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Original articles

Autologous tumor killing and natural cytotoxic activity of tumor-associated macrophages in cancer patients

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Summary. *Tumor-associated macrophages (TAM) isolated from pleural effusions and ascites fluids of cancer patients were tested for cytotoxicity against freshly isolated autologous tumor cells and K562 in a 4-h ^{51}Cr-release assay, and in vitro effects of OK432 (a streptococcal preparation) and partially purified human leukocyte interferon (IFN) on their cytotoxicities were examined. Positive cytotoxicities against K562 were recorded for TAM samples from 2 of 23 pleural effusions and 3 of 10 ascites specimens. Tumor-associated macrophages were not cytotoxic to autologous tumor cells, while low but significant lysis was observed with tumor-associated lymphocytes (TAL) samples from 2 of 13 pleural effusions and 1 of 6 ascites specimens. In vitro treatment with OK432 resulted in an enhancement of natural cytotoxicity in 4 of 13 TAM and 10 of 15 TAL samples. An induction or augmentation of autologous tumor killing activity by OK432 was observed in 2 of 10 TAM and 8 of 11 TAL samples. In contrast, IFN failed to induce autologous tumor killing activity, although IFN-enhanced lysis of K562 was detected in 1 of 7 TAM and 2 of 9 TAL samples. These results indicated that autologous tumor killing and natural cytotoxic activities were defective in macrophages and lymphocytes at the site of the tumor growth, and both activities were strongly enhanced by OK432 rather than IFN.*

Introduction

Tumor-associated lymphocytes (TAL) isolated from solid tumors [12], ascites fluids [19], and pleural effusions [15, 16] have been shown to express low or no natural killing activity. Similarly, low natural cytotoxic activity of tumor-associated macrophages (TAM) has been documented in ovarian cancer patients [10, 13]. It is, however, difficult to interpret the data on cytotoxic activity against tumor cell lines, since tumor cells have been shown to acquire susceptibility to natural killer (NK) cells through growth in vitro [2, 3]. For better evaluation of antitumor activity of cancer patients, studies on autologous combinations of effector and target cells have been performed. Fresh tumor cells are relatively resistant to lysis by autologous peripheral blood lymphocytes (PBL) and TAL [1, 14, 17, 24]. However, little is known about autologous tumor killing activity of TAM [6, 10, 23].

It has been reported that both PBL and TAL treated with interferon (IFN) are frequently cytotoxic to autologous fresh tumor cells in patients with ascitic tumors in vitro [1]. In contrast, IFN treatment of PBL has enhanced lysis of allogeneic, but not autologous, fresh tumor cells from solid neoplasms [7, 21]. We have reported that OK432, a heat- and penicillin-treated lyophilized powder of the Su strain of *Streptococcus pyogenes* A3, augments NK and autologous tumor killing activity of TAL from pleural effusions [16, 18, 19]. The present study was designed to investigate autologous tumor killing and natural cytotoxic activities of macrophages isolated from carcinomatous fluids and in vitro effects of OK432 and IFN on their cytotoxicities.

Materials and methods

Patients. Fluid specimens were obtained from 23 patients with carcinomatous pleural effusions and 10 patients with malignant ascites. Of these patients 16 had lung cancer, 8 breast cancer, 5 malignant melanoma, 2 rectal cancer, and 2 had uterine cancer. The patients had not received prior treatment with any anticancer agents at the time of the study. Peripheral blood specimens from 22 healthy donors were used as controls.

Preparation of effector cells. Effector cells were prepared as described in detail elsewhere [15, 16]. Mononuclear cells were isolated from heparinized peripheral blood by centrifugation on Ficoll-Hypaque gradients. The cells at the interface were washed and suspended in RPMI-1640 supplemented with 25 mM HEPES, 2 mM L-glutamine, 100 U penicillin/ml, 100 µg streptomycin/ml, and 10% heat-inactivated fetal calf serum (FCS; Gibco, Glasgow, Scotland); this is referred to subsequently as complete medium. Specimens of pleural effusions and ascites fluids were centrifuged, then the cells were washed, suspended in complete medium, and layered on discontinuous gradients of 75% and 100% of Ficoll-Hypaque. After centrifugation at 400 *g* for 30 min, 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. Mononuclear cells having less than 5% tumor cells as judged by morpho-

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logical examination of Wright-Giemsa-stained smears were accepted for use.

