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Generation of T Cell Growth Factor (TCGF)-dependent Splenic Lymphoid Cell Line with Cell-mediated Immunosuppressive Reactivity against Syngeneic Murine Tumor*

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Abstract—*Splenic T cells obtained from tumor-bearing mice could be cultured with T cell growth factor (TCGF) for over* 12 months. *The TCGF-dependent lymphoid cell line strongly inhibited cell-mediated anti-tumor immunity directed against syngeneic tumor. However, the suppression was non-specific for the given tumor. The cell line expanded with TCGF expressed a phenotypic characterization of T cells defined by monoclonal anti-Thy-*1.2 *antibody.*

INTRODUCTION

THE DISCOVERY of T cell growth factor (TCGF), which permits continuous T lymphocyte cultures *in vitro* [1], provides a potential opportunity for establishing T cell lines *in vitro* derived from cells sensitized to a variety of antigen. Many investigators have reported that a cytotoxic T cell line against tumor could be established by using TCGF [2-9]. However, there are only a few reports concerning a TCGF-dependent suppressor cell line in human [10, 11] and in mouse [12, 13] tumors.

In this report we noted that a TCGF-dependent splenic lymphoid cell line obtained from tumor-bearing mice could be serially grown *in vitro* in large numbers with maintenance of cell-mediated immunosuppressive function. Furthermore, several phenotypic characteristics and descriptions of some of the functional properties of one cell line are also reported.

MATERIALS AND METHODS

Animals

A/J mice maintained in our animal facility were used throughout this study at 6-8 weeks of age.

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Tumors

Methylcholanthrene-induced fibrosarcoma S1509a and SaI of A/J mice origin were used. The cell lines were maintained *in vitro* in stationary suspension culture in RPMI-1640 medium (Grand Island Biological Co., Grand Island, NY) supplemented with 5% FCS (Flow Laboratories, McLean, VA) and kanamycin (100 μg/ml; Meiji Seika Co., Tokyo, Japan).

Preparation of TCGF

Preparations containing TCGF were obtained from 48-hr tissue culture medium (RPMI-1640 with 5% FCS and 5×10^{-5}M 2-mercaptoethanol) and cultures of concanavalin A (Con A, 5 μg/ml, Sigma) stimulated spleen cells of normal rats (Sprague-Dawley). The preparations combined with a final concentration of 20 mg/ml of α-methyl-D-mannoside (Sigma) were filtered through a 0.45-μm filter (Millipore Corp.) to remove subcellular particles before use in long-term culture. These preparations were used for experiments as TCGF.

Cell culture with TCGF

A/J mice were injected subcutaneously (s.c.) with 1×10^6 S1509a cells. Twenty-one days after inoculation, lymphoid cells obtained from spleen were passed through a nylon-wool column, according to the method of Julius *et al.* [14], to enrich a T-cells population. Approximately 2×10^6 cells passing through the column were placed

in tissue culture flasks (Nunc, No. 16337, Denmark). RPMI-1640 medium supplemented with 10% FCS, 100 μg/ml of kanamycin and 50% TCGF preparation, which will be referred to hereafter as the complete medium, was used to initiate the cultures. All cultures were incubated in 5% CO_2 in air at 37°C and fed three times a week by changing half of the complete medium.

Assay for suppressor cell activity in vitro

This has been previously described in detail [15-18]. Target cell lysis was expressed as percentage of ^{51}Cr release calculated by the following formula:

$$\%\ \text{of}\ ^{51}Cr\ \text{release} = \frac{\text{release in test} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

Winn test and its inhibition test

In vivo suppressive activity of TCGF-dependent lymphoid cells was made by an inhibition test of tumor neutralization, the Winn test [19]. Briefly, groups of mice were injected s.c. with 0.5×10^6 viable cells of S1509a or 0.5×10^6 S1509a cells with 20×10^6 cytotoxic lymphoid cells. Preparation of the cytotoxic lymphoid cells has previously been described in detail [15-18]. Another group of mice was inoculated s.c. with 0.5×10^6 S1509a cells mixed together with 20×10^6 cytotoxic lymphoid cells and 20×10^6 TCGF-dependent lymphoid cells. Each group consisted of seven animals. Tumor size was measured by Vernier calipers and expressed as the product of two perpendicular diameters in cm^2. The mean tumor size in each group and the standard error of the mean are indicated in Fig. 1.

