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Lysis of fresh human tumour cells by autologous tumour-associated lymphocytes: Two distinct types of autologous tumour killer cells induced by co-culture with autologous tumour

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Summary. The specific and natural killer (NK)-restricted nature of auto-tumour cytotoxicity of tumour-associated lymphocytes was studied in cancer patients with malignant pleural effusions. Large granular lymphocytes (LGL) and small T lymphocytes were isolated from carcinomatous pleural effusions by centrifugation on discontinuous Percoll gradients. Tumour cells freshly isolated from pleural effusions were classified according to their susceptibility to lysis by Percoll-purified LGL from the blood of normal donors in a 4-h ^{51}Cr release assay. Of 12 NK-sensitive tumour samples, 11 were killed by autologous fresh effusion LGL, whereas only 2 were lysed by autologous T cells. Neither LGL nor T cells were cytotoxic to NK-resistant autologous tumour cells. T cells and LGL were each cultured in vitro with autologous tumour cells for 6 days. Effusion LGL maintained their auto-tumour killing activity in 10 of 12 autologous mixed lymphocyte-tumour cultures (MLTC) with NK-sensitive tumour, while LGL lost the activity when cultured alone. Removal of high-affinity sheep eryhtrocyte-rosetting cells from Percoll-purified LGL enriched effector cells. Autologous MLTC-derived LGL could also kill NK-sensitive allogeneic effusion tumour cells and K562 cells, as did fresh LGL. In autologous MLTC LGL failed to acquire lytic function to NK-resistant autologous tumour cells. In contrast, in vitro activation of effusion T cells with autologous tumour cells induced auto-tumour killer cells in 9 of 12 NK-sensitive tumour samples and 3 of 6 NK-resistant tumour cases. However, cultured T cells were incapable of killing allogeneic tumour cells and K562 cells. In the autologous MLTC effusion T cells proliferated vigorously in response to autologous tumour cells, whereas LGL showed no proliferation. The enrichment of blasts from cultured T cells on discontinuous Percoll gradients resulted in an enhancement of auto-tumour cytotoxicity, with no reactions recorded in blast-depleted, small, resting T cells. These results indicate that two distinct types of auto-tumour-recognising lymphocytes, LGL and T cells, are present in carcinomatous pleural effusions of cancer patients and that each effector type recognises different membrane moieties of autologous effusion tumour cells.

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Introduction

In vitro cell-mediated cytotoxicity is considered as an expression of the host immune defense mechanisms. For an evaluation of the cytotoxicity of lymphocytes against tumour in cancer patients, studies on autologous combinations of fresh effector and target cells have been performed. Blood lymphocytes from about 25% of cancer patients express lysis of autologous, freshly isolated tumour cells in a short-term assay [1, 9, 19, 29, 34]. Our recent studies have shown that lytic potential to autologous fresh tumour cells is present in the peripheral blood and pleural effusions of cancer patients and that it is associated with a minor proportion of large granular lymphocytes (LGL) and is restricted to the cell population that can lyse natural killer (NK)-sensitive K562 cells [15, 20]. By using the two-target conjugate cytotoxicity assay we have provided direct evidence that the subset of NK cells is involved in auto-tumour cytotoxicity [22]. Other studies have shown slightly different data that lysis of fresh autologous tumour cells is observed with LGL and/or high-density T cells [29].

In a high proportion of cases blood lymphocytes from cancer patients have been demonstrated to proliferate when they are exposed in vitro to autologous tumour cells in the autologous mixed lymphocyte-tumour culture (MLTC) [7, 25]. In most such autologous MLTC cytotoxic potential to autologous tumour cells was generated [26, 27, 28, 31, 32, 33, 34]. The data from these experiments imply that blood lymphocytes recognise autologous tumour cells in cancer patients. Cold target competition studies have revealed that lytic potential generated in vitro by co-cultivation with autologous tumour cells is specific to the identical tumour [26, 28]. However, the induction of cytotoxicity against allogeneic tumour cells was also documented in some autologous MLTC [27, 32, 34]. Conversely, blood lymphocytes from cancer patients have been shown to lyse autologous fresh tumour cells as well as allogeneic fresh tumour cells and various cultured tumour cell lines when they are activated in vitro by allosensitization [14, 36]. By lectins [12], by interleukin 2 (IL 2) [5, 6], and by the streptococcal prepation OK432 [21, 23, 24]. In these studies, T cells, null cells and NK cells are activated depending on the stimulus given. Since a variety of lymphoid cells develop cytotoxicity against autologous fresh tumour cells, it is important to obtain the initial selection of the lymphoid subset that responds and functions as killer cells in the au-

tologous MLTC. Our recent studies indicate that lytic potential restricted to autologous tumour cells can be generated when small T cells are first isolated from blood lymphocytes and then co-coultured with autologous tumour cells [21]. In these experiments blood T cells proliferated vigorously in response to autologous tumour cells, whereas LGL showed no proliferation but maintained their autologous tumour killing and NK cell activity. Also, LGL populations devoid of auto-tumour cytotoxicity failed to develop it in the autologous MLTC.