After incubation of mononuclear cells for 1 h at 37 °C in plastic dishes precoated with FCS, nonadherent cells were collected. The preparation contained more than 96% lymphocytes as judged by Wright-Giemsa staining and morphology. After the dishes had been thoroughly washed, adherent cells were collected by incubating the dishes with Versene (1 : 5,000, Gibco) for 15 min at room temperature and by gentle scraping with a rubber policeman. The recovered cells contained more than 96% monocytes/macrophages as assessed by nonspecific esterase staining and morphology. Every fraction was more than 97% viable according to the trypan blue dye exclusion test.

Tumor cells from carcinomatous fluids. Cell suspension enriched tumor cells obtained above were contaminated by mesothelial cells, monocytes/macrophages, and lymphocytes. The cell suspension was layered on discontinuous gradients of 25%, 15%, and 10% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) in complete medium, then centrifuged at 25 *g* for 7 min at room temperature [17, 18]. Tumor cells and depleted lymphoid cells were collected from the bottom, suspended in complete medium, and incubated in plastic dishes for 30–60 min at 37 °C. After incubation nonadherent cells were collected, washed, and resuspended in complete medium. Usually, the nonadherent cells contained mainly tumor cells with less than 5% contaminating nonmalignant cells as judged by morphological examination of Wright-Giemsa smears, and were more than 95% viable according to the trypan blue dye exclusion test. The cells having less than 5% contamination with nonmalignant cells were accepted for use as tumor cells.

Treatment with OK432 and IFN. OK432 was supplied by Chugai Pharmaceutical Co., Tokyo, Japan. The unit of 'KE' is used to express the strength of the preparation, 1 KE corresponding to 0.1 mg dried streptococci. Human leukocyte IFN, partially purified and frozen, was obtained from Immunoloski Zabod, Zagreb, Yugoslavia. The specific activity was 2×10^6 U/mg protein. Effector cells at a concentration of 1×10^6/ml in complete medium were incubated alone or with OK432 (0.5 KE/ml) or IFN (10^3 U/ml) for 20 h at 37 °C, as described previously [18, 25, 26]. After incubation the cells were washed and resuspended in complete medium. The viabilities of incubated cells were more than 95%, and no significant difference was observed in the recovery of viable cells incubated alone and with OK432 or IFN.

Target cells. Fresh tumor cells and K562 human myeloid leukemia cell line [8] were used as targets. Target cells were incubated overnight with 100 μCi $Na_2{}^{51}CrO_4$ (specific activity 100–350 μCi/μg Cr; Radiochemical Centre, Amersham, Buckinghamshire, England) at 37 °C, as previously described [17, 18]. After incubation the cells were washed four times, suspended in complete medium and further incubated for 3 h at 37 °C. The cells were then washed twice and resuspended at a concentration of 5×10^4/ml in complete medium. The specimens more than 90% viable according to the trypan blue dye exclusion test were accepted for use as target cells.

Cytotoxicity assay. A 4-h ^{51}Cr-release assay was performed as described in detail elsewhere [17, 18], unless otherwise stated. Briefly, 100 μl labeled target cells (5×10^3) and 100 μl effector cells in different numbers were added to wells of round-bottomed microtiter plates (Nunc, Roskilde, Denmark). After a 4-h incubation the supernatant was collected and the radioactivity was counted in an automaticgamma counter. The specific percentage lysis for each assay was calculated from the following formula for triplicate samples: Specific percentage lysis = [(test cpm – spontaneous cpm) ÷ (maximum cpm – spontaneous cpm)] × 100. The ranges of spontaneous release from K562 and fresh tumor cells were 2%–16% and 6%–36% of the total isotope count, respectively.

Statistical analysis. The results were evaluated for statistical significance by Student's *t*-test and Fisher's exact probability test. A specific percentage lysis greater than 8.0% was always statistically significant at $P < 0.05$ and considered to be positive.

