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Suppression by Sorted CD8$^+$CD11b$^-$ Cells from T-cell Growth Factor-activated Peripheral Blood Lymphocytes on Cytolytic Activity against Tumour in Patients with Gastric Carcinoma

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To confirm the phenotypic characteristics of lymphokine-activated suppressor (LAS)effector cells, we isolated CD8$^+$CD11b$^-$ and CD8$^-$CD11b$^-$ cells from T-cell growth factor (TCGF)-activated peripheral blood lymphocytes (PBL) in 7 patients with gastric carcinoma (4 non-resectable and 3 resectable carcinoma) and 3 healthy controls. Sorted CD8$^+$CD11b$^-$ cells from 3 of the patients with non-resectable carcinoma and from 1 of the patients with resectable carcinoma showed LAS cell activity. However, the LAS cell activity could not be observed in CD8$^+$CD11b$^-$ cells from healthy controls. In addition, a sorted CD8$^-$CD11b$^-$ subset of cells from both cancer patients and control did not express any suppressive activity. These facts clearly show that the cell populations responsible for suppression of cell-mediated antitumour immunity reside within CD8$^+$CD11b$^-$ T-cells, at least in patients with advanced carcinoma.
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INTRODUCTION

WE HAVE shown that dominantly expressed CD8$^+$CD11b$^-$ cells within T-cell growth factor (TCGF)-activated peripheral blood lymphocytes (PBL) in patients especially those with non-resectable gastric carcinoma have lymphokine-activated suppressor (LAS) cell activity, irrespective of the presence of lymphokine-activated killer (LAK) cell activity of CD8$^+$ CD11b$^-$ cells in TCGF-activated PBL from normal healthy control subjects [1]. A functional diversity was therefore suggested to exist within CD8$^+$CD11b$^-$ cell populations of TCGF-activated PBL. In order to resolve this question, cell sorting techniques have been requested.

In this study to define the cells responsible for suppression of cytolytic activity against tumour, we carried out sorting techniques to isolate CD8$^+$CD11b$^-$ cells from TCGF-activated PBL in patients with gastric carcinoma. The results showed that the sorted CD8$^+$CD11b$^-$ cells, at least those from cancer patients, exerted an inhibition of cytolytic activity against tumour.

PATIENTS AND METHODS

Patients

7 patients with advanced gastric carcinoma were studied. They were divided into two groups: group I consisted of 4 patients who had advanced non-resectable carcinoma with systemic metastasis (stage IV); and group II of 3 patients who had resectable carcinoma with or without regional lymph-node metastasis and were eligible for radical or non-radical resection (stage III, 1 case; stage IV, 2 cases). Blood samples were obtained from patients before surgery and/or chemotherapy (mitomycin and/or tegafur). The patients ranged in age from 35 to 78 (mean 58.3) years. The control group consisted of 3 healthy volunteers, ranging in age from 44 to 64 years (mean 54.7).

Preparation of PBL and TCGF-containing medium

PBL from heparinised blood samples were separated by standard Ficoll–Hypaque (Pharmacia, Uppsala) density gradient centrifugation described previously [1, 2]. As the TCGF source, we used cultured supernatant from human spleen cells stimulated with 0.08% phytohaemaggulutinin (PHP-P, Difco, Detroit). The details concerning TCGF preparation were presented in a previous paper [1–4].

Cultivation of PBL in the presence of either rIL-2 or TCGF

Approximately 2×10^6 PBL were placed in tissue culture flasks (Falcon, #3013, Becton Dickinson). RPMI-1640 medium supplemented with 10% FCS, 100 μg/ml kanamycin and 50% TCGF preparations or 100 U/ml recombinant interleukin-2 (rIL-2; Shionogi, Osaka) were used to initiate the cultures. The IL-2 activity of 50% TCGF preparation used throughout the experiment was approximately equivalent to the activity of 100 U/ml rIL-2 described previously [1–4]. All cultures were maintained for 14 days in 5% CO_2 in air at 37°C and fed three times per week by change of the culture medium.