The lymphocytes with the most potent anti-tumour activity may logically be expected to be present within or around the site of tumour growth. The study of tumour-associated lymphocytes in carcinomatous pleural effusions of cancer patients could offer an approach to understanding the role of lymphocytes in the host defense mechanism against malignancy [1, 16, 18], especially since both lymphocytes and tumour cells can be easily isolated without enzyme treatment, which is required for the isolation of effector and target cells from solid tumours and may affect the function of effector cells and the sensitivity of target cells. We have reported that tumour-associated LGL from malginant pleural effusions of cancer patients lyse autologous fresh effusion tumour cells, while no reactions are recorded for tumour-associated T lymphocytes [15, 20, 22, 23, 24]. It is, however, unknown whether these tumour-associated lymphoid subsets can be stimulated in vitro with autologous tumour cells to proliferate and develop autologous tumour killing activity. In the present study we have evaluated the role of tumour-associated LGL and T cells in the lysis of autologous tumour cells freshly isolated from carcinomatous pleural effusions of cancer patients before and after in vitro co-cultivation with autologous tumour cells.

Materials and methods

Patients. Peripheral blood and pleural effusions were obtained from 18 patients with carcinomatous pleural effusions. Histological diagnosis revealed that 10 patients had adenocarcinoma and 8 had squamous cell carcinoma of the lung. The patients, ranging in age from 33 to 71 years, were receiving no anti-cancer therapy at the time of the study. Blood samples from 45 healthy normal donors were used as roughly matched age and sex controls.

Blood and effusion effector cells. Effector cells were prepared as described in detail elsewhere [15–24]. Mononuclear cells were isolated from heparinized peripheral blood by Ficoll-Hypaque gradient centrifugation and were suspended in RPMI-1640 medium supplemented with 25 mM HEPES, 2 mM L-glutamine, 100 units penicillin/ml, 100 µg streptomycin/ml, and 10% heat-inactivated human AB serum (Gibco Bio-Cult, Glasgow, Scotland) (hereafter referred to as "complete medium").

Specimens of pleural effusions were obtained from the patients by thoracentesis. Specimens were immediately centrifuged at 400 *g* for 5 min. The pellet was then wahsed, and the cells were adjusted to a concentration of 1×10^6/ml in complete medium. The cell suspension was layered on discontinuous gradients of 75% and 100% Ficoll-Hypaque, and centrifuged at 400 *g* for 30 min. Lymphocyte-rich mononuclear cells were collected from the 100% interface, tumour cells and mesothelial cells from the 75% interface, and polymorphonuclear cells, erythrocytes and aggregated tumour cells were collected from the bottom of the tube. The procedure was repeated if separation was not successful, as judged by morphological examination. Mononuclear cells having less than 5% tumour cells, as judged by morphological 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 pre-coated with autologous serum. After incubation, non-adherent cells were collected and passed through Sephadex G10 columns at 37 °C to remove further contaminating monocyte/macrophages and tumour cells. The G10-eluted lymphocytes were incubated in nylon-wool columns for 1 h at 37 °C, after which they were eluted with warm complete medium. The non-adherent lymphocytes (5×10^7) were placed on the top of seven-step discontinuous gradients of 40%–55% Percoll (Pharmacia, Fine Chemicals, Uppsala, Sweden) in medium by 2.5% increments in a 15-ml conical tube, and the tube was centrifuged at 550 *g* for 30 min as described previously [15, 20–24]. The cells collected from low-density fractions 2 and 3 usually consisted of more than 65% LGL, as judged by morphological examination of Giemsa stained cytocentrifuged smears, and are hereafter referred to as "LGL". In some experiments these Percoll-purified LGL were further purified by depletion of high-affinity sheep erythrocyte (E)-rosette-forming cells at 29 °C on Ficoll-Hypaque gradients. The LGL-enriched fraction contained more than 85% LGL. The cells from high-density fractions 6 and 7 consisted mainly of small T cells, as judged by E-rosette formation, with less than 2% LGL and are hereafter referred to as "T lymphocytes". Every fraction was more than 96% viable according to the trypan blue dye exclusion test.

Effusion tumour cells. Cell suspensions enriched for tumour cells obtained as described above were contaminated by monocyte/macrophages, mesothelial cells and lymphoid cells. To eliminate these contaminating non-malignant cells, the cell suspension was layered on discontinuous gradients of 25%, 15%, and 10% Percoll in complete medium in a 15-ml plastic tube, and the tube was centrifuged at 25 *g* for 7 min, as described previously [19, 20, 22, 23]. Tumour cells depleted of lymphoid cells were collected from the bottom, washed and suspended in complete medium. To eliminate further contamination from monocyte/macrophages and mesothelial cells, the cell suspension was inucbated in plastic dishes for 30–60 min at 37 °C. After incubation, non-adherent cells were recovered, washed and suspended in complete medium. Usually, the non-adherent cells consisted mainly of tumour cells with less than 5% contaminating non-malignant cells, as judged by morphological examination of Wright-Giemsa stained smears, and were more than 95% viable according to the trypan blue dye exclusion test. Cells that had less than 5% contamination with non-malignant cells were accepted for use. Tumour cells either were used immediately or were cryopreserved in 90% human AB serum plus 10% dimethyl subphoxide for further use.