Analysis of phenotype of the cells by monoclonal antibody

Immunofluorescence staining of TCGF-dependent lymphoid cells with mouse monoclonal antibody was performed directly using fluorescein isothianate (FITC)-conjugated anti-Thy-1.2 antibody (catalog No. 1333, Becton Dickinson, FACS system, Mountain View, CA). Flow cytometry of stained cells was performed on a fluorescence-activated cell sorter (FACS-IV, Becton Dickinson, FACS system, Mountain View, CA), as described by Herzenberg [20].

RESULTS

Splenic cells obtained from tumor-bearing mice have been grown over 12 months and continue to thrive. Once growth was established, cell numbers expanded by about 7- to 10-fold every 7 days. The cells grown in complete medium died within 48 hr if switched to fresh medium not containing TCGF or if switched to fresh medium supplemented with Con A alone (data not shown). These results reveal that continued growth of the cells is dependent on the presence of TCGF. After the maintenance of cultures in exponential growth for over 12 months, suppressor cell activity of TCGF-dependent lymphoid cells was assayed. As shown in Table 1, when the cells were added to the mixture of ^{51}Cr-labeled target cells and cytotoxic lymphoid cells the cells significantly suppressed the cytotoxic T cell activity compared with normal cells ($P < 0.001$). The results indicate that the cells grown in our system possess a cell-mediated immunosuppressive function. This was proved true in *in vivo* experiments (Fig. 1). When S1509a cells mixed with cytotoxic T cells were given to normal syngeneic mice the growth of tumor significantly decreased compared with that in mice injected s.c. with S1509a cells without cytotoxic T cells. However, the growth of tumor in mice receiving a mixture of S1509a cells, cytotoxic T cells and TCGF-dependent lymphoid cells was identical to the growth in mice receiving only S1509a cells.

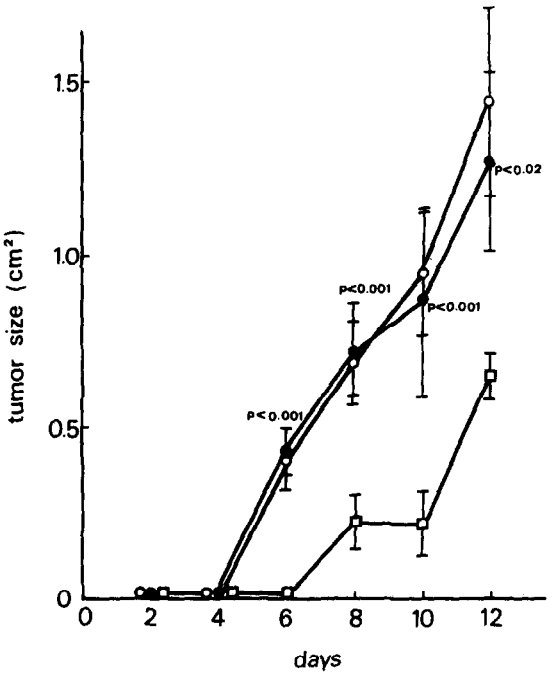

Fig. 1. Tumor neutralization test (Winn assay) and its inhibition by TCGF-dependent lymphoid cells. S1509a cells (5×10^5) *were transferred either alone* (●) *or with cytotoxic T lymphocytes (Tc)* (□), *or with Tc and TCGF-dependent lymphoid cells* (○) *to normal A/J mice. Tumor:Tc:TCGF-dependent lymphoid cells ratio* = 1:40:40. *Each group consisted of seven animals. Tumor size was measured by Vernier calipers and expressed as the product of two perpendicular diameters in* cm^2. *The mean tumor size* ± *S.E. in each group was plotted. Statistical significances between the tumor-Tc group and tumor-Tc-TCGF-dependent lymphoid cells were calculated by the Student's* t *test and expressed by* P *values.*

Table 1. Suppression of cytotoxic T cell activity by TCGF-dependent lymphoid cells