Monoclonal antibodies (Mab) and two-colour flow cytometric analysis

Fluorescein isothiocyanate (FITC)-conjugated anti-Leu 2a (CD8), anti-Leu 3a (CD4), anti-Leu 7 (CD57), and phycoerythrin (PE)-conjugated anti-Leu15 (CD11b), anti-Leu 8 and anti-Leu 11 (CD16) were provided by Becton Dickinson. KOLT 2 (CD28) was obtained from Nichirei (Tokyo). Secondary antibody staining was with the PE-conjugated goat anti-mouse IgG antibody (Biomeda, Foster City, California). Freshly isolated and cultured lymphoid cells with TCGF were stained with fluorescein-conjugated Mab for 30 min at 4°C and were washed twice, as described previously [1–4]. The cells stained with

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FITC and PE-conjugated Mab were analysed for two-colour flow cytometry (FACScan, Becton Dickinson).

Two-colour FACS sorting

The activated PBL cultivated in the presence of TCGF for 12 days were separated aseptically into $CD8^+CD11b^-$ and $CD8^-CD11b^-$ subsets of cells using FACS-IV (Becton Dickinson). Purity of each separated cell population was in excess of 95%. Both unfractionated and fractionated cells were cultured in the presence of TCGF for a further 2 days, and then tested for suppressive activity. After the additional incubation, purity of each cell population was rechecked and was in excess of 90%.

Assay for cytolytic activities

Cytolytic activities of rIL-2 activated PBL were tested against human natural killer (NK)-resistant Daudi cells. The cell lines were kindly donated by the Japanese Cancer Research Resources Bank, National Institute of Hygienic Sciences, Tokyo. The rIL-2 induced cytolytic effects of cultured PBL on tumour targets was examined with the use of standard 4 h ^{51}Cr-release assay essentially as described previously [1–4]. Specific lysis was expressed according to the following formula: % of specific ^{51}Cr release ^{51}Cr release = experimental release − spontaneous release) ÷ (maximum release − spontaneous release) × 100. The maximum releasable counts were determined by repeated freezing and thawing of the labelled cells, and amounted to 80–90% of the total radioactivity incorporated into the cells. Spontaneous release determined from target cells incubated in the culture medium was always 5% or less of the maximum release.

Assay for suppressor cell activities of TCGF-activated PBL from patients on the cytotoxic reaction

This was performed according to the method described previously [1, 3]. Briefly, as the source of suppressor cells, the desired numbers of TCGF-activated PBL from patients (for experiments) and normal individuals (for controls) were added to the mixture of 7.5×10^4 rIL-2-activated PBL for effector cells and 1.5×10^4 ^{51}Cr-labelled targets in a final volume of 0.2 ml on the microtitre plates. The mixture was incubated at 37°C for 4 h, and the radioactivity released from the target cells was measured. All assays were of autologous cultivation, namely, the TCGF-activated PBL were obtained from the blood of the same patients as the rIL-2-activated PBL. To standardise results and permit evaluation of the degree of suppression, the following formula was used: suppression (%) = $(1 - \Delta^a/\Delta^b)100$, where Δ^a is the change in the percentage cytotoxicity after the addition of suppressor cell source from patients to the culture of target cells admixed with effector cells, and Δ^b is the change in percentage cytotoxicity of target cells admixed with effector cells without cells of the suppressor cell source.