Autologous MLTC. Autologous MLTC were performed using flat-bottomed tissue culture plates (Costar, Cambridge, Mass., USA) with 2×10^6 lymphocytes and 4×10^5 mitomycin C-treated (50 µg/ml for 30 min at 37 °C) auto-

logous effusion tumour cells in a total volume of 2 ml complete medium, as described previously [21]. Preliminary experiments had revealed that a responder-to-stimulator cell ratio of 5:1 was optimal for the generation of cytotoxicity against autologous tumour cells. Control cultures consisted of lymphocytes alone. Cultures were incubated for 6 days at 37 °C in a humidified 5% CO_2 atmosphere. After culture the cells were harvested, washed and suspended in complete medium. The recovery of lymphoid cells from a 6-day culture varied from 30% to 90%.

Isolation of autologous MLTC-derived effector cells. Cells recovered from autologous MLTC were separated as described previously [21]. Lymphoid cells recovered from autologous MLTC were placed on the top of three-step discontinuous gradients of 50%, 35%, and 25% Percoll in medium in a 15-ml conical tube, and the tube was centrifuged at 550 *g* for 30 min. The cells collected from the 35% interface contained more than 90% blasts, as judged by morphological examination of Giemsa stained smears, and the fraction is hereafter referred to as the "blast-enriched fraction". The cells recovered from the 50% interface were composed mainly of small- and medium-sized lymphoid cells with less than 5% contamination of blasts, and the fraction is hereafter referred to as the "blast-depleted fraction". The total cell recovery was more than 80% of the original input. Each fraction was more than 95% viable according to the trypan blue dye exclusion test.

Lymphocyte proliferation assay. The proliferative response of lymphocytes in autologous MLTC was measured in a ^3H thymidine incorporation assay, as described previously [17, 21]. Briefly, 1×10^5 lymphocytes were cultured alone or with 2×10^4 mitomycin C-treated autologous tumour cells in a total volume of 0.2 ml in wells of microtiter places (Costar) for 6 days at 37 °C in a humidified 5% CO_2 atmosphere. To each well 1 μCi ^3H thymidine (5 Ci/mmol, Radiochemical Centre, Amersham, Buckinghamshire, England) was added for the last 20 h of the culture. The culture was then harvested, and the incorpotation of thymidine into DNA was measured with a liquid scintillation counter. The results are expressed as the difference between the counts per minute of responding cells in the presence and absence of stimulating autologous tumour cells (Δ cpm).

Cytotoxicity assay. A 4-h ^{51}Cr release assay was performed using effusion tumour cells (see above) and the K562 human myeloid leukaemia cell line [10] as targets, as described in detail elsewhere [15, 16, 18–24]. Briefly, 100 μl ^{51}Cr-labelled target cells (5×10^3) and 100 μl effector cells were assigned at different effector-to-target cell ratios (E:T) to wells of round-bootomed microtiter plates (Nunc, Roskilde, Denmark), and were incubated for 4 h at 37 °C in a humidified 5% CO_2 atmosphere. After incubation the supernatant was collected, and the activity was counted in an autogamma scintillation counter. The percentage cytotoxicity for each assay was calculated by means of the following formula: Percentage cytotoxicity = [(test cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm)] × 100. The ranges of spontaneous ^{51}Cr release from K562 cells and from effusion tumour cells were 3%–12% and 5%–39%, respectively, of the total isotope count. The maximum release determined by addition of Triton X-100 varied from 80% to 90%.

Statistical analysis. All determinations were performed in triplicate, and results were calculated as the mean plus or minus the standard deviation. Cytotoxicity greater than 8% was always statistically significant at $P < 0.05$ by Student's *t*-test and was considered as being positive.

Results

Lysis of NK-sensitive effusion tumour cells by fresh and cultured autologous lymphocytes

Tumour cells freshly isolated from pleural effusions of cancer patients were classified according to their susceptibility to lysis by purified NK cells. Of 18 (67%) effusion tumour samples 12 were lysed by allogeneic Percoll-purified LGL of at least one normal donor; therefore these effusion tumour cells (tumour 1–12) were considered as being NK-sensitive. The other 6 effusion tumour samples (tumour 13–18) were not killed by normal LGL from any of three different donors and thus were considered as being NK-resistant.

