *g* for 7 min, as previously described [28, 29]. Tumor cells depleted of lymphoid cells were collected from the bottom, washed, and suspended in complete medium. To remove residual contaminating mesothelial cells and monocyte/macrophages, the cell suspension was incubated for 30–60 min at 37° C in plastic dishes. After incubation, nonadherent cells were recovered, washed, and suspended in complete medium. The nonadherent cells usually consisted mainly of tumor cells, with less than 5% contaminating nonmalignant cells as judged by morphologic examination of Wright-Giemsa-stained smears, and were more than 93% viable according to the trypan blue dye exclusion test. Cells having less than 5% contamination with nonmalignant cells were accepted for use. Tumor cells were either used immediately or frozen according to a temperature-controlled freezing program and stored at $2–5 \times 10^6$/vial in 90% human AB serum plus 10% dimethyl sulfoxide in liquid nitrogen for further use. After rapid thawing, cells were gradually diluted to 10 ml with complete medium, centrifuged, and suspended in complete medium.

In vitro treatment with OK432 and IFN. Effector cells at a concentration of 1×10^6/ml in complete medium were preincubated alone or with 50 μg OK432/ml or 1,000 IU IFN/ml (human leukocyte IFN, specific activity 2×10^6 IU/mg protein, Immunoloski Zavod, Zagreb, Yugoslavia) for 20 h at 37° C in a humidified 5% CO_2 atmosphere, as described previously [23, 27, 29]. After incubation, the cells were harvested, washed, and suspended in complete medium.

Labeling of target cells. Freshly isolated effusion tumor cells (above) and the K562 human erythromyeloid leukemia cell line [10] were used as targets. Target cells were labeled by addition of 100 μCi $Na_2{}^{51}CrO_4$ (sp. act. 100–350 μCi/μg Cr; Radiochemical Centre, Amersham, Buckinghamshire, England) and incubated overnight at 37° C in a humidified 5% CO_2 atmosphere, as previously described [28, 29]. After incubation, the cells were washed four times, resuspended in complete medium, and further incubated for 3 h at 4° C. The cells were then washed twice and suspended at a concentration of 5×10^4/ml in complete medium.

Cytotoxicity assay. A 4-h ^{51}Cr-release assay was performed as described in detail elsewhere [20, 21, 23–32]. Briefly, 100 μl ^{51}Cr-labeled target cells (5×10^3) and 100 μl effector cells in different numbers were added to each well of round-bottomed microtiter plates (Nunc, Roskilde, Denmark) and incubated for 4 h at 37° C in a humidifed 5% CO_2 atmosphere. After incubation, the supernatant was collected, and the specific ^{51}Cr release as percentage cytotoxicity was calculated by the formula for triplicate samples: Percentage cytotoxicity = (test cpm − spontaneous cpm) / (maximum cpm − spontaneous cpm) X 100. The ranges of spontaneous ^{51}Cr release from effusion tumor cells and K562 were 5%–35% and 5%–10% of the total isotope count, respectively. The maximum release, determined by Triton X-100, varied from 80% to 90%.

Statistical analysis. The results were evaluated for statistical significance by Student's *t*-test. Cytotoxicity greater than 8% was always statistically significant at $P < 0.05$ and was considered positive.

Results

Clinical effects and toxicity of i.pl. OK432 administration

Clinical parameters were evaluated by thoracentesis and chest x-ray examinations 7 days after i.pl. injection of OK432. Eight of ten patients who received i.pl. OK432 injections showed a reduction or disappearance of tumor cells in their pleural effusions and a decrease in effusions within 7 days, and thus were considered as responders. The other two patients, however, had no reduction or disappearance of tumor cells in their pleural effusions or any reduction of the effusions with i.pl. OK432 and were considered nonresponders. Along with the decrease in effusion tumor cells the frequency of lymphoid cells increased in pleural effusions of responder patients. Toxic reactions to i.pl. OK432 treatment included fever in seven patients and chest pain in three subjects. Both symptoms appeared soon after i.pl. injection of OK432, but was tolerable and subsided within 24 h without medication. No other side-effects were recorded, and in no case was treatment fatal.