Tc*	Suppressor source from:	% ^{51}Cr release		
		1:40:20†	1:40:40†	1:40:80†
+	none	44.8 ± 1.7‡	44.8 ± 1.7	44.8 ± 1.7
+	normal spleen cells	36.6 ± 4.1	32.2 ± 0.1	36.3 ± 2.0
+	TCGF-dependent lymphoid cells	14.9 ± 0.8	4.6 ± 1.8	2.2 ± 0.9

*Cytotoxic T cells (Tc) against S1509a cells were prepared from spleen cells of mice immunized with 1 × 10^6 mitomycin C-treated S1509a cells 10 days before and reactivated by cultivating with mitomycin C-treated homologous tumor *in vitro* for 5 days.

†Target:Tc:TCGF-dependent lymphoid cells ratios.

‡Mean ± S.E. of quadruplicate cultures.

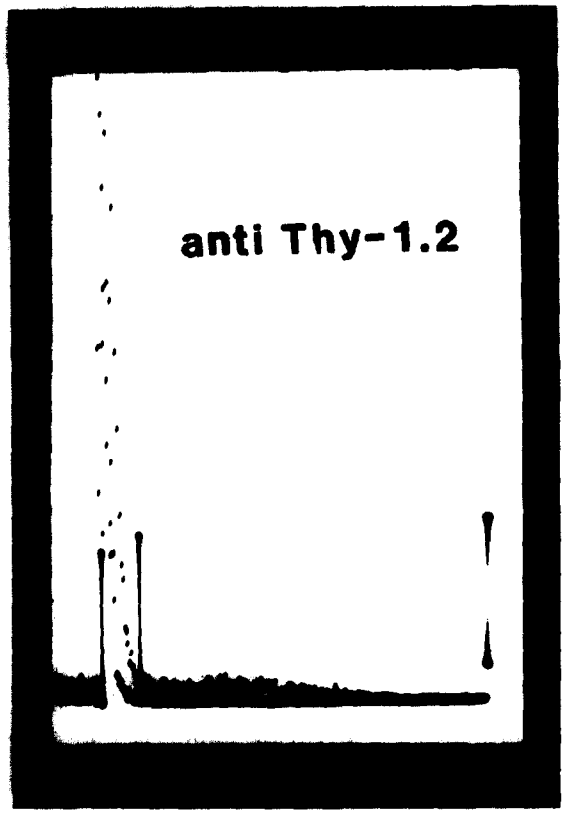

Fig. 2. Immunofluorescence staining of TCGF-dependent splenic lymphoid cells with anti-Thy-1.2. The cells were stained with directly fluorescein-conjugated monoclonal anti-Thy-1.2 antibody. The background (unstained cells) is also superimposed in the panel. Fluorescence distribution was analyzed on FACS-IV fitted with a linear unit. A vertical axis shows a relative number of cells and a horizontal axis shows fluorescence intensity.

The results show that the TCGF-dependent lymphoid cells strongly inhibit the suppression of tumor growth caused by cytotoxic cells. Specificity of the immunosuppressive activity of the cells was studied. As shown in Table 2, the cytotoxic reaction of both S1509a and SaI in the cytotoxic T cells activated by S1509a was strongly inhibited by the TCGF-dependent lymphoid cells. In addition, when the TCGF-dependent lymphoid cells were added to the mixture of cytotoxic T cells against SaI and target SaI cells the cytotoxic reaction was also suppressed. These results suggest that the suppression is non-specific for the given tumor.

Experiments were further carried out to determine whether the cells possess T cell surface markets. Figure 2 shows FACS analysis of Thy-1.2 determinant expression on the cultured cells detected by direct staining with FITC-conjugated monoclonal antibody.