Having established the classification of fresh effusion tumour cells, tumour-associated lymphocytes from malignant pleural effusions of cancer patients were fractionated into LGL and small T cells on discontinuous Percoll gradients and tested for cytotoxicity against NK-sensitive, fresh effusion tumour cells from the same patients, with or without in vitro 6-day co-culture with autologous tumour cells. Of 12 NK-sensitive effusion tumour samples, 11 (92%) were lysed by fresh autologous effusion LGL, whereas only 2 (17%) were killed by fresh autologous effusion T cells (Table 1). Next, LGL and T cells were each cultured alone or with autologous tumour cells for 6 days and were then tested for lysis of cryopreserved autologous tumour cells. Effusion LGL lost their lytic activity to autologous tumour cells when they were cultured alone. In contrast, LGL that were co-cultured with autologous tumour cells maintained their auto-tumour killing activity in 10 of 11 (91%) cases where fresh LGL had showed positive reactivity. One LGL sample devoid of auto-tumour cytotoxity failed to develop it in autologous MLTC. There was no significant difference in the mean percentage cytotoxicity of fresh LGL and autologous MLTC-derived LGL ($21.0 \pm 4.0\%$ vs $21.9 \pm 3.9\%$ at an E:T of 20:1; mean ± SEM). On the other hand, in vitro activation of effusion T cells with autologous effusion tumour cells resulted in the generation of auto-tumour killer cells. An induction of auto-tumour cytotoxicity was observed with 7 of 10 (70%) previously non-reactive T cell samples, and an enhancement of the activity was recorded for 2 previously cytotoxic samples. When cultured alone, T cells failed to maintain or develop autologous tumour killing activity. No significant difference was seen with the mean percentage cytotoxicity to autologous tumour cells of cultured LGL and cultured T cells ($21.9 \pm 3.9\%$ vs $17.9 \pm 5.3\%$ at an E:T of 20:1), although cultured LGL showed higher levels of cytotoxicity than cultured T cells in 4 of 12 (33%) cases. After 6 days of autologous MLTC with NK-sensitive effusion tumour cells, the lysis of autologous tumour cells was recorded for 10 of 12 (83%) effusion LGL samples and 9 of 12 (75%) effusion T cell samples.

Table 1. Lysis of NK-sensitive effusion tumour cells by fresh and cultured autologous, tumour-associated LGL and T cells

Patient	% Cytotoxicity against autologous tumour cells[a]					
	LGL[b]			T cells[c]		
	Fresh	Cultured alone[d]	Cultured with auto-tumour[e]	Fresh	Cultured alone	Cultured with auto-tumour
1	26.5[f]	2.4	26.6[f]	2.0	5.2	16.8[f]
2	19.6[f]	0.7	13.5[f]	5.1	1.2	12.6[f]
3	9.7[f]	1.8	16.2[f]	1.5	1.1	1.2
4	48.6[f]	7.2	50.1[f]	16.1[f]	0.9	46.8[f]
5	15.4[f]	2.2	22.1[f]	5.5	0.1	14.9[f]
6	8.4[f]	3.7	18.8[f]	3.0	−1.9	10.7[f]
7	39.3[f]	3.1	37.4[f]	12.1[f]	4.3	53.8[f]
8	32.3[f]	5.8	25.1[f]	2.9	2.0	29.6[f]
9	10.6[f]	NT[g]	1.3	5.5	2.3	1.1
10	3.5	2.2	5.6	−1.6	−1.4	−1.0
11	13.4[f]	NT	14.3[f]	1.9	4.9	10.8[f]
12	25.4[f]	3.2	32.0[f]	3.9	3.8	33.3[s]

[a] % Cytotoxicity against fresh or cryopreserved effusion tumour cells that were lysed by Percoll-purified LGL from normal donors at an E : T of 20 : 1
[b] LGL-enriched fractions 2 and 3 from discontinuous Percoll gradients
[c] Small T cell-enriched fractions 6 and 7 from discontinuous Percoll gradients
[d] Lymphocytes cultured alone for 6 days
[e] Lymphocytes cultured with autologous tumour cells for 6 days
[f] Value is significant ($P < 0.05$)
[g] Not tested

Table 2. Lysis of K562 cells by fresh and cultured LGL and T cells

Patient	% Cytotoxicity against K562[a]					
	Fresh		Control culture[b]		Autologous MLTC[c]	
	LGL	T cells	LGL	T cells	LGL	T cells
1	11.6[d]	1.7	4.7	1.6	12.6[d]	0.9
2	24.4[d]	1.5	0.7	0.8	18.7[d]	1.3
3	8.2[d]	2.3	3.1	−0.5	9.6[d]	3.5
4	19.8[d]	0.9	4.5	3.8	23.5[d]	5.2
5	9.4[d]	2.2	1.0	−1.1	9.0[d]	4.1

[a] % Cytotoxicity against K562 cells at an E : T of 5 : 1
[b] Lymphocytes cultured alone for 6 days
[c] Lymphocytes cultured with autologous tumour cells for 6 days
[d] Value is significant ($P < 0.05$)

Lysis of K562 cells by fresh and cultured lymphocytes

Since both LGL and T cells recovered from autologous MLTC were found to kill NK-sensitive autologous tumour cells, an attempt was made to ascertain whether autologous MLTC-derived effector cells can kill highly NK-sensitive K562 cells. Lysis of K562 cells was exerted predominantly by fresh effusion LGL, with no reactions recorded against K562 cells in LGL-depleted, fresh effusion T cells (Table 2). When LGL were co-cultured with autologous tumour cells for 6 days, they maintained their NK cell activity against K562 cells. However, LGL lost the activity when they were cultured alone for 6 days in human serum. In contrast, T cells failed to aquire anti-K562 cytotoxic activity even after 6 days of stimulation with autologous tumour cells, although these cultured T cells were capable of killing NK-sensitive autologous effusion tumour cells.