RESULTS

PBL from cancer patients and normal healthy control groups were be cultured in the presence of TCGF. Figure 1 shows an example of a FACS profile defined by CD8 and CD11b on TCGF-activated PBL from a patient with non-resectable carcinoma (patient 1 in Table 1). The cultured PBL were characterised by markedly increased proportions of $CD8^+CD11b^-$ cells and decreased proportions of $CD8^+CD11b^+$ cells (Fig. 1, upper). The sorting procedure yielded $CD8^+CD11b^-$ populations of at least 95% purity (Fig. 1, lower). TCGF-activated $CD8^+CD11b^-$ and $CD8^-CD11b^-$ cells isolated were tested for their suppressive

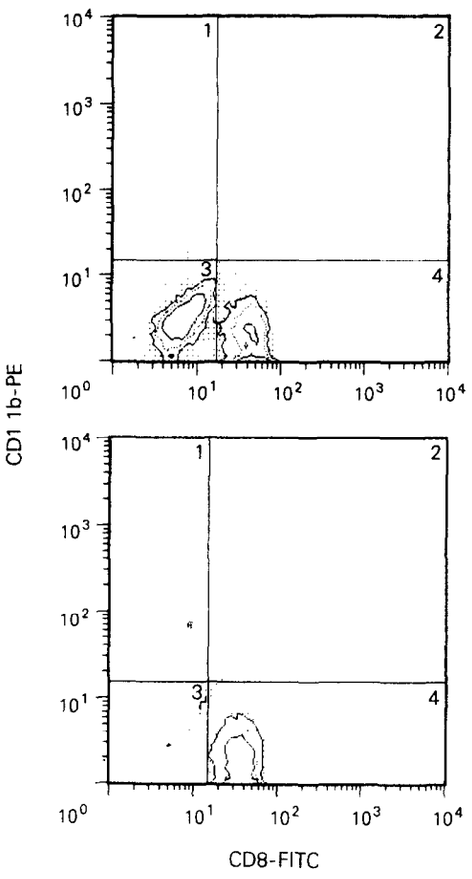

Fig. 1. FACS profiles of cell surface CD8 and CD11b expression in TCGF-activated PBL (upper) and sorted $CD8^+CD11b^-$ cells (lower) from TCGF-activated PBL in patients with gastric carcinoma. Integration of the four subsets: (upper) $CD8^+CD11b^-$, 47.2%; $CD8^+CD11b^+$, 1.2%; $CD8^-CD11b^+$, 1.0%; $CD8^-CD11b^-$, 50.6%; (lower) $CD8^+CD11b^-$, 97.1%; $CD8^+CD11b^+$, 0.4%; $CD8^-CD11b^+$, 0.0%; $CD8^-CD11b^-$, 2.5%.

activity on the tumour lysis by LAK cells which had been activated *in vitro* by rIL-2.

Table 1 shows the summary of results of suppressor cell activity of sorted $CD8^+CD11b^-$ and $CD8^-CD11b^-$ cells for TCGF-activated PBL in patients with carcinoma and healthy controls. The suppressor cell activity was considered to be positive when cytolytic activity with suppressor cell source was significantly ($P < 0.05$, t test) lower than the activity without suppressor cell source. Of 4 patients with non-resectable carcinoma, 3 (patients 1, 2 and 4) showed significant depression of cytolytic activities by the sorted $CD8^+CD11b^-$ cells. In addition, the sorted cells were generally more suppressive than unfractionated TCGF-activated PBL. However, $CD8^-CD11b^-$ cells from patients with non-resectable carcinoma did not show any suppressive reactivity. In patients with resectable carcinoma, sorted $CD8^+CD11b^-$ from 1 case (patient 5) showed suppressor activity, whereas the cells from another 2 cases (patients 6 and 7) were incapable of suppressing the LAK cell activity. The $CD8^-CD11b^-$ cells could not act as suppressor cells. The sorted $CD8^+CD11b^-$ and $CD8^-CD11b^-$ cells from normal controls did not show any ability to inhibit the cytolytic activity of LAK cells.

Unfractionated TCGF-activated PBL from cancer patients and normal healthy control were analysed by FACS (Table 2).

