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A Murine Thymic Stromal Cell Line which Supports the Development of $CD4^+8^-$ Helper T Cells

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Abstract TSt-4 is a fibroblastoid cell line derived from fetal thymus tissue of C57BL/6 mice. TSt-4 cells express neither class I nor class II major histocompatibility (MHC) antigens but expresses class II antigens after stimulation with γ-interferon. When $CD4^-CD8^-$(DN) cells of young adult thymuses were cultured on the monolayer of TSt-4, some of the cells attached on or crawled under the layer. About 15% of recovered cells after cultures were $CD4^+CD8^-$ while the rest remained to be DN, and almost all of these cells expressed CD3 antigen. These cells showed helper activity in polyclonal immunoglobulin production by B cells. TSt-4 cells were found to express interleukin-7 (IL-7) mRNA among interleukins examined. It was also found that the addition of recombinant IL-7 into the cultures of DN cells resulted in a marked increase of cell recovery and the development of $CD4^+8^-$ cells, showing that IL-7 is mainly involved in the generation or proliferation of $CD4^+CD8^-$ cells from DN cells. These results strongly suggest that TSt-4 retains an activity of the thymus to support the differentiation of immature DN thymocytes into functional mature T cells, in which IL-7 produced by TSt-4 is involved.

INTRODUCTION

The development of T cells from progenitors of extrathymic origin is crucially dependent upon the thymic microenvironment (Moore and Owen,1967; Marrack et al., 1988; Katsura et al., 1989). Although recent studies have succeeded to identify the cells responsible for important events in the late phase of T cell development, which include positive (Sha et al., 1988; Marusic et al., 1989) and negative (Spiser et al., 1989; Mazda et al., 1991) selection, only little is clarified about the mechanisms underlying the early phase of T cell development such as commitment to the T cell lineage or induction of differentiation. An important step to reveal the mechanism is to identify the stromal cells involved. Normal development of T cells from thymic as well as prethymic progenitors occurs in deoxyguanosine-treated fetal thymic lobes (Ready et al., 1984; Watanabe et al., 1989), which is mainly composed of epithelial and fibroblastoid cells. Primary cultures of thymic stromal cells as well as epithelial and fibroblastoid cell lines derived from these cultures have been investigated for their inducibility of T cell development (Denning et al., 1988; Ranson et al., 1987; Eshel et al., 1990; Tatsumi et al., 1990; de la Hera et al., 1989).

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No evidence, however, has been found that these cells are able to induce normal T cell development from progenitors in *in vitro* cultures, suggesting the importance of the thymic architecture constructed by different types of cells. Nevertheless, some of the fibroblastoid or epithelial lines have been shown to support restricted step(s) of T cell differentiation. For example, Tatsumi et al. (1990), showed that cultivation of $CD4^-CD8^-$ (double negative, DN) cells on the monolayer of a stromal cell line resulted in the generation of $CD4^-8^+$ single positive cells followed by the generation of $CD4^+8^+$ (double positive, DP) cells. A similar result was also shown by Imhof et al. (1988). Recent work by Nishimura et al. (1990) indicated that a nurse cell-like cell line in combination with recombinant interleukin (IL)-2 and anti-CD3 mAb was able to induce the maturation of DP cells into $CD4^-8^+$ cells. On the other hand, no stromal cell line is reported which supports the generation of $CD4^+8^-$ cells.

Recently, Watanabe et al. established a thymic stromal cell line named TSt-4 (Watanabe et al., submitted). In the present work, we used this fibroblastoid cell line, which expresses neither class I nor class II antigen but expresses class II antigen after exposure to γ-interferon. Cultivation of DN cells on the monolayer of TSt-4 resulted in the generation of functional $CD4^+8^-$ T cells but not $CD4^-8^+$ T cells. The role of thymic fibroblast in T cell differentiation was discussed.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from the Japan SLC Co. Ltd. (Shizuoka, Japan).

Culture medium

For cell suspensions and treatment of cells with antibodies plus complement, Eagle's minimal essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 5% fetal calf serum (FCS) (M. A. Bioproducts, MD, USA) was used. For culturing thymic stromal cells RPMI-1640 medium (Gibco Laboratories, NY, USA) supplemented with 5% FCS, 50 μg/ml streptomycin, 50 U/ml penicillin, and 2-mercaptoethanol (50μM) was used. The same medium was also used for assaying the helper activity.