Generation of cytotoxicity to NK-resistant autologous tumour cells in autologous MLTC

Six effusion tumour samples that were found to be resistant to lysis by purified NK cells of normal donors served as targets in a 4-h Cr release assay of autologous effusion LGL and T cells, before and after 6 days of autologous MLTC. Neither fresh LGL nor fresh T cells showed cytotoxic reactions to these NK-resistant autologous tumour cells (Table 3). Co-culture of LGL with NK-resistant auto-

Table 3. Generation of cytotoxicity against NK-resistant autologous tumour cells in autologous MLTC

Patient	% Cytotoxicity against autologous tumour cells[a]					
	LGL			T cells		
	Fresh	Cultured alone[b]	Cultured with auto-tumour[c]	Fresh	Cultured alone	Cultured with auto-tumour
13	2.4	−1.0	4.8	0.8	3.3	6.0
14	1.7	2.0	5.9	1.6	0.2	17.4[d]
15	2.3	0.8	0.3	2.4	−2.7	24.8[d]
16	4.8	4.5	6.6	−3.8	2.4	5.6
17	3.7	3.1	1.5	3.5	0.4	−1.1
18	3.1	1.4	1.2	4.4	3.1	33.1[d]

[a] % Cytotoxicity against fresh or cryopreserved effusion tumour cells that were not lysed by Percoll-purified LGL from normal donors at an E : T of 20 : 1
[b] Lymphocytes cultured alone for 6 days
[c] Lymphocytes cultured with autologous tumour cells for 6 days
[d] Value is significant ($P < 0.05$)

logous tumour cells was ineffective in inducing cytotoxicity to the identical tumour. In contrast, in vitro activation of effusion T cells with autologous NK-resistant effusion tumour cells resulted in the development of auto-tumour cytotoxicity in three of six (50%) cases. When T cells were cultured alone, however, no lytic potential was induced. These results indicate that tumour-associated T cells, but not tumour-associated LGL, can be triggered to become killer cells capable of lysing NK-resistant autologous tumour cells in the autologous MLTC. The data also indicate that NK-resistant effusion tumour cells are not totally resistant to cell-mediated lysis.

Enrichment of auto-tumour cytotoxicity by depletion of high-affinity E-rosette forming cells from Percoll-purified LGL

Since LGL fractions that were isolated on discontinuous Percoll gradients were contaminated by low-density T lymphocytes, the low-density fractions 2 and 3 from the gradients were depleted of high-affinity E-rosette forming cells before the initiation of autologous MLTC. These LGL-enriched effusion cell fractions expressed increased levels of cytotoxicity against autologous effusion tumour cells (Table 4). When these highly purified LGL were co-cultured with autologous tumour cells, they showed strong lysis of autologous tumour cells, which was higher than that exerted by Percoll-purified LGL. Similar enrichment of effector cells was seen when Percoll-purified effusion LGL were treated with the OKT3 monoclonal antibody directed against T cells plus complement (data not shown). Thus, the lysis of autologous tumour cells observed with fresh and autologous MLTC-derived low-density tumour-associated lymphocytes is unlikely to be derived from contaminating T cells but is attributed to the lytic function of LGL.

Table 4. Enrichment of autologous tumour killing activity by depletion of high-affinity E-rosetting cells from low-density fractions

Patient	% Cytotoxicity against autologous tumour[a]			
	Fresh		Cultured[b]	
	LGL_c	LGL E_{HA}-[d]	LGL	LGL E_{HA}-
1	26.5	38.4[e]	26.6	35.5[e]
2	19.6	25.3[e]	13.5	23.4[e]
3	9.7	17.5[e]	16.2	28.1[e]

[a] % Cytotoxicity against autologous tumour cells at an E : T of 20 : 1
[b] Lymphocytes cultured autologous tumour cells for 6 days
[c] LGL-enriched fractions 2 and 3 from discontinuous Percoll gradients
[d] Highly purified LGL populations depleted of high-affinity E-rosetting cells from low-density fractions 2 and 3 prior to autologous MLTC
[e] Value is significantly higher than that of corresponding Percoll-purified LGL ($P < 0.05$)

Specificity of cytotoxicity generated in autologous MLTC

In an attempt to examine the specificity of the cytotoxicity of effector cells recovered from autologous MLTC, allogeneic fresh effusion tumour cells also served as targets in a cytotoxicity assay. Autologous MLTC-derived effusion LGL lysed allogeneic NK-sensitive effusion tumour cells as well as autologous NK-sensitve tumour cells in four of five (80%) cases (Table 5). No cytotoxic reactions, however, were recorded against allogeneic NK-resistant tumour cells in cultured LGL from autologous MLTC. In contrast, T cells that were co-cultured with autologous tumour cells for 6 days expressed specific lysis of only autologous tumour cells, with no reactivity to allogeneic effusion tumour cells regardless of their susceptibility to NK cells. Autologous MLTC-derived LGL and T cells showed no cytotoxicity against autologous mesothelial cells and T lymphoblasts (data not shown). These results indicate that in vitro stimulation of tumour-associated lymphocytes with autologous tumour cells results in the appearance of two distinct types of cytotoxic cells, cultured LGL with NK-restricted cytotoxicity to autologous and allogeneic NK-sensitive effusion tumour cells and cultured T cells with specific cytotoxicity to autologous tumour cells independently of NK sensitivity of target cells.