Table 1. Suppressor cell activity of sorted CD8$^+$CD11b$^-$ cells from TCGF-activated PBL in patients with advanced (non-resectable and resectable) gastric carcinoma

Case*	Age/sex/stage†	Cytolytic activity Without SCS	Cytolytic activity With SCS‡	% Suppression	Cytolytic activity with SCS§	% Suppression	Cytolytic activity with SCS‖	% Suppression
1	44/M/IV	37.0 (0.2)**	27.8 (0.8)††	24.9	25.1 (2.0)	32.2	33.4 (2.3)	13.2
2	75/F/IV	18.1 (0.3)	18.1 (0.5)	0.0	4.1 (0.3)	78.4	21.1 (0.3)	−11.1
3	35/F/IV	36.4 (0.7)	33.5 (1.8)	8.0	31.7 (2.4)	12.9	ND	
4	59/M/IV	16.3 (0.3)	18.0 (0.5)	−10.4	11.0 (1.7)	32.5	22.0 (1.9)	−35.0
5	43/F/IV	39.2 (1.4)	40.7 (1.8)	−3.8	30.2 (1.0)	23.0	ND	
6	78/M/III	34.2 (0.8)	37.9 (1.7)	−10.8	34.5 (3.1)	−0.9	33.4 (1.5)	2.3
7	74/M/IV	42.3 (1.0)	40.8 (0.3)	3.5	43.6 (0.5)	−3.1	43.4 (0.7)	−2.6
8	44/F	35.5 (1.2)	33.1 (2.0)	6.8	37.2 (1.3)	−8.4	ND	
9	56/M	38.5 (1.6)	37.1 (1.1)	3.6	40.8 (1.3)	−6.0	38.0 (3.3)	1.3
10	64/M	43.4 (1.4)	47.2 (1.7)	−8.8	44.6 (0.8)	−2.8	51.6 (3.6)	−17.5

SCS = suppressor cell source, ND = not done.
Mean (S.E.).
*Case nos 1–4: non-resectable gastric cancer, 5–7: resectable gastric cancer, 8–10: healthy controls.
†Histopathological stage defined by the General Rules for Gastric Cancer Study [11].
‡Unfractionated TCGF-activated PBL, §sorted CD8$^+$CD11b$^-$ cells from TCGF-activated PBL and ‖sorted CD8$^-$CD11b$^-$ cells from TCGF-activated PBL were used as suppressor cell sources.
**Target cell:killer cell ratio, 1:5; ††target cell:killer cell:suppressor cell source ratio, 1:5:20.

Table 2. Two-colour flow cytometric analysis of PBL and TCGF-activated PBL from healthy controls and patients with advanced gastric carcinoma (resectable and non-resectable)

Phenotype	Non-resectable (n=4) PBL	Non-resectable (n=4) TCGF-activated PBL	Resectable (n=3) PBL	Resectable (n=3) TCGF-activated PBL	Control (n=3) PBL	Control (n=3) TCGF-activated PBL
CD8$^+$CD11b$^-$	15.2 (2.9)	44.1 (2.8)	14.1 (1.9)	51.5 (1.2)	11.8 (4.7)	52.1 (10.0)
CD8$^+$CD11b$^+$	12.5 (1.9)	2.2 (0.9)	10.3 (1.8)	2.3 (0.5)	16.8 (6.2)	1.8 (0.23)
CD8$^-$CD11b$^+$	17.3 (0.8)	5.1 (2.0)	20.3 (1.8)	2.0 (0.4)	18.1 (1.0)	2.1 (0.8)
CD4$^+$Leu8$^-$	7.4 (2.1)	7.3 (1.9)	5.6 (1.4)	14.3 (1.3)	5.2 (0.8)	11.8 (2.8)
CD4$^+$Leu8$^+$	36.9 (5.9)	48.5 (5.5)	36.7 (3.6)	39.3 (5.9)	28.6 (4.1)	33.7 (2.8)
CD4$^-$Leu8$^+$	30.6 (6.4)	24.3 (2.5)	38.0 (3.1)	22.4 (1.4)	39.5 (5.3)	35.1 (9.6)
CD57$^+$CD16$^-$	14.0 (2.4)	2.2 (0.4)	10.2 (2.9)	2.8 (0.1)	6.2 (2.1)	2.4 (0.2)
CD57$^+$CD16$^+$	11.2 (1.2)	0.8 (0.3)	10.2 (2.2)	1.1 (0.2)	16.6 (3.4)	1.8 (0.5)
CD57$^-$CD16$^+$	4.3 (1.8)	0.5 (0.3)	4.5 (1.1)	3.8 (0.5)	4.3 (0.8)	1.7 (0.1)
CD8$^+$CD28$^-$	18.7 (4.8)	32.1 (5.6)	12.7 (3.7)	30.9 (2.4)	17.7 (4.4)	18.2 (3.9)
CD8$^+$CD28$^+$	10.5 (0.9)	16.9 (0.3)	16.8 (4.3)	22.7 (2.1)	13.7 (4.6)	33.5 (11.1)
CD8$^-$CD28$^+$	51.0 (6.5)	43.6 (5.3)	52.3 (4.0)	38.1 (0.4)	46.3 (2.8)	42.8 (9.4)