Effects of i.pl. administration of OK432 on autologous tumor-killing activity of blood lymphocytes

Peripheral blood lymphocytes from untreated patients with carcinomatous pleural effusions were tested for cytotoxicity against tumor cells freshly isolated from the pleural effusions of the same patients in a 4-h ^{51}Cr-release assay. Freshly isolated effusion tumor cells were relatively resistant tolysis by autologous blood lymphocytes. A significant increase in ^{51}Cr release above baseline was observed in only two of ten (20.0%) cases (Table 1). On the other hand, positive NK cell activity against K562 cells was recorded for blood lymphocytes from all patients. Seven days after i.pl. injection of OK432 autologous tumor killing activity was induced in blood lymphocytes from one responder patient who had previously shown no reactivity. However, autologous tumor killing activity remained unchanged in the other seven responder patients and two nonresponders. During the observation period of 8 days, the day-to-day coefficient of variation (CV) of the cytotoxicity assay was 0.13 ± 0.09 (mean ± SD) for normal controls tested repeatedly, and any change of more than 0.31 CV in a patient was considered significant. On day 7, blood NK cell activity increased in two patients, remained unchanged in seven patients, and decreased in one subject. These results suggest that cytotoxic activity to autologous tumor cells and K562 of blood lymphocytes is not consistently modified by i.pl. administration of OK432.

Table 1. Lysis of autologous tumor and K562 by blood lymphocytes obtained before and after intrapleural administration of OK432

Patient[a]	% Cx[b] to autotumor		% Cx to K562	
	Day 0	Day 7	Day 0	Day 7
1	3.9	2.9	22.1	24.1
2	5.8	14.7[c]	43.9	57.2
3	− 3.3	− 2.0	29.5	19.4
4	15.4	18.7	36.8	62.2[c]
5	2.2	4.0	18.8	36.9[c]
6	0.8	5.2	9.7	12.3
7	4.6	5.8	14.8	18.2
8	10.9	12.8	33.3	45.8
9	− 0.4	1.0	16.4	15.8
10	4.5	3.7	24.4	13.0[d]

[a] Peripheral blood mononuclear cells from responder patients (patients 1–8) and nonresponders (patients 9 and 10) were tested for cytotoxicity before and 7 days after i.pl. injection of OK432
[b] % Cytotoxicity against fresh or cryopreserved autologous effusion tumor cells obtained before i.pl. OK432 at an effector-to-target cell ratio (E : T) of 40 : 1 in a 4-h ^{51}Cr-release assay
[c] Value is significantly higher than that on day 0 ($P < 0.05$)
[d] Value is significantly lower than that on day 0 ($P < 0.05$)

Augmentation of autologous tumor killing activity of effusion lymphocytes by i.pl. administration of OK432

Since the most potent antitumor activity may be expected to be present within or around the site of tumor growth, effusion lymphocytes were tested for their ability to lyse autologous effusion tumor cells before and 7 days after i.pl. administration of OK432. Significant levels of cytotoxicity against autologous tumor cells were observed with effusion lymphocytes from two of ten (20.0%) untreated patients (Table 2). Effusion lymphocytes also expressed low or no NK cell activity against K562. Clinical responsiveness to i.pl. OK432 treatment was not associated with pretreatment values of autologous tumor killing activity and NK cell activity of effusion lymphocytes. Seven days after i.pl. injection of OK432 effusion lymphocytes were again tested for the lysis of autologous effusion tumor cells that had been isolated before i.pl. OK432 injection and cryopreserved. Autologous tumor killing activity was induced in five of six (83.3%) responder patients who had previously shown no reactivity and was enhanced in the other two responder patients in whom activity had already been present before i.pl. OK432 therapy. In contrast, effusion lymphocytes

Table 2. Lysis of autologous tumor and K562 by effusion lymphocytes obtained before and after intrapleural administration of OK432