Table 5. Specificity of cytotoxicity generated by autologous MLTC

Patient	Effector cells[a]	% Cytotoxicity against[b] NK-sensitive autologous tumour	NK-sensitive allogeneic tumour	NK-resistant allogeneic tumour
4	LGL	50.1[c]	36.4[c]	5.2
	T cells	46.8[c]	7.4	4.8
5	LGL	22.1[c]	35.6[c]	0.5
	T cells	14.9[c]	4.5	1.3
6	LGL	18.8[c]	14.8[c]	4.9
	T cells	10.7[c]	1.6	0.6
8	LGL	25.1[c]	6.9	−1.7
	T cells	29.6[c]	2.7	3.6
12	LGL	32.0[c]	28.1[c]	0.8
	T cells	33.3[c]	4.3	3.9

[a] LGL and T cells each were cultured with autologous tumour cells for 6 days
[b] % Cytotoxicity at an E : T of 20 : 1
[c] Value is significant ($P < 0.05$)

Table 7. Distribution of auto-tumour killer T cells from autologous MLTC on discontinuous Percoll gradients

Patient	% Cytotoxicity to autologous tumour[a] Unseparated[b]	Blast-enriched	Blast-depleted
4	46.8	60.3[c]	7.8[d]
5	14.9	27.0[c]	2.5[d]
6	10.8	20.8[c]	1.0[d]
14	17.4	28.3[c]	4.8[d]
15	24.8	38.8[c]	3.7[d]

[a] Cytotoxicity was tested against autologous tumour cells at an E : T of 20 : 1
[b] Small T cell-enriched fractions 6 and 7 from seven-step discontinuous Percoll gradients were cultured with autologous tumour cells for 6 days and then fractionated into blast-enriched and blast-depleted fractions on three-step discontinuous Percoll gradients
[c] Value is significantly higher than that of unseparated cells ($P < 0.05$)
[d] Value is significant lower than that of unseparated cells ($P < 0.05$)

Lymphoproliferative response to autologous tumour cells

The lymphoproliferative response to autologous tumour cells was evaluated using a ^{3}H thymidine incorporation assay. Effusion T cells responded vigorously to autologous effusion tumour cells in 6 days of autologous MLTC. Significant incorporation of ^{3}H thymidine above base line was observed in eight of ten (80%) T cell cultures, with the mean response of 1,827 ± 511 Δ cpm (Table 6). Positive reactions were recorded in all six cases where cultured T cells showed auto-tumour cytotoxicity. No proliferation was seen with cultured T cells with no auto-tumour killing activity. In two autologous MLTC, however, T cells expressed proliferative response, but no cytotoxic reaction.

Table 6. Lymphoproliferative response and cytotoxicity to autologous tumour cells of LGL and T cells[a]

Patient	^{3}H TdR incorporation (Δcpm)[b] LGL	T cells	% Cytotoxicity[c] LGL	T cells
1	35 ± 54	2,666 ± 249[d]	26.6[d]	16.8[d]
2	16 ± 84	1,745 ± 271[d]	13.5[d]	12.6[d]
4	280 ± 182	4,951 ± 269[d]	50.1[d]	46.8[d]
5	101 ± 109	2,874 ± 378[d]	22.1[d]	14.9[d]
6	−39 ± 98	1,728 ± 199[d]	18.8[d]	10.7[d]
9	53 ± 83	−94 ± 77	1.3	1.1
13	86 ± 72	103 ± 116	4.8	6.0
14	96 ± 120	3,223 ± 318[d]	0.3	24.8[d]
16	NT	602 ± 212[d]	−3.8	5.6
17	118 ± 114	476 ± 163[d]	1.5	−1.1

[a] Lymphocytes were cultured with autologous tumour cells for 6 days
[b] Proliferative response was measured by the incorporation of ^{3}H thymidine into DNA. Results are expressed as means ± SD of triplicate samples
[c] Cytotoxicity was tested against autologous tumour cells in a ^{51}Cr release assay at an E : T of 20 : 1
[d] Value is significant ($P < 0.05$)

The mean lymphoproliferative response was significantly higher in cytotoxic cultures than in non-cytotoxic cultures (2,865 ± 485 Δ cpm vs 272 ± 162 Δ cpm). In contrast, no stimulation of ^{3}H thymidine incorporation on day 6 was observed with effusion LGL that were co-cultured with autologous effusion tumour cells, with the average response being 83 ± 30 Δ cpm. There was no difference in the background counts per minute of effusion LGL and T cells (data not shown). Thus, in the autologous MLTC the generation of auto-tumour cytotoxicity of effusion T cells is mainly associated with a positive lymphoproliferative response to autologous effusion tumour cells, and such association was not observed in effusion LGL culture.

Distribution of auto-tumour killer T cells from autologous MLTC on discontinuous Percoll gradients.