TCGF preferentially expanded CD8$^+$ cells, especially CD8$^+$CD11b$^-$ cells, but it did not enhance growth of the CD4$^+$, CD57$^+$ and CD16$^+$ cells. However, another prominent feature was the increased proportion of CD8$^+$CD28$^-$ cells in TCGF-activated PBL from patients with carcinoma. The percentage of CD8$^+$CD28$^-$ cells in patients significantly increased compared with that in normal healthy controls ($P < 0.05$). The CD8$^+$CD28$^-$ cells from normal healthy controls were not increased by cultivation in the presence of TCGF. In contrast, the percentage of control CD8$^+$CD28$^+$ cells was increased by cultivation in the presence of TCGF, while that of TCGF-activated CD8$^+$CD28$^+$ cells from patients with non-resectable carcinoma was not greatly increased.

DISCUSSION

In the present study, we isolated CD8$^+$CD11b$^-$ cells from TCGF-activated PBL from patients with gastric carcinoma, and showed that the sorted CD8$^+$CD11b$^-$ cells from patients, especially those with non-resectable carcinoma, have LAS cell activity.

Our previous studies were consistent with the observation that CD8$^+$CD11b$^-$ cells from PBL and spleen cells responded preferentially to TCGF, and that the CD8$^+$CD11b$^+$ subset of the cells responded poorly [1–3]. Furthermore, functional studies showed that TCGF-activated PBL with the main proportion of CD8$^+$CD11b$^-$ from patients with cancer had LAS cell activity [1]. Suppression was not seen in TCGF-activated

PBL from healthy controls [1] and in TCGF-activated splenic lymphoid cells [3] with a main surface phenotype of $CD8^+CD11b^-$ from patients with advanced carcinoma. In addition, TCGF-activated malignant pleural and peritoneal effusion-associated lymphoid (EAL) cells mixed with tumour cells from advanced carcinoma showed cytolytic activities against allogeneic and/or autologous tumour [4]. It was suggested that cytolytic activities of the EAL cells activated in the presence of TCGF might be mediated by $CD8^+CD11b^-$ effector cells. Great caution, of course, must be paid to assessing the relationship between the lymphocyte phenotype and its function. Maximally expanded populations of cells with a surface phenotype of $CD8^+CD11b^-$ in TCGF-activated cells cannot simply be assumed to have cytotoxic or suppressor function.