Results

Lysis of K562 cells

Macrophages isolated from carcinomatous fluids expressed low or no cytotoxicity against K562. Positive reactions were recorded for TAM in 2 of 23 pleural effusion samples and 3 of 10 ascites fluid specimens, with the mean specific percentage lysis of 4.3 ± 0.9% (mean ± SEM) and 6.6 ± 2.6% at an effector-to-target (E : T) ratio of 20 : 1, respectively (Fig. 1 A). The levels of cytotoxicities of TAM were comparable to that (4.5 ± 0.6%) of normal peripheral blood monocytes (PBM), and the frequency of positive reactions was similar to that (9%) of normal PBM. On the other hand, TAL showed significant lysis of K562 in 8 of 23 pleural effusion and 3 of 10 ascites samples, while the mean values of cytotoxicities by TAL in both pleural effusion (8.6 ± 1.9%) and ascites (10.5 ± 4.3%) samples were lower than that (37.1 ± 3.1%) of normal PBL ($P < 0.005$).

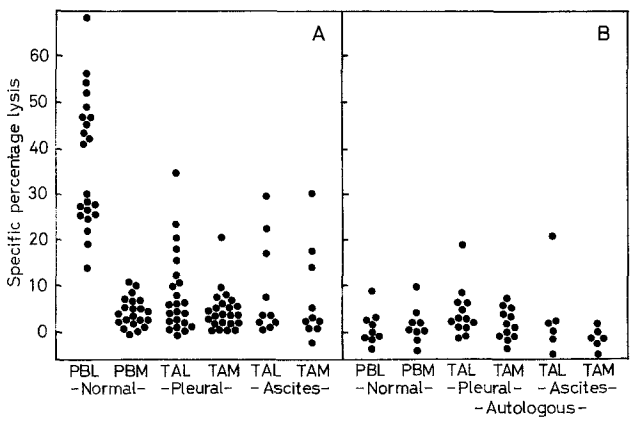

Fig. 1 A, B. Cytotoxic activity against K562 **(A)** and fresh tumor cells **(B)** of unstimulated effector cells. Cytotoxic activity was measured at an effector-to-target ratio of 20 : 1 in a 4-h assay. Specific percentage lysis over 8.0% was estimated to be positive ($P < 0.05$)

Table 1. Effects of OK432 and IFN on cytotoxicity of pleural effusion cells against autologous tumor cells and K562

Experiment	Effector	Specific percentage lysis[a]					
		Autologous tumor			K562		
		Medium	OK432	IFN	Medium	OK432	IFN
1	TAM	0.9	2.2	0.6	4.1	1.0	2.1
	TAL	−0.5	23.4[b]	−0.2	20.3	35.2[b]	19.1
2	TAM	2.9	5.3	3.3	5.7	13.4[b]	6.4
	TAL	4.5	20.2[b]	6.3	3.6	13.0[b]	4.1
3	TAM	−3.9	10.6[b]	−3.7	4.6	13.7[b]	4.3
	TAL	2.3	18.8[b]	−1.3	5.6	29.7[b]	6.3

[a] Effector cells were incubated with medium, OK432 (0.5 KE/ml) or IFN (10^3 U/ml) for 20 h, then washed and tested for cytotoxicity at an effector-to-target ratio of 20:1 in a 4-h assay

[b] The values are significantly higher than that of cells cultured with medium alone ($P<0.05$)

Table 2. Effects of OK432 and IFN on cytotoxicity of ascites fluid cells against autologous tumor cells and K562

Experiment	Effector	Specific percentage lysis[a]					
		Autologous tumor			K562		
		Medium	OK432	IFN	Medium	OK432	IFN
1	TAM	1.8	3.1	−2.2	1.9	1.4	1.1
	TAL	1.9	15.1[b]	4.3	7.2	15.4[b]	7.4
2	TAM	0.2	2.3	0.9	17.2	21.5	22.8
	TAL	−1.2	4.5	−0.2	29.4	38.9[b]	29.2
3	TAM	−1.4	3.4	2.8	29.7	48.5[b]	46.2[b]
	TAL	2.3	19.8[b]	0.3	22.0	54.9[b]	40.8[b]

[a] Effector cells were incubated with medium, OK432 (0.5 KE/ml) or IFN (10^3 U/ml) for 20 h, then washed and tested for cytotoxicity at an effector-to-target ratio of 20:1 in a 4-h assay

[b] The values are significantly higher than that of cells cultured with medium alone ($P<0.05$)

Lysis of fresh tumor cells

Fresh tumor cells isolated from 6 pleural effusions and 3 ascites fluids were relatively resistant to lysis by allogeneic normal PBM as well as PBL which showed strong NK activity against K562 in a 4-h cytotoxicity assay (Fig. 1 B). No lysis of autologous fresh tumor cells was shown by TAM at an E:T ratio of 20:1, although significant lysis was recorded for TAL from 2 of 13 pleural effusion and 1 of 6 ascites specimens. No positive cytotoxicity was induced by TAM even at higher E:T ratios of up to 80:1. Moreover, with a prolonged assay time of up to 20 h fresh tumor cells were still resistant to lysis by autologous TAM as well as TAL (data not shown). Incubation over 20 h was not successful because of high spontaneous chromium release.