Patient[a]	% Cx[b] to autotumor		% Cx to K562	
	Day 0	Day 7	Day 0	Day 7
1	1.4	9.9[c]	4.3	36.7[c]
2	5.7	31.6[c]	5.9	59.8[c]
3	− 1.9	5.3	2.8	16.3[c]
4	17.3	57.2[c]	23.4	70.1[c]
5	6.2	22.3[c]	9.7	38.1[c]
6	1.6	10.8[c]	1.8	19.5[c]
7	3.7	15.5[c]	6.6	29.6[c]
8	12.4	33.4[c]	17.1	60.8[c]
9	− 1.2	− 0.3	1.0	6.2
10	3.7	2.9	4.1	6.5

[a] Pleural effusion mononuclear cells from responder patients (1–8) and nonresponder patients (9 and 10) were tested for cytotoxicity before and after i.pl. injection of OK432

[b] % Cytotoxicity against fresh or cryopreserved effusion tumor cells obtained from the same patient on day 0 at an E : T of 40 : 1

[c] Value is significantly higher than that on day 0 ($P < 0.05$)

Table 3. Characterization of autotumor killer cells in pleural effusions of patients before and after intrapleural administration of OK432

Patient	% Cytotoxicity against autologous tumor[a]			
	LGL[b]		T cells[c]	
	Day 0	Day 7	Day 0	Day 7
1	9.8	17.9[d]	0.7	2.4
2	19.5	52.2[d]	3.5	4.5
3	NT[e]	NT	2.2	− 1.6
4	36.1	75.7[d]	6.7	7.7
5	12.4	41.4[d]	3.6	2.6
6	NT	NT	0.5	− 3.3
7	14.0	35.1[d]	− 1.7	0.9
8	27.8	64.1[d]	3.1	3.6
9	3.0	1.7	1.1	1.8
10	NT	NT	− 1.4	0.2

[a] % Cytotoxicity against fresh or cryopreserved autologous effusion tumor cells at an E : T of 20 : 1

[b] LGL-enriched fractions 2 and 3 from discontinuous Percoll gradients

[c] Small T lymphocyte-enriched fractions 6 and 7 from Percoll gradients

[d] Value is significantly higher than that on day 0 ($P < 0.05$)

[e] Not tested

from two nonresponder patients were still not cytotoxic to autologous effusion tumor cells even after i.pl. injection of OK432. Similarly, i.pl. administration of OK432 resulted in an induction or enhancement of effusion NK cell activity in all responder patients, whereas effusion NK cell activity remained negative in nonresponder patients 7 days after i.pl. OK432 treatment. These results indicate that i.pl. administration of OK432 brings about an induction or augmentation of cytotoxic activity against autologous effusion tumor cells and K562 cells in the pleural effusions of cancer patients who have a reduction or disappearance of tumor cells in their pleural effusions.

Susceptibility of effusion tumor cells isolated from untreated and OK432-treated patients

The next experiments were performed to ascertain whether effusion lymphocytes from OK432-treated patients could also lyse autologous tumor cells freshly isolated from the pleural effusions on day 7. Effusion tumor cells from OK432-treated patients were equally killed by autologous effusion lymphocytes. No significant differences were observed in percentage cytotoxicities against effusion tumor cells isolated on day 0 and 7 (14.6% ± 5.5% vs 18.3% ± 4.6%, mean ± SD of 4 patients). Effusion lymphocytes from nonresponder patients were cytotoxic to neither cryopreserved nor freshly isolated autologous effusion tumor cells. These results indicate that the enhanced lysis of autologous effusion tumor cells by effusion lymphocytes from OK432-treated patients is unlikely to be due to the cryopreservation of tumor cells, but rather results from the activation of effectors.