To clarify the phenotypic analysis of the LAS cells within TCGF-activated PBL, we undertook to sort $CD8^+CD11b^-$ cells and assayed their LAS cell function. Analysis and separation using the FACS involve difficult problems in retaining viability, and preserving subsistent function of the lymphocytes. In order to obtain the high purity of the desired cell population in excess of 95% presented here, a low cell flow rate was designed. However, it increased the time needed to obtain the necessary number of sorted cells. Furthermore, intense laser illumination was irradiated over the part of the jet through which cells pass. This resulted in serious problems in maintaining the cells' viability during the processing. Indeed, sorted $CD8^+CD11b^-$ cells which had been isolated by FACS-IV did not show any LAS cell activity, in spite of expression of LAS cell activity by unfractionated TCGF-activated PBL from the patient (data not shown). The sorted cells, therefore, were cultured in the presence of TCGF for a further 2 days to recover and the LAS cell function was determined. Since desired numbers of sorted $CD8^+CD11b^-$ and $CD8^-CD11b^-$ cells could not always be examined sufficiently, we could not use a 1:40 target: suppressor cell ratio, as described previously [1, 3]. For these reasons, 1:20 target:suppressor cell ratios were employed to assay the LAS cell activity. Consequently, it appears to be limited by the modest degree of suppression.

Clement and colleagues [5, 6] reported that sorted $CD8^+CD11b^+$ cells inhibited the proliferative responses to soluble antigens and/or mitogens and also suppressed the PWM-induced B-cell differentiation. In this study, we could not isolate $CD8^+CD11b^+$ cells nor examine the LAS cell activity, since $CD8^+CD11b^+$ in PBL could not expand, and decreased in proportion during the cultivation in the presence of TCGF (Table 2). Takeuchi *et al.* [7] showed that suppression of PWM-induced IgG synthesis could be elicited in both $CD8^+CD11b^-$ and $CD8^+CD11b^+$ cells. They also observed that Con A-induced suppressor cells were primarily found in the $CD8^+CD11b^-$ subsets of cells. Thus, some of their findings accords with our data that suppressor–effector cells are mediated by the $CD8^+CD11b^-$ cells. However, Clement and colleagues [5, 6] and Takeuchi *et al.* [7] did not determine whether or not $CD8^+CD11b^-$ and/or $CD8^+CD11b^+$ cells suppress the cytolytic activity directed against tumour cells. $CD8^+CD11b^+$ cells have been shown to have NK characteristics in their function [7]. Also, $CD8^+CD11b^+$ cells contain a vast majority of the cells with NKHl phenotype and large granular morphology [7]. Furthermore, Uchida and Klein [8] stated that large granular lymphocytes (LGL) suppressed T-cell response in autologous mixed lymphocyte-tumour culture (MLTC) and that LGL-mediated suppression was totally abrogated by CD16 plus complement. However, once autotumour cytotoxic T-cells were generated in autologous MLTC, their cytotoxicity was no longer inhibited by LGL. It might be therefore considered that $CD8^+CD11b^+$ cells do not have a possibility to inhibit the cell-mediated cytotoxicity of the *in vitro* system presented here. Further studies are needed to clarify the cellular basis of the generation of suppressor cells within $CD8^+CD11b^+$ cells. Nevertheless, our data demonstrate that sorted $CD8^+CD11b^-$ cells but not $CD8^-CD11b$ cells inhibit the effector process of tumour cell lysis by LAK cells which had been activated *in vitro* by rIL-2. Thus, the cell populations responsible for suppression of LAK cell activity belong to the T-cell subset of $CD8^+CD11b^-$.

$CD8^+CD28^-$ cells have been postulated as markers of suppressor T-cells [9, 10] which exert suppression of immunoglobulin production. Indeed, the suppressor cell activity of cells as related to the increased $CD8^+CD28^-$ cells as well as $CD8^+CD11b^-$ populations was shown in patients with carcinoma (Tables 1 and 2). In contrast, the proportion of $CD8^+CD28^-$ cells from healthy controls remained unchanged by cultivation in the presence of TCGF (Table 2). The facts suggest that the majority of suppressor-effector cells which act as inhibitors of cell-mediated cytolytic activity also reveal the $CD8^+CD28^-$ phenotype at population level. In order to be convincing, however, cell-sorting techniques must be needed further.

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