Effects of OK432 and IFN

Results of representative experiments of the effects of OK432 and IFN on autologous tumor killing and natural cytotoxic activities in pleural effusion specimens are shown in Table 1. After in vitro treatment with OK432, autologous tumor killing activity was induced in some TAM and TAL samples which initially showed no reactivity. Lysis of K562 was induced or augmented by OK432 in some TAM and TAL samples. In contrast, IFN failed to induce autologous tumor killing activity in both TAM and TAL samples. Moreover, significant enhancement of lysis of K562 by IFN was not recorded in either TAM or TAL samples. In ascites fluid specimens, OK432 did not induce autologous tumor killing activity of TAM, while TAL-mediated lysis of autologous tumor cells was induced by OK432 in some TAL samples (Table 2). On the other hand, lysis of K562 was enhanced by OK432 in some TAM and TAL samples. However, IFN-treated TAM and TAL did not show autologous tumor killing activity, although lysis of K562 was enhanced by IFN in some TAM and TAL samples. An induction of autologous tumor killing activity by OK432 was not associated with that of natural cytotoxicity in both pleural effusions and ascites fluids.

Results of in vitro treatment with OK432 and IFN studied are shown in Table 3. An induction or augmentation of autologous tumor killing activity by OK432 was observed in 2 of 10 (2 of 5 pleural and 0 of 5 ascites) TAM and 8 of 11 (5 of 7 pleural and 3 of 4 ascites) TAL samples.

Table 3. Augmentation of cytotoxicity of tumor-associated cells against autologous tumor cells and K562 by OK432 and IFN

Effector	Incidence of significant augmentation[a]			
	Autologous tumor		K562	
	OK432	IFN	OK432	IFN
Pleural TAM	2/5 (40)	0/3 (0)	3/7 (43)	0/4 (0)
Pleural TAL	5/7 (71)	0/4 (0)	6/9 (67)	0/5 (0)
Ascites TAM	0/5 (0)	0/3 (0)	1/6 (17)	1/3 (33)
Ascites TAL	3/4 (75)	0/3 (0)	4/6 (67)	2/4 (50)

[a] Numbers of augmented samples/numbers of tested samples (%)

Lysis of K562 was enhanced by OK432 in 4 of 13 (3 of 7 pleural and 1 of 6 ascites) TAM and 10 of 15 (6 of 9 pleural and 4 of 6 ascites) TAL samples. In contrast, IFN failed to induce autologous tumor killing activity in 3 pleural and 3 ascites TAM and in 4 pleural and 3 ascites TAL samples. However, IFN-enhanced lysis of K562 was recorded in 1 of 7 (0 of 4 pleural and 1 of 3 ascites) TAM and 2 of 9 (0 of 5 pleural and 2 of 4 ascites) TAL samples.

Discussion

The present study demonstrated that TAM isolated from carcinomatous pleural effusions and ascites fluids expressed low but significant cytotoxicity against K562 in approximately 15% of samples. Other investigators have observed similar results that TAM from human ascitic and solid ovarian carcinomas show low cytotoxic activity against murine tumor cells in a long-term assay [10, 13]. However, there is the possibility that contaminating lymphocytes, especially NK cells, are responsible for the observed cytotoxicity in the present study, since K562 are highly sensitive to lysis by NK cells. It has been reported that spontaneous killing of K562 is greatly reduced after removal of NK cells from blood adherent cells using the methods based on light scatter properties or reactivity with monoclonal antibodies [5]. In contrast, we have recently shown that cytotoxicity of blood adherent cells against K562 is abolished after treatment with OKM1 monoclonal antibody and complement, but not after treatment with Leu-11a and complement [20]. In preliminary experiments we also found that treatment with Leu-11a and complement did not abrogate the lysis of K562 by adherent cells obtained from carcinomatous fluids. In addition, peroxidase-positive cells have been shown to bind and kill K562 in a single cytotoxicity assay [4]. Furthermore, even after treatment with Leu-11a and complement adherent cells bound and killed K562 at a single cell level (data not shown). We can therefore propose that lysis of K562 by adherent cells from both blood and carcinomatous fluid is mediated by monocytes/macrophages.