Antibodies

Anti-Thy-1.2 mouse monoclonal antibody (mAb) (HO-13-4), anti-L3T4 mouse mAb (GK1.5), anti-major histocompatibility complex (MHC) class I mouse mAb specific for H-$2K^b$ (B8-24-3), anti-class II mouse mAb specific for I-$A^{b,d,q}$ and I-$E^{d,k}$ (M5/114.15.2), rat mAb directed to a surface antigen of mouse macrophage and dendritic cell (Mac-1) were from the American Type Culture Collection (MD, USA). Rat mAb specific for cortical epithelial cells of mouse thymus (Th-3) were donated by Dr. K. Hirokawa, Tokyo Metropolitan Institute for Gerontorogy (Hirokawa et al., 1986). Anti-L3T4 rat mAb (RL172.4) (Ceredig et al., 1985) was donated by Dr. H. R. MacDonald (Ludiwig Institute for Cancer Reserch, Lausanne, Swizerland), and anti-CD3 hamster mAb (145-2C11) (Bluestone et al., 1987) was donated by Dr. J. A. Bluestone (Ben May Institute, University of Chicago, USA). Anti-Thy-1.2 and anti-hamster Ig were used as purified fluoresceinated (FITC) proteins which were prepared as described previously (Katsura et al., 1985). FITC-goat anti-mouse Ig and FITC-goat anti-rat Ig were purchasad from Cappel Laboratories

(PA, USA). Phycoerythrin (PE)-conjugated rabbit anti-rat Ig was purchased from Biomeda Co., CA, USA.

Cell suspensions and treatment of cells with antibodies plus complement

DN thymocytes was obtained by treating the thymus cells twice with a mixture of anti-L3T4 (culture supernatant of hybridoma RL172.4) and anti-Lyt2.2 (culture supernatant of hybridoma HO-2.2) plus selected rabbit complement (1/20). Cell recovery was about 0.5%. More than 99% of the lymphoid cells recovered after the treatment are DN. Stromal cells were suspended in Eagle's minimum essential medium containing EDTA (1mM), washed twice, and then used for staining with mAb.

Fluorescence staining and fluorescence-activated cell analysis

The basic methods for staining and analysis have been described in detail elsewhere (Katsura et al., 1989). All procedures were carried out at an ice-water temparature. Cells to be stained (2 to $5x10^5$ cells) were pelleted, suspended in 50μl of first stage antibodies (the culture supernatant of hybridomas), and incubated for 30 min in small conical plastic tubes. They were washed, resuspended in medium containing fluorescinated second stage antibodies. In the case of anti-Thy1.2, cells were directly stained with FITC-conjugated anti-Thy-1.2. Stained cells were washed, passed through nylon mesh (pore size; 40μm), and analysed with a flowcytometer FACScan (Becton-Dickinson Co. Ltd., Mountain View, CA) or ABCAS-100 (Showa Denko Co. Ltd., Tokyo, Japan) gated to exclude nonviable cells.

Assay for helper activity

Helper activity was assayed as Concanavalin A-mediated polyclonal Ig production by B cells as described previously (Kina et al., 1987). Cells used for helper source were mixed with $5x10^4$ splenic B cells from C57BL/6 mice, and cultured for 5 days in the presence of Concanavalin A (5μg/ml). IgM concentration in the culture supernatant was determined by radioimmunoassay (Tsubata et al., 1988).

Hemopoietic colony assay

Colony assay was carried out as described by Nakahata et al. (Nakahata et al., 1982). Semisolid methylcellulose cultures consisted of 0.8% methylcellulose (4,000 cps, Muromachi Kagaku Kogyo, Tokyo, Japan) in alpha medium (Gibco Laboratories) supplemented with 20% FCS. One ml of the culture medium containing $2x10^4$ bone marrow cells and 100 units of recombinant IL-3 (donated by Dr. Y. Hirai, Otsuka Phamaceutical Co., Tokushima, Japan) or the culture supernatant of TSt-4 (20%) was placed into a 35 mm petri dish. Colonies were scored on day 8 under a inverted microscope.