Lysis of autologous tumor cells by effusion LGL before and after i.pl. administration of OK432

In an attempt to determine whether the reactivity to autologous effusion tumor cells is mediated by NK cells or other effector cells, effusion lymphocytes were passed throug nylon-wool columns and further fractionated by discontinuous Percoll density gradient centrifugation. In untreated patients cytotoxicity against autologous effusion tumor cells was performed predominantly by the low-density, LGL-enriched populations (Table 3). Significant lysis of effusion tumor cells by autologous effusion LGL was observed even in case where unseparated lymphocytes expressed no cytotoxicity (patients 1, 2, 5, and 7). Enrichment of LGL resulted in an increase in autologous tumor killing activity in case where unseparated lymphocytes had positive reactivity (patients 4 and 8). In contrast, LGL-depleted, small T lymphocyte populations were unable to kill autologous effusion tumor cells in all ten cases. Seven days after i.pl. administration of OK432 effusion LGL and T cells were again tested for cytotoxicity against cryopreserved autologous effusion tumor cells. In all six responder patients monitored, effusion LGL expressed increased autologous tumor-killing activity in response to i.pl. OK432 on day 7. Effusion LGL from a nonresponder patient, however, were still not cytotoxic to autologous effusion tumor cells even after i.pl. OK432 treatment. On the other hand, effusion T lymphocytes again failed to show cytotoxicity against autologous tumor cells on day 7. These results suggest that i.pl. administration of OK432 to cancer patients results in augmentation of autologous tumor killing activity of effusion LGL.

Frequency of LGL in pleural effusions before and after i.pl. administration of OK432

Autologous tumor killing activity is found to be strongly associated with the typical morphology of LGL/NK cells [20, 22, 28, 30] (Table 3). To examine whether the augmentation of cytotoxicity against autologous effusion tumor cells is attributable to an increase in LGL in pleural effusions, effusion lymphocytes were morphologically examined by Giemsa staining before and 7 days after i.pl. injection of OK432. There were no signifiant differences in percentages of LGL among effusion lymphocytes obtained on day 0 and day 7 (8.2% ± 5.2% vs 8.8% ± 5.1%), although the frequency of lymphoid