DISCUSSION

In this paper we describe the establishment of a TCGF-dependent lymphoid cell line that exhibits *in vitro* and *in vivo* immunosuppressor cell activity against syngeneic tumor. Fleisher *et al.* [10] reported that PBL obtained from a patient with a non-leukemic form of myocosis fungoids could be cultured with condition medium. They stated that an established suppressor T cell line inhibited human polyclonal B cell immunoglobulin biosynthesis, and a possible mechanism of the suppressor T cells was by the generation of a lectin-like suppressor lymphokine. On the other hand, Lamb and Feldmann [11] showed the generation of TCGF-dependent human suppressor T cell clone which recognized and inhibited the function of a helper T cell clone, and argued that the specificity of the suppressor cell clone was for the antigen receptor of the helper cell. Fresno *et al.* [12] reported that Ly-2^+ suppressor T cell clones expressed surface receptors specific for glycoprotein from sheep erythrocytes (SRBC). They held that the suppression by the antigen-binding peptides reflected direct inhibition of T helper activity. The inhibitory mechanisms of the TCGF-dependent suppressor T cells reported by these investigators [10-12] did not involve cell cytotoxicity. In our data splenic lymphoid cells maintained in the long term with complete medium strongly

Table 2. TCGF-dependent lymphoid cell-suppressed cytotoxic T cells activity of various cytotoxic T cells-target combinations

Target cells	Cytotoxic T cells activated by:	Suppressor source from:	% ^{51}Cr release* (mean ± S.E.)
S1509a	S1509a	none	70.3 ± 3.2
		normal spleen cells	66.0 ± 2.2
		TCGF-dependent lymphoid cells	0.0 ± 0.9
SaI	S1509a	none	62.3 ± 0.13
		normal spleen cells	41.3 ± 0.9
		TCGF-dependent lymphoid cells	0.0 ± 1.5
SaI	SaI	none	30.7 ± 3.6
		normal spleen cells	40.6 ± 4.1
		TCGF-dependent lymphoid cells	0.0 ± 1.4

*Target:cytotoxic T cells:suppressor source = 1:40:40.

abrogate the effect of cytotoxic T cells on target tumors. Activity of suppressor cells are about 5-10 times stronger than previously reported results of activity of spleen cells in tumor-bearing mice [15-18]. The results are essentially different from other studies [10-12].

Recently, Roberts *et al.* [13] established a continuous murine suppressor T cell line (UV1 cells) which was capable of suppressing anti-tumor immune responses both *in vivo* and *in vitro*, and the UV1 cells showed a number of T lymphocyte differentiation antigens and did not reveal any detectable amounts of surface immunoglobulin, I-A or E/C antigens, Fc receptors or macrophage antigens. However, this study did not determine a complete characterization of the functional properties and antigenic specificity of the UV1 cell line. The TCGF-dependent lymphoid cells in our study were derived from a T cell-enriched spleen cell population from S1509a cell-bearing mice. The cells did not ingest latex particles *in vitro* and also were not histochemically stained for non-specific esterase as detected by the method of Yam *et al.* [21] (data not shown). Thus the cells are not non-phagocytic and non-macrophagic. By flow cytometric analysis it was determined that our cell line showed Thy-1.2 antigen. These data reveal that the cell line is derived from T cell lineage.

It has been shown previously by one of the authors [15] that with two closely related sarcomas, S1509a and SaI, the cytotoxic T cells activated by either S1509a or SaI could kill both tumors equally well, whereas suppressor T cells generated in the hosts bearing either one of these tumors specifically inhibited the cytotoxic reaction against the homologous but not cross-reactive tumor target cells. In the present study, however, TCGF-dependent lymphoid cells showed functional suppressor activity directed toward tumor antigens shared by S1509a and SaI cells. The results suggest that specificity and characteristics of TCGF-dependent lymphoid cells with immunosuppressive reactivity are different from those of suppressor T cells observed in S1509a-bearing mice. The cell line established in ours may be heterogeneous in nature regarding its ability to respond to a wide range of tumor antigens shown by S1509a cells. In addition, although the cell line reveals a Thy-1.2 antigen, it may lose useful markers for defining functional subsets of T cells as a result of long-term cell culture using a TCGF. Consequently, the cell might be unable to recognize unique antigenic determinants possessed by the individual tumors and might non-specifically suppress the cytotoxic reaction for the syngeneic tumors. Clearly, further experimentation is required to establish a complete antigenic specificity of our cell line. Nevertheless, data presented here demonstrate that our T cell line strongly suppresses cell-mediated anti-tumor immunity *in vitro* and *in vivo*. Accordingly, the cell line established in our laboratory would be useful for research on the function of suppressor cells in tumor immunology.

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