Fresh tumor cells isolated from carcinomatous fluid were relatively resistant to lysis by allogeneic normal PBL and autologous TAL in agreement with our previous observations [14, 17, 18] and those of others [1, 7, 9, 21, 22]. Furthermore, it was documented in this study that fresh tumor cells were also resistant to lysis by allogeneic normal PBM and not lysed by autologous TAM. Mantovani et al. [10] have reported that primary cultured ovarian tumor cells established from cancer ascites are lysed by allogeneic mononuclear phagocytes in 7 of 11 preparations, and lysis by autologous TAM is observed in 2 of 4 cases at high E:T ratios in a 48-h assay. Vose [23] has shown that macrophages isolated from solid neoplasms lyse allogeneic and autologous fresh tumor cells in approximately 50% of patients at low E:T ratios in an 18-h assay. Similarly, Haskill et al. [6] have reported that macrophages isolated from ascitic and solid ovarian tumors have autologous tumor killing activity in many cases. However, in the present study no lysis of fresh tumor cells by autologous TAM was detected even at high E:T ratios or in a long-term assay. Our adherent cells contained more than 96% monocytes/macrophages, although the above authors reported that more than 90% of the adherent cell preparations were monocytes/macrophages as assessed by nonspecific esterase staining and morphology. The purity of monocytes/macrophages and the source of tumor target cells might contribute to these discrepancies.

Interferon has been shown to play a major role in the activation of natural cytotoxic activity of PBL and PBM in normal individuals [4, 11]. We have recently reported that IFN can enhance natural cytotoxicity of blood effectors, but not TAL, in cancer patients [16, 19, 25, 26]. The present study has indicated that macrophages at the site of the tumor growth are unresponsive to IFN. Our previous report has demonstrated that IFN fails to enhance NK activity of TAL because of the presence of suppressive macrophages in carcinomatous pleural effusions [16]. Similar mechanisms could be operating for TAM. Studies of separation of suppressive and cytolytic macrophages are in progress. However, it has been reported that the presence of β-IFN during a 48-h cytotoxicity assay causes enhanced tumoricidal activity of macrophages from human ascitic and solid ovarian carcinomas against murine tumor cells [13]. These conflicting results might be due to different types of IFN and methods of treatment of effector cells. The difference of tumor target cells might also contribute to these discrepancies. On the other hand, NK activity of TAL has been demonstrated to be enhanced by OK432 [16, 18, 19]. Similarly, OK432 enhanced natural cytotoxicity in approximately 30% of TAM and 70% of TAL samples in this study. Collective data indicate that OK432 has a stronger stimulatory effect on natural cytotoxicity in both TAM and TAL.

It has been shown that in vitro treatment with IFN is rarely capable of activating PBL to kill autologous tumor cells from solid neoplasms [7, 21, 22]. Similarly, we have recently reported that neither blood nor effusion lymphocytes are activated by IFN to be cytotoxic to autologous effusion tumor cells [18, 19]. The data presented here have extended these findings to show that overnight exposure of TAM and TAL to IFN resulted in no induction of autologous tumor killing activity. In a previous report β-IFN caused slight enhancement of cytotoxicity against fresh ovarian tumor cells by PBL and TAL [1]. However, our preliminary studies have revealed that β-IFN and γ-IFN were also incapable of inducing autologous tumor killing activity. In contrast, lytic activity of PBL and TAL to autologous tumor cells has been shown to be induced or augmented by OK432 [18, 19]. In the present study OK432 frequently enhanced the autologous tumor killing activity of TAL and sometimes of TAM. Collective data indicate that cytotoxicity against fresh tumor cells is not modified by

IFN, while the reason why autologous tumor killing activity is enhanced by OK432, but not by IFN, is not clear. Intrapleural administration of OK432 has indeed resulted in a reduction of tumor cells [16]. Therefore, the reduction of tumor cells caused by intrapleural administration of OK432 may be responsible for in vivo activation of TAL and TAM.

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