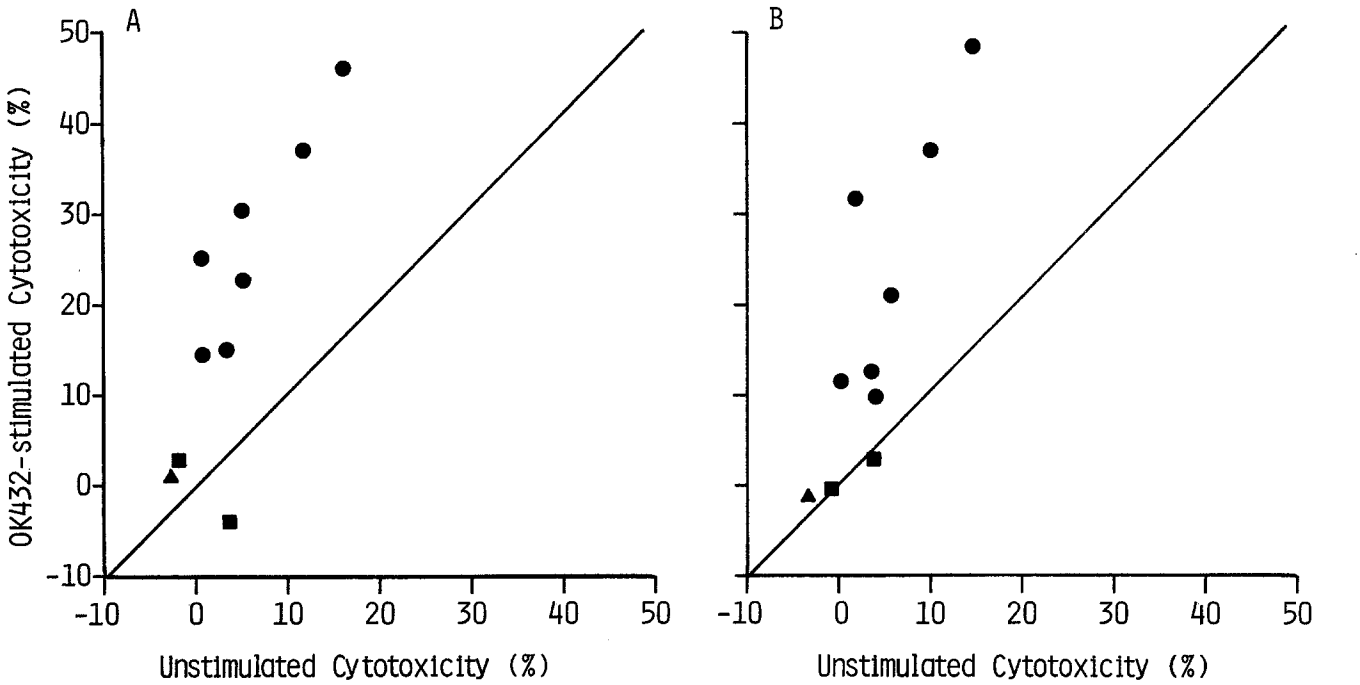

Fig. 1A, B. In vitro effects of OK432 on autologous tumor-killing activity of effusion lymphocytes (**A**) and blood lymphocytes (**B**). Lymphocytes from untreated patients were cultured alone or with OK432 (50 μg/ml) for 20 h, then washed and tested for cytotoxicity against autologous effusion tumor cells at an E : T of 40 : 1. ●, responder patients who showed a reduction or disappearance of effusion tumor cells and an augmentation of effusion autotumor killing activity on day 7 of i.pl. OK432 treatment; ▲, responder patients who had a reduction of effusion tumor cells but no enhancement of effusion autotumor killing activity on day 7; ■, nonresponder patients who showed neither reduction of effusion tumor cells nor enhancement of effusion autotumor killing activity on day 7

cells increased in pleural effusions following i.pl. administration of OK432. These results indicate that the enhanced autologous tumor killing observed in pleural effusions of OK432-treated patients is not derived from redistribution of effector cells but may rather result from activation of autotumor killer cells.

In vitro augmentation of autologous tumor killing activity by OK432

To ascertain whether the responsiveness of cancer patients to i.pl. administration of OK432 could be predicted by in vitro tests, blood and effusion lymphocytes from untreated patients were stimulated in vitro with OK432. There were good correlations between in vivo and in vitro responsiveness of effusion lymphocytes to the autologous tumor killing activity enhancing effects of OK432 (Fig. 1A). In vitro exposure to OK432 resulted in an augmentation of autologous tumor killing activity of effusion lymphocytes from patients who subsequently showed an enhancement of autotumor killing activity and a reduction or disappearance of tumor cells in their pleural effusions in response to i.pl. administration of OK432. In contrast, no positive in vitro reactions were recorded in one patient who subsequently showed a reduction of effusion tumor cells but no induction of autotumor killing activity and in two subjects who subsequently failed to respond both clinically and immunologically to i.pl. OK432 treatment. In vitro treatment with OK432 of blood lymphocytes also induced an enhancement of cytotoxicity against autologous tumor cells in clinical responder patients, but not in nonresponders, although blood lymphocytes from these patients were not activated in vivo with i.pl. injection of OK432 (Fig. 1B). On the other hand, in vitro overnight treatment with IFN failed to induce or augment autologous tumor killing activity in both responders and nonresponders (data not shwon).

In vitro effects of OK432 on autologous tumor killing activity of LGL and T lymphocytes

For confirmation of the suggestion that i.pl. administration of OK432 enhances autologous tumor killing activity of effusion LGL with no effects on effusion T cells, effusion nonadherent lymphocytes from untreated patients were fractionated by discontinuous Percoll gradient centrifugation, and each fraction was stimulated in vitro with OK432. Overnight treatment of effusion LGL with OK432 resulted in an enhancement of cytotoxicity against autologous effusion tumor cells, whereas effusion T cells failed to express significant lysis of autologous tumor cells in response to OK432 in (Table 4). Positive reactions were recorded for effusion LGL from all four responder patients tested. Effusion LGL from a nonresponder patient were still not cytotoxic to autologous effusion tumor cells even after in vitro treatment with OK432. These results indicate that OK432 enhances autologous tumor killing activity of LGL and that T cells are not involved in lysis of autologous tumor cells.