To better evaluate the raltionship between cytotoxicity and lymphoproliferation, cultured effusion T cells recovered from autologous MLTC were fractionated into blast-enriched and blast-depleted fractions on discontinuous three-step Percoll density gradients, and each fraction was tested for lysis of autologous effusion tumour cells. The blast-enriched fraction expressed increased levels of cytotoxicity against autologous tumour cells, whereas the blast-depleted fraction was devoid of auto-tumour cytotoxicity (Table 7). When autologous MLTC-derived effusion LGL were isolated on the discontinous Percoll gradients, cytotoxic reactions were recorded for the blast-depleted fraction (data not shown). The blast-enriched fraction was not tested because so few cells were recovered in the fraction. These results indicate that the specific cytotoxicity against autologous tumour cells seen with cultured effusion T cells is confined to the T-blast fraction.

Discussion

In the present report, several observations have been made concerning the cytotoxic activity and proliferative capacity of tumour-associated lymphoid subsets, LGL and T

ells, in cancer patients with carcinomatous pleural effusions. In agreement with our previous observations [15, 20, 2, 23], lysis of autologous, freshly isolated effusion tumour cells was exerted predominantly by fresh effusion GL, with low or no reactivity in fresh effusion small T mphocytes. However, when effusion T cells were co-cultured with autologous effusion tumour cells in vitro, they roliferated and expressed cytotoxicity against the identical tumour. The development of auto-tumour cytotoxicity as recorded in approximately 70% of patients, whereas ie lysis of autologous tumour cells by unstimulated effuon T cells was documented in about 10% cases. The data ndicate that the lysis by fresh effector cells reveals the resence of tumour-associated T lymphocytes with recepors for antigens expressed on autologous tumour cells and iat during in vitro culture with autologous tumour cells ie clone may expand. This evidence is derived from our ndings that cytotoxicity generated in autologous mixed T ell tumour culture is specific to autologous tumour. In the utologous MLTC, however, lytic potential to autologous mour is not confined to cultured T cells, since cultured ffusion LGL are also found to mediate lysis of autologous ffusion tumour cells. Furthermore, autologous MLTC-deved LGL could kill allogeneic NK-sensitive tumour cells. imilar observations have been made with blood T cells nd LGL that are isolated prior to autologous MLTC [21]. n the other hand, when unseparated blood lymphocytes f cancer patients are exposed in vitro to autologous tuour cells, they also develop cytotoxicity to the identical mour [26, 27, 28, 34]. These investigators have reported iat cytotoxicity induced in autologous MLTC is specific the autologous tumour. However, cytotoxic potential to llogeneic tumour cells was also generated during autolous MLTC in some of their experiments [27, 34]. In addion, recent studies have shown that autologous MLTC-deved effector cells without blast isolation express cytotox reactivity to autologous tumour cells but also high NK ell activity and considerable cytotoxicity against allogeeic tumour cells and that T blasts isolated from autolous MLTC have cytotoxicity restricted to the autologous mour [31, 32]. T cells are known to exceed LGL in numers among blood lymphocytes; in addition, T cells, but ot LGL, are found to proliferate in response to autolous tumour cells [21, Table 6]. Collectively, it seems likely iat when unfractionated lymphocytes are cultured with utologous tumour cells, specific cytotoxic T cells domiate the autologous MLTC to the exclusion of LGL-deived NK-resistricted killer cells.

It has recently been demonstrated that mixed lymphoyte culture (MLC) induces alloreactive cytotoxic T lymhocyte clones that can mediate both specific and NK-like ytolysis and that two independent recognition structures re involved in this dual reacitivty [13]. On the basis of hese observations, these investigators have proposed that he NK cell is assigned to the T cell lineage. However, the wo tumour-associated effector cell populations, auto-tuour speicific killer cells and NK-restricted killer cells, hat are recovered from autologous MLTC in the present tudy appear to be distinctive and not stages in the differntiation of a given lymphoid population for the following easons. Cultured T cell-mediated lysis is restricted to aulogous tumour cells and is observed regardless of NK ensitivity of target cells, whereas cultured LGL-mediated ytotoxicity is directed against both autologous and allogeneic tumour cells and is observed provided that target cells have the NK recognition structure. Cultured LGL also lyse NK-sensitive K562 cells, whereas cultured T cells lack anti-K562 reactivity despite killing of NK-susceptible autologous tumour cells. Lysis of autologous tumour cells seen with cultured T cells is confined to the blast-enriched fraction, whereas auto-tumour cytotoxicity observed with cultured LGL is attributed to the non-blast fraction. Collective data suggest that in the autologous MLTC tumour-associated T cells probably recognise tumour-associated antigens expressed on autologous tumour cells and proliferate, which leads to the generation of auto-tumour-specific killer cells. We have previously reported that tumour-associated T cells proliferate vigorously in response to autologous non-T cells in cancer patients with malignant pleural effusions [17]. However, the reactions observed in the autologous MLTC are unlikely to result from auto-recognition of HLA-DR antigens, since neither the proliferative response [30] nor development of auto-tumour cytotoxicity was blocked by monoclonal antibodies against HLA-DR antigens (unpublished observation). Furthermore, positive autologous MLTC reactions were recorded irrespective of the expression of class I and class II major histocompatibility (MHC) antigens on effusion tumour cells (data not shown). In contrast, HLA-DR antigens have been shown to play a central role in stimulation of autologous blood lymphocytes with primary melanoma cells [4]. The discrepancy may be explained by the difference in the tumour cell type studied.