Northern blot analysis

Total cellular RNA was prepared from TSt-4 cells, TEC1-4C18 cells, and poke-weed mitogen stimulated spleen cells according to the method described by Chirgwin et al. (1979). Poly(A)$^+$RNA was purified by passing through a oligo-dT-cellulose column as described in the standard protocol (Sambrook et al., 1989). Poly(A)$^+$RNA samples (2μg each) were subjected to electrophoresis on a formaldehyde/1% agarose gel and transferred

to a Gene-Screen Plus nylon membrane (New England Nuclear, Boston, MA). The filter was hybridized with cDNA probes (kindly donated by Dr. T. Sudo). Filter hybridization and washing conditions were as described (Gyotoku et al., 1991).

RESULTS

Characterization of stromal cell lines

As a thymic stromal cell line, we used TSt-4 in this study, while as a control, TSt-10 derived from thymus was also used. Both cell lines were established in the Chest Desease Research Institute of Kyoto University (Watanabe et al., submitted) and donated by Dr. Watanabe.

The morphology of these cell lines are shown in Fig. 1. Both are apparently very

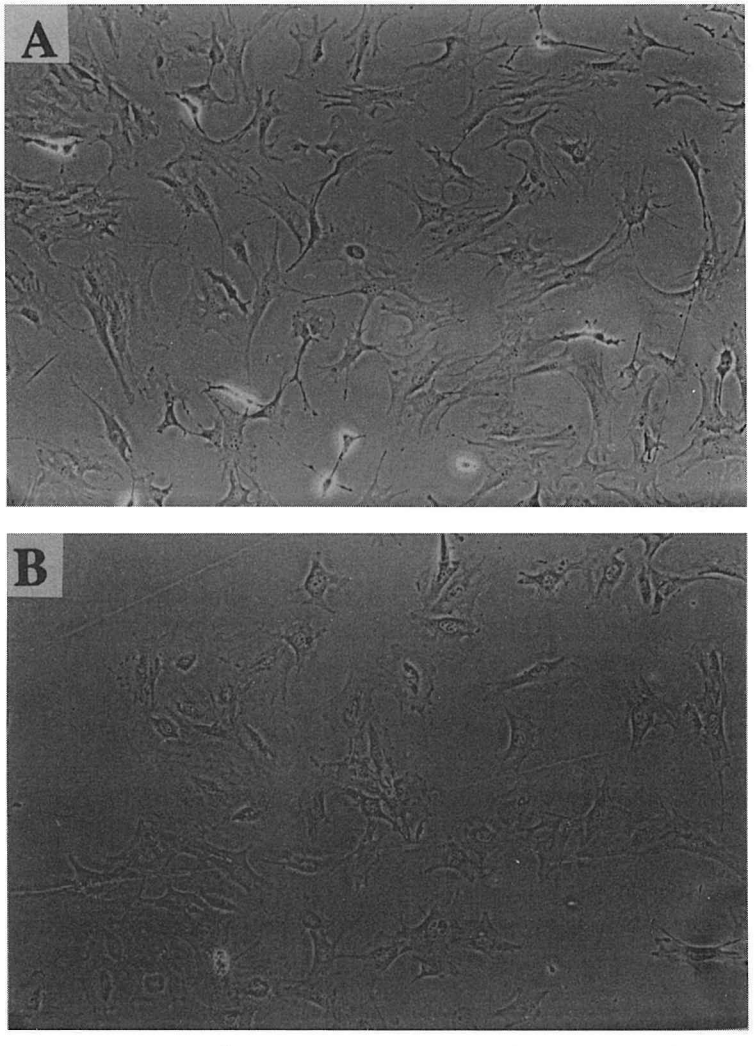

Fig. 1. Phase contrast micrographs of TSt-4 (A) and TSt-10 (B) cultures (X200).

similar, and judged to be fibroblastoid. Immunohistochemical and electron microscopical investigation of TSt-4 indicated that this cell line is negative for cytokeratin, desmosome and tonofilament (K. Hirokawa, unpublished observation), and positive for vimentin (S.

Table 1. Phenotypes of thymic stromal cell lines[1)]

Surface antigen	TSt-4	TSt-10
Thy-1	−	+
class-1	−(−)[2)]	+(+)
class-2	−(+)	−(+)
Th-3	−	−
Th-4	+	+
Mac-1	−	−
NLDC-145	−	−
Sca-1	+	+
TER	−	−
M1/69	+	+
pgp-1	+	+
N-CAM	+	+

[1)] Phenotypic characterization of TSt-4 and TSt-10 was done by flow cytometry analysis

[2)] Values parentheses represent those determined after treatment with recombinant γ-interferon (100 units/ml for 3 days) which was donated by Dr. T. Sudo (Biomaterial Research Institute, Kanagawa, Japan).

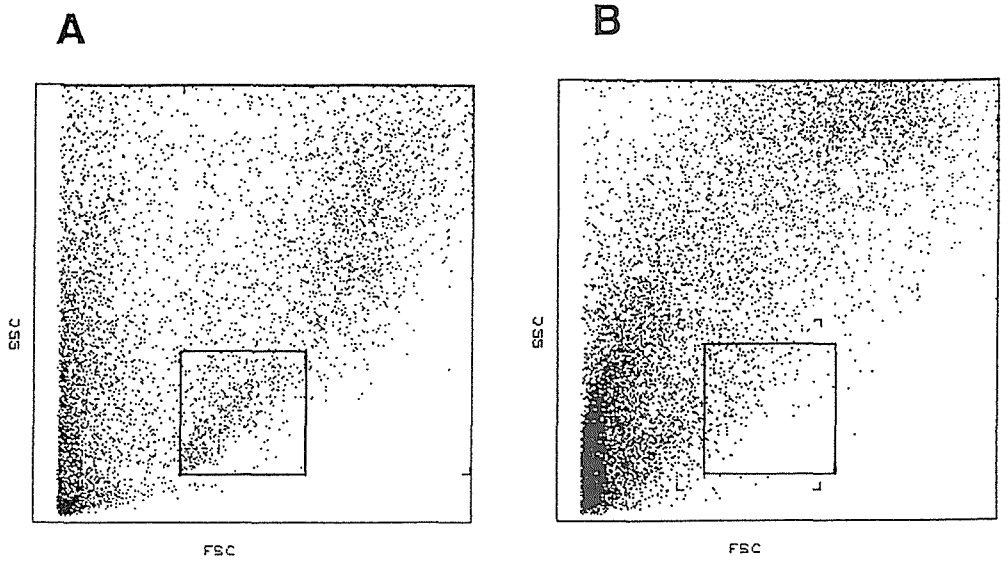

Fig. 2. Foward and side scatter analysis of cells recoverd from cultures of thymic DN cells on TSt-4 or TSt-10. DN cells were seeded on the monolayers of stromal cells in 24-well plates and cultured for 1 week before assay. A large proportion of living cells recovered from cultures on TSt-4 distributed on the lymphoid fraction (A), whereas the majority of living cells recovered from cultures on TSt-10 distributed on the macrophage fraction (B).

Table 2. Cell recovery in cultures of adult DN cells on the monolayer of stromal cell lines[1)]

Culture days	Recovered cells (%)		
	Medium alone	TSt-4	TSt-10
1	100	50	36
3	7	10	12
6	0.5	6	2

[1)] DN cells were cultured alone or on the monolayer of stromal cell lines TSt-4 or TSt-10 in 24-well plates. Lymphoid cells were recovered by pipetting, washed, and viable lymphoid cells were enumerated by trypan blue dye exclusion.

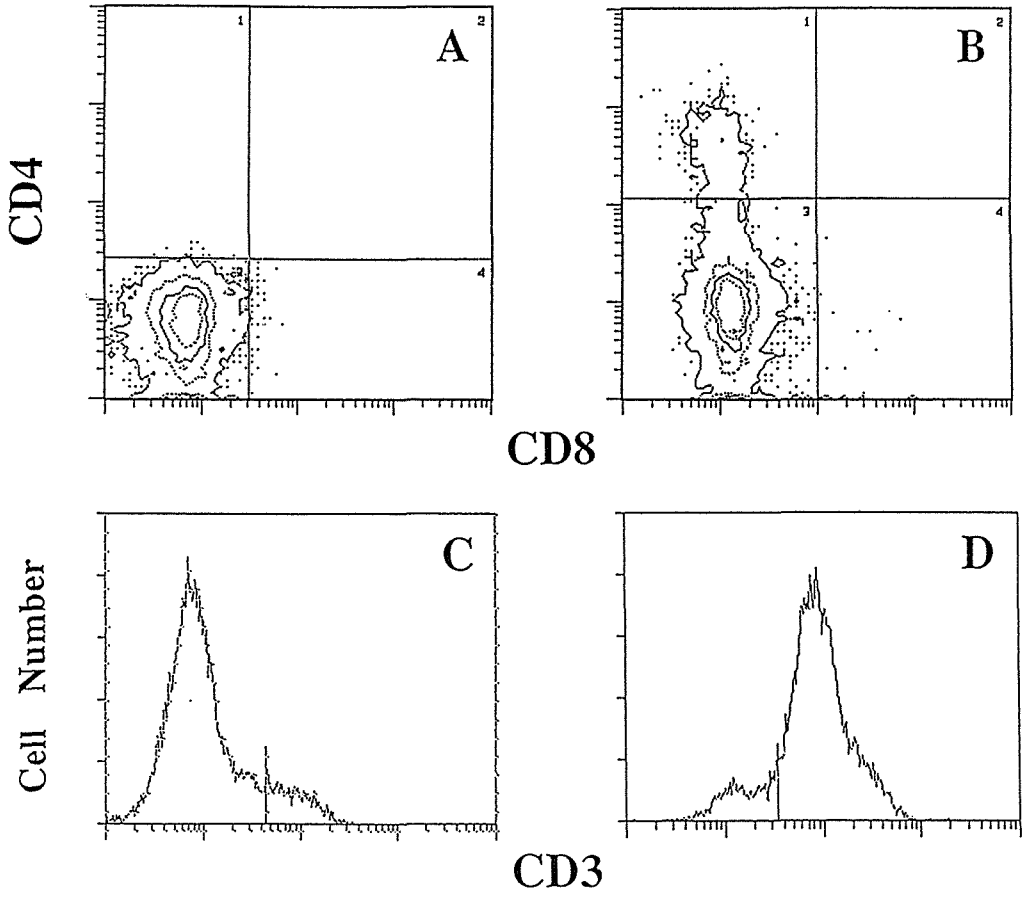

Fig. 3. Development of $CD4^{+}8^{-}$ cells by culturing thymic DN cells on the monolayer of TSt-4. DN fraction of thymocytes used for seeding on TSt-4 layrer (A, C), and cells recovered 1 week after culturing DN cells on TSt-4 monolayer (B, D) were analysed for CD4 vs CD8 expression (A, B) and CD3 expression (C, D).

Ideyama, unpublished observation).

Cell surface properties of TSt-4 and TSt-10 were investigated using a panel of monoclonal antibodies (mAb), and the results are summarized in Table 1. It is unlikely that TSt-4 and TSt-10 are macrophages or dendritic cells, because they do not express antigens specific for these cells, and are negative for nonspecific esterase activity (data not shown). These lines are negative for mAb Th3 specific for thymic cortical epithelial cells. TSt-4 expresses neither class I nor class II antigen, whereas TSt-10 expresses class I but not class II antigen. Both cell lines can be induced to express class II antigen by stimulating with γ-interferon.

TSt-4 supports the generation of $CD4^+8^-$ cells from DN cells

DN fraction of thymocytes were cultured on the monolayer of TSt-4 or TSt-10 in 24-well plates. Within few days after seeding DN cells on a TSt-4 layer, a portion of cells attached on or crawled under the layer. This was also the case when DN cells were seeded on a TSt-10 layer. Seven days after the initiation of culture, the cells on TSt-4 or TSt-10 layer were collected. The recovered cells were assayed with a flowcytometer for forward vs. side light scatter. Major fraction of cells recovered from cultures on TSt-4 was lymphoid cells (Fig. 2A). On the other hand, the cells recovered from the cultures on TSt-10 were predominantly macrophage-like cells (Fig. 2B), which was confirmed by a finding that most of these cells were positive for Mac-1 (data not shown). 1, 3 and 6 days later, cells were harvested by pipetting and the numbers of viable lymphoid cells were enumerated

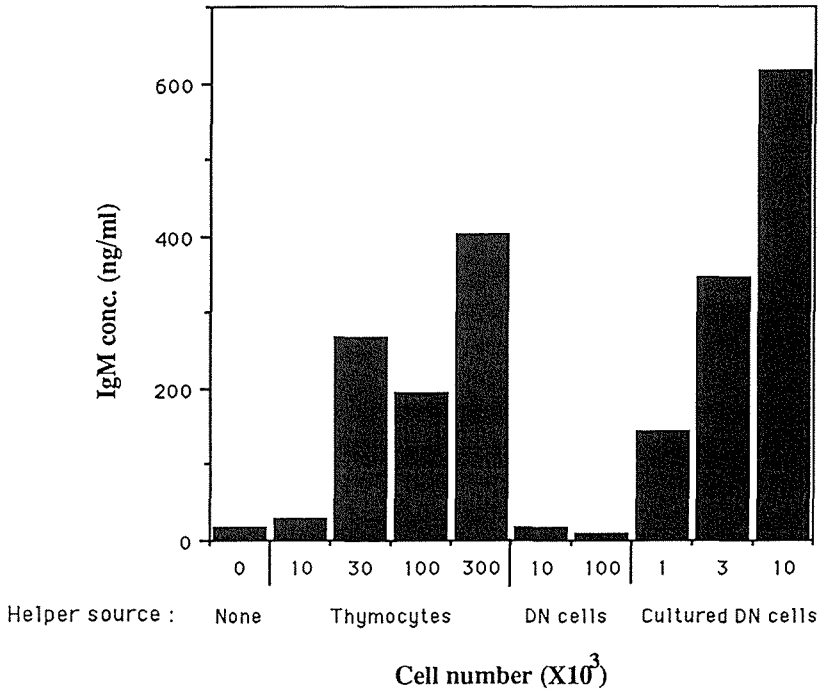

Fig. 4. Helper activity of DN cells cultured on TSt-4 monolayer . DN cells were cultured on TSt-4 for 1 week, and the helper activity of the recovered cells was determined by polyclonal IgM production of B cells.

(Table 2). Without stromal cells, it was difficult to maintain viable cells for 6 days. The condition did not improve by culturing on TSt-10. On the other hand, lymphocytes were maintained in cultures on TSt-4. Surface phenotypes of these cells are shown in Fig 3. About 15% of the cells expressed CD4 antigen, whereas the rest remained DN. $CD4^-8^+$ or double positive (DP) cells were very few, if any (Fig 3B). Fig 2D shows that quite a large proportion of cells express CD3 molecules on their surface.

Helper activity of DN cells cultured on TSt-4 monolayer

DN cells were cultured on TSt-4 layer for 7 days, and the helper activity of the recovered cells was determined by polyclonal IgM production of B cells. As shown in Fig.4, 10^3 recovered cells, about 15% of which was $CD4^+8^-$, were effective in helping IgM production. Since $3x10^4$ unfractionated normal thymus cells are required to express a similar level of helper activity, cultured cells are much more effective than normal thymus cells in helping B cells to produce Ig. These results suggest that TSt-4 not only supports the generation of $CD4^+8^-$ cells from DN cells but also stimulates the cells to mature functionally.

Culture supernatant of TSt-4 did not support the T cell generation but supported the generation of macrophages

We have tried to induce the T cell differentiation by culturing DN cells in the presence of culture supernatant of TSt-4 (TSt-4 sup.). However, T cell generation have not been observed in such cultures, suggesting that the T cell differentiation or proliferation is

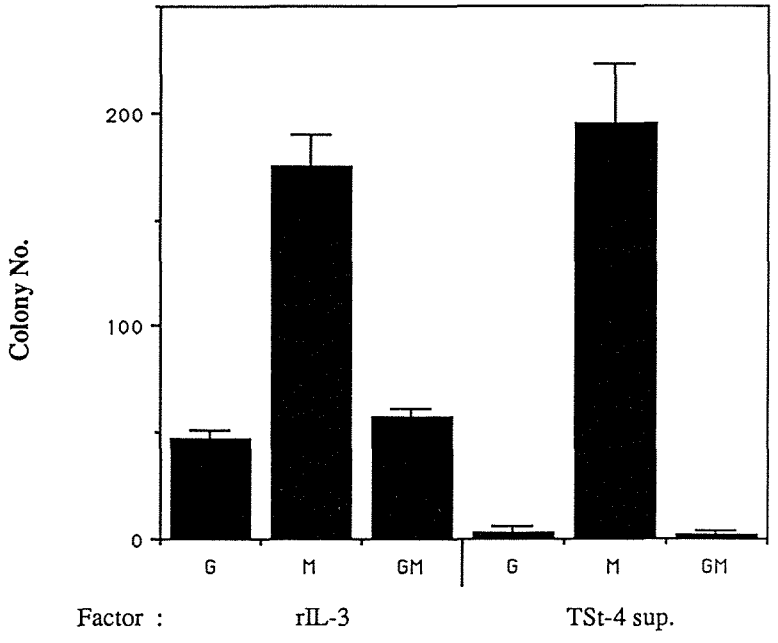

Fig. 5. Macrophage colony stimulating activity in culture supernatant of TSt-4 . Colonies were scored on day 8, and mean + SE of triplicate cultures are shown. Only colonies containing more than 100 cells were counted. G, granulocyte; M, macrophage; GM, mixture of granulocyte and macrophage.

induced through interaction between DN cells and TSt-4 cells. Instead, all such cultures were eventually predominated by macrophages. Thus, colony stimulating activity of TSt-4 sup. was investigated. As shown in Fig.5, TSt-4 sup. selectively supported the production of macrophages colonies, the production of mixed colonies or granulocyte colonies being not supported.

Northern blot analysis for production of growth factors by TSt-4

Poly(A)$^+$ mRNA was extracted from TSt-4 and as a control from poke-weed mitogen (PWM)-stimulated spleen cells and a murine thymus-epithelial cell line TEC1-4C18, and assayed for the presence of various growth factor mRNAs. The data are shown in Figure 6 and Figure 7. TSt-4 dose not express mRNA for IL-1, IL-2, IL-3, IL-4, IL-5, IL-6 (data not shown for IL-3 and IL-5), granulocyte colony stimulating factor (CSF) or granulocyte/ macrophage-CSF, but expresses macrophage-CSF mRNA and a small amount of IL-7 mRNA. To ensure that the equivalent amount of RNA was loaded in each lane of the blots, they were stripped and probed with β-actin cDNA. It was confirmed that similar amounts of RNA were loaded (data not shown).

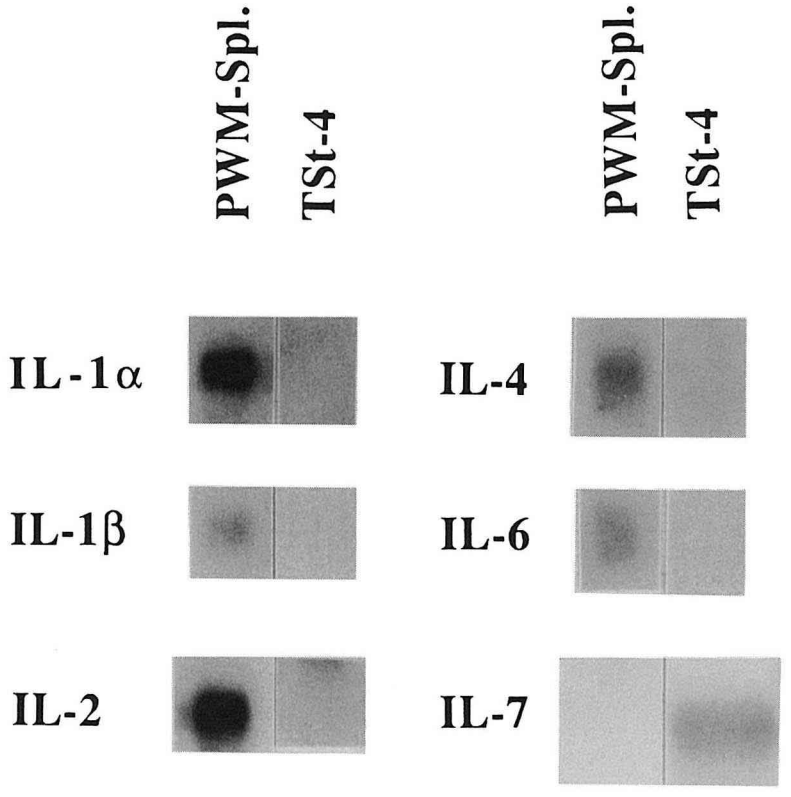

Fig. 6. Northern blot analysis of IL-1α, IL-1β, IL-2, IL-4, IL-6 and IL-7 mRNAs in TSt-4 and PWM-stimulated spleen cells (PWM-Spl.). The poly(A)$^+$RNA was hybridized with each probe. All lanes contained 2μg of poly(A)$^+$RNA.

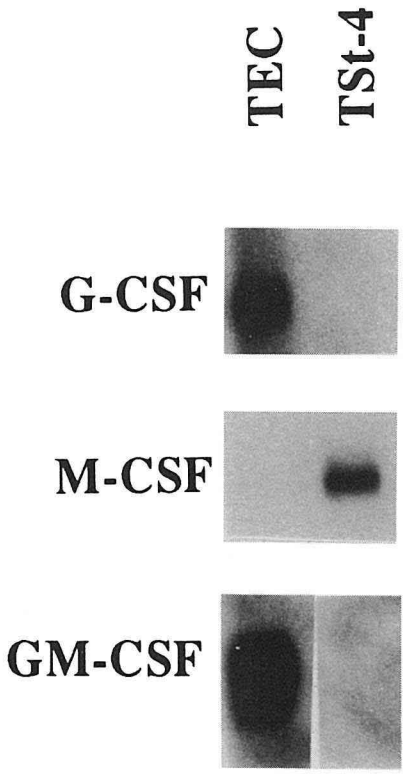

Fig. 7. Northern blot analysis of G-CSF, M-CSF and GM-CSF mRNAs in TSt-4 and TEC1-4C18. The poly(A)$^+$RNA was hybridized with each probe. All lane contained 2μg of poly(A)$^+$RNA.

Table 3. Cell recovery in cultures of adult DN cells supplemented with IL-7[1)]

Culture days	Recovered cells (%)			
	Medium		TSt-4	
	none	IL-7	none	IL-7
1	80	84	44	48
3	6	26	26	68
5	ND[2)]	35	5	48
7	ND	52	5	64

[1)] DN cells were cultured alone or on the monolayer of TSt-4, with or without IL-7 (100 units/ml) in 24-well plates. Lymphoid cells were recovered by pipetting,washed, and viable lymphoid cells were enumerated by trypan blue dye exclusion.

[2)] Not detected.

Supplementation with recombinant IL-7 promoted the growth of $CD4^+8^-$ cells

Since it was shown above that TSt-4 expresses IL-7 mRNA among interleukins examined, we next investigated whether IL-7 might be involved in the generation or proliferation of $CD4^+8^-$ cells from DN cells. DN cells were cultured on TSt-4 layer in the presence of recombinant IL-7 (100 units/ml). In addition, DN cells were cultured with IL-7 without TSt-4 layer. Lymphoid cells were harvested and enumerated on day 1, 3, 5 and 7, and assayed for cell surface antigens on day 7. The cell recovery on day 7 in IL-7 supplemented cultures was about 10 times as high as that in cultures on the TSt-4 layer without exogenous IL-7 (Table 3). As shown in Figure 8, CD4 vs CD8 or CD3 profiles of cells generated in IL-7 supplemented cultures are similar to those observed in cultures on TSt-4 without IL-7 (Figure 3B). These results suggest that IL-7 is mainly involved in generation or proliferation of $CD4^+8^-$ cells from DN cells.