Effects of i.pl. administration of OK432 on cytotoxic activity of adherent effusion cells

Since OK432 has been demonstrated to activate macrophages to kill certain tumor target cells in animal experimental models [14, 17], adherent cells (more than 93% monocyte/macrophages) were isolated from pleural effusions and tested for their ability to lyse fresh autologous effusion tumor cells before and 7 days after i.pl. administration of OK432. Adherent

Table 4. In vitro effects of OK432 on autotumor killing activity of LGL and T cells

Patient	% Cytotoxicity to autologous tumor[a]			
	LGL[b]		T cells	
	Medium	OK432[c]	Medium	OK432
1	8.9	20.7[d]	0.7	1.6
2	19.5	32.4[d]	3.5	5.6
4	36.1	62.5[d]	6.7	7.4
7	14.0	31.4[d]	− 1.7	− 3.5
9	3.0	1.9	1.1	0.4

[a] % Cytotoxicity at an E : T of 20 : 1
[b] Effusion LGL and T cells obtained from discontinuous Percoll gradients
[c] Effector cells were incubated alone or with OK432 (50 μg/ml) for 18 h, then washed and tested
[d] Value is significantly higher than that obtained with medium ($P < 0.05$)

Table 5. Cytotoxicity to autologous tumor of adherent effusion cells obtained before and after intrapleural administration of OK432

Patient	% Cytotoxicity to autologous tumor[a]	
	Day 0	Day 7
1	3.5	2.4
2	0.7	4.5
3	− 4.4	1.3
4	3.7	7.6
5	4.6	− 1.2
6	0.6	2.9
7	− 3.8	2.2
8	2.2	0.1
9	2.4	5.2
10	− 2.6	2.8

[a] Adherent cells obtained from pleural effusions of patients before and 7 days after i.pl. injection of OK432 were tested for cytotoxicity against autologous effusion tumor cells in a 4-h Cr-release assay at an E : T of 20 : 1

effusion cells from untreated patients expressed no significant levels of cytotoxicity against autologous tumor cells in a 4-h ^{51}Cr-release assay (Table 5). With longer incubation periods (up to 18 h) no positive reactions were recorded (data not shown). Administration of OK432 i.pl. resulted in no induction of autologous tumor killing activity of adherent effusion cells from patients, regardless of their clinical responsiveness to i.pl. OK432. Adherent effusion cells were unable to kill autologous effusion tumor cells even after in vitro activation with OK432 (data not shown). These results suggest that effusion monocyte/macrophages are not responsible for the lysis of autologous effusion tumor cells in pleural effusions observed after i.pl. injection of OK432.

Discussion

In the present report, several observations have been made concerning the biologic role of NK cells and antitumor activity of OK432. In agreement with our previous observations [27], i.pl. administration of OK432 to cancer patients has resulted in a reduction or complete disappearance of tumor cells in the pleural effusions and in a decrease in pleural effusions. The clinical effects were maintained for more than 4 weeks by repeated i.pl. injection of OK432 at weekly intervals (data not shown). Similar clinical findings have been reported previously [15]. While the therapeutic usefulness of i.pl. OK432 treatment has been established, the mechanism responsible for the antitumor activity of i.pl. OK432 was not fully clarified in these studies. The present study has confirmed previous observations that effusion NK cell activity is low or absent in untreated patients [24, 25] and that i.pl. injection of OK432 results in an enhancement of effusion NK cell activity and a reduction or disappearance of tumor cells in the pleural effusions [27].