The antigens involved in the cytotoxic reaction of autologous MLTC-derived T cells is not clarified in the present study. The reactivity seems to be confined to tumour cells since cultured T cells from autologous MLTC failed to kill autologous non-malignant cell types such as fresh effusion mesothelial cells and mitogen-induced T lymphoblasts (data not shown). The data also indicate that effusion tumour cells, in contrast to non-malignant effusion or blood cells, express the determinants that are recognised by autologous effusion effector cells recovered from autologous MLTC. It has recently been demonstrated at the clonal level that cytotoxic T lymphocyte clones derived from autologous MLTC lyse autologous tumour cells such as melanoma cells [3] and lymphoma cells [35] and that the cytotoxic reactions are independent of class I and class II MHC antigens. These investigators have also shown that the T cell differentiation antigens T3, T4, and T8 are not involved in auto-tumour cytotoxicity of T cell clones, although these clones express surface phenotype of T3 (+) T4 (+) or T3 (+) T8 (+). Our preliminary experiments in which monoclonal antibodies against class I and class II MHC antigens are used to block auto-tumour cytotoxicity of autologous MLTC-derived effector cells have demonstrated no involvement of class I and class II HLA antigens in the cytotoxic reactions.

When blood lymphocytes are cultured in vitro with autologous tumour cells [26, 28] or with allogeneic lymphocytes [2, 11, 13], they are shown to lyse K562 cells in addition to specific target cells. For an enrichment of auto-tumour-specific killer cells in autologous MLTC, the isolation of reactive blasts on discontinuous gradients has been used [31, 32, 33]. Similar observations have been made in allogeneic MLC where specific cytotoxic alloreactivity is confined to T cell blasts and NK-like activity is expressed by both blasts and non-blasts [11]. Furthermore, cytotoxic

T cell clones from autologous MLTC have been reported to have auto-tumour killing activity, but no reactivity to K562 cells [3, 35]. In our previous [21] and present (Table 2) studies no anti-K562 killer cells were generated in mixed cultures of Percoll-purified T cells and autologous tumour cells where auto-tumour cytotoxicity was induced. In addition, the T blast-enriched fraction had cytotoxicity restricted to autologous tumour cells, with no reactivity in the blast-depleted fraction. On the other hand, medium-sized lymphocytes have been shown to be activated in vitro by IL 2 to express strong cytotoxicity to various target cells [5]. Thus, it seems likely that NK-like activity observed in mixed cultures of unseparated lymphocytes is exerted by medium-sized lymphocytes trans-stimulated through lymphokines produced during the autologous MLTC reaction.

It has been shown that blood lymphocytes lose their NK cell activity when they are cultured alone [2]. Data presented in this report have extended these findings to indicate that the co-culture of tumour-associated LGL with autologous tumour cells is required for the maintenance of their auto-tumour killing and NK cell activity. This confirms our previous observations with blood LGL [21]. The mechanism responsible for the persistent auto-tumour killing activity of LGL is not yet understood. Effusion LGL are found not to proliferate in response to autologous effusion tumour cells in vitro, indicating that proliferation is not required. In addition, autologous MLTC-derived LGL showed the same target specificity and morphology as fresh LGL. Taken together, auto-tumour cytotoxicity observed with cultured LGL is unlikely to be derived from an expansion of the specific lymphocyte clone that recognises the tumor-associated antigen expressed on autologous tumour cells but may be due to auto-tumour killer cells (a subset of NK cells) that are present in carcinomatous pleural effusions and alive at the end of autologous MLTC. An alternative possibility is that new auto-tumour killer cells are recruited from the same LGL population without proliferation during autologous MLTC. As LGL have been demonstrated to produce a variety of lymphokines [8], it seems possible that LGL produce lymphokines in response to autologous tumour cells, which in turn are involved in the persistence of auto-tumour killer cells or the recruitment of new cytotoxic cells.

In conclusion, the data presented in this report indicate the existence of two distinct types of auto-tumour-recognising lymphocytes in carcinomatous pleural effusions of cancer patients. Tumour-associated LGL that recognise the NK relevant structure of autologous tumour cells can lyse the target cells in the NK-restricted manner, and their activity can be maintained by co-culture with autologous tumour. Tumour-associated T cells that recognise the tumour-associated antigen on autologous tumour cells cannot kill the target cells, but the clone can expand to express substantial auto-tumour-specific cytotoxicity during in vitro culture with autologous tumour. The mechanism by which effusion tumour cells escape the attack by these tumour-associated effector cells is not understood. Preliminary experiments have revealed the presence of two distinct types of suppressor cells, one type capable of inhibiting auto-tumour cytotoxicity of LGL and another type capable of blocking the expansion of auto-tumour-recognising T lymphocytes.

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