It has been demonstrated that freshly isolated human tumor cells from solid neoplasms, ascitic carcinoma, and carcinomatous pleural effusions are relatively resistant to lysis by autologous peripheral blood and tumor-associated lymphocytes [1, 9, 20, 28–30]. The data presented in this communication have extended these findings to show that i.pl. administration of OK432 to cancer patients induces an enhancement of autologous tumor killing activity of tumor-associated lymphocytes. Seven days after i.pl. injection of OK432, cytotoxicity against autologous effusion tumor cells of effusion lymphocytes was augmented in responder patients who had a reduction or complete disappearance of tumor cells in their pleural effusions. In contrast, autologous tumor killing activity remained negative in pleural effusions of nonresponder patients who showed no clinical evidence of therapeutic benefit from i.pl. OK432 treatment. It should be noted that the augmentation of autologous tumor killing activity of effusion lymphocytes is closely associated with the enhancement of effusion NK cell activity and the reduction or disappearance of tumor cells in the pleural effusions of cancer patients who received i.pl. OK432 therapy. Preliminary kinetics studies have revealed that the enhanced autologous tumor killing activity of effusion lymphoid cells is detectabel 3 days after i.pl. injection of OK432 and is maintained for more than 4 weeks by repeated i.pl. injections of OK432 at weekly intervals. In contrast, both autologous tumor killing activity and NK cell activity of blood lymphocytes was not modified in a consistent pattern by i.pl. administration of OK432 on day 7. The possibility that an early, transient increase in autotumor killing activity of blood lymphocytes following i.pl. OK432 injection is missed cannot be exluded in the present study, since the earliest test for cytotoxicity was conducted on day 7 of OK432 treatment.

Our recent studies, using the population level assay and the single-cell level assay, have shown that a minor proportion of blood and tumor-associated LGL lyse autologous fresh tumor cells [20, 28]. More recently, we have provided direct evidence that a single LGL simultaneously binds and kills both a fluorescent K562 cell and a nonfluorescent autologous tumor cell in the two-target conjugate cytotoxicity assay, while small T lymphocytes lack any such capacity [30]. Data presented in this report have extended these findings to demonstrate that i.pl. administration of OK432 to cancer patients results in an augmentation of autologous tumor killing activity of effusion LGL, whereas effusion T cells are not induced to kill autologous effusion tumor cells by i.pl. OK4332. This has been further confirmed by preliminary depletion studies in which treatment of Percoll-purified LGL with OKT3 monoclonal antibody plus complement eliminates contaminating T cells and enhances autotumor killing activity and treatment with Leu 11a monoclonal antibody plus complement eliminates LGL and abrogates both autologous tumor killing activity and NK cell activity. In addition, in vitro treatment with OK432 has

enhanced autologous tumor killing activity of LGL, while T cells have no reactivity even after activation with OK432. The nonspecific nature of OK432-activated killer cells is suggested by the findings that in vitro OK432-activated lymphocytes [26, 29] and effusion LGL from OK432-treated patients (data not shown) are cytotoxic not only to autologous tumor cells but also to allogeneic fresh tumor cells. It is of importance that the enhancement of autologous tumor killing activity of effusion LGL is closely associated with the decrease or complete disappearance of tumor cells in the pleural effusions of patients given i.pl. OK432. Taken together, it seems likely that LGL/NK cells may interact in vivo with autologous tumor cells in the pleural effusions of cancer patients who receive OK432 i.pl. and that this could lead to the reduction or disappearance of effusion tumor cells in OK432-treated patients. In contrast to our findings, other investigators have observed that lysis of autologous biopsy tumor cells is exerted by blood LGL in some patients, by high-density T cells in some cases, and by both types of effector cells in yet others [35]. The reason for this discrepancy is not yet known. It might be due to differences in the source of tumor cells and the method of preparing effector cells.

The mechanism responsible for the augmentation of autologous tumor killing activity of effusion LGL with i.pl. OK432 treatment is not clearly understood, although these in vivo effects of i.pl. OK432 have been confirmed by in vitro treatment of effusion LGL with OK432. There were good correlations between in vitro and in vivo responsiveness to autotumor killing activity-enhancing effects of OK432, suggesting that similar mechanisms are involved in both in vivo and in vitro enhancement of autologous tumor killing activity with OK432. OK432 has been reported to induce IFN in mice [12, 17]. It might be possible that cytotoxicity against autologous tumor cells is enhanced through IFN induced by i.pl. injection of OK432. However, both IFN-α and IFN-γ have been shown not to augment in vitro autologous tumor killing activity [6, 7, 29, 31, 32, 34]. Furthermore, cell-free supernatants from pleural effusions of OK432-treated patients had no enhancing effects on autologous tumor killing activity and contained no detectable amounts of IFN (data not shwon). The reason why IFN augments NK cell activity, but not autologous tumor killing activity, is not understood. It could be that the autotumor killer subset of NK cells is already activated in vivo and thus does not respond to exogenous IFN, and that the stronger stimulus is required for its activation. The possibility that autologous tumor killing activity is enhanced in vivo by IFN, however, cannot be ruled out in the present study. Lymphoid cells activated in vitro by allosensitization [5, 18], by lectins [13], and by IL 2 [6, 7, 37] have been demonstrated to lyse autologous fresh tumor cells, and it has been suggested that IL 2 plays a central role in the activation of effector cells [6]. OK432 i.pl. however, seems to augment cytotoxic activity against autologous effusion tumor cells of effusion LGL independently of IL 2 induction for the following reasons: (1) OK432 enhances in vitro autologous tumor killing activity independently of IL 2 [29, 31, 32]; (2) lymphokine-activated killer cells are of blastoid morphology and serologically positive for T cell markers (OKT3, OKT8) and negative for NK cell markers (Leu 7, OKM1) [6, 7], while effusion LGL isolated from OK432-treated patients are morphologically and phenotypically indistinguishable from those of untreated patients (unpublished observations); and (3) in preliminary experiments pleural effusion supernatants from OK432-treated patients fail to augment autologous tumor killing activity and do not contain any detectable amounts of IL 2. The present study, however, does not exclude the possibility that IL 2 could be induced by i.pl. OK432 and activate in vivo autotumor killer cells. Recent mouse studies have revealed that OK432 induces the production of a new lymphokine, termed natural killer cell-activating factor (NKAF), which is clearly different from IFN, IL 1, and IL 2 and is capable of augmenting NK cell activity [8]. Such lymphokines may be involved in the activation of autotumor killer cells with i.pl. administration of OK432.

OK432 has been demonstrated to augment natural cytotoxicity against cell line targets of human blood monocytes [39] and cytotoxic or cytostatic activity of peritoneal or spleen macrophages from rodents [14, 17]. On the other hand, human tumor-associated macrophages from solid neoplasms have previously been shown to be cytotoxic to autologous tumor cells in an 18-h assay [36]. In the present study, however, effusion monocyte/macrophages were unable to lyse autologous effusion tumor cells even after i.pl. administration of OK432, when tested in a 4 h assay. Prolonged incubation (up to 18 h) has also failed to induce positive reactions (data not shown). The reason for this discrepancy is not understood. It might be due to the differences in the source of tumor cells and macrophages. An initial decline in the cytotoxicity of tumor-associated macrophages against cell line targets has been documented in patients with ovarin ascitic carcinoma after IP administration of *Corynebacterium parvum,* although autologous tumor killing activity was not examined in this study [11].

In conclusion, the data presented in this report clearly indicate that i.pl. administration of OK432 could augment autologous tumor killing activity and reduce tumor cells in the pleural effusions of cancer patients. This appears to result from the direct activation of tumor-associated LGL capable of lysing autologous tumor cells. While the mechanism by which OK432 enhances autologous tumor killing activity of effusion LGL is not yet understood, i.pl. OK432 therapy will afford subsequent benefit to patients with carcinomatous pleural effusions. In fact, the augmentation of autologous tumor killing activity of effusion LGL with i.pl. administration of OK432 has been well correlated with the reduction or disappearance of tumor cells in the pleural effusions. From collective data we propose that a minor proportion of NK cells interact in vivo with autologous tumor cells at least in pleural effusions of cancer patients treated with i.pl. OK432 treatment.

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Received May 1, 1984/Accepted June 12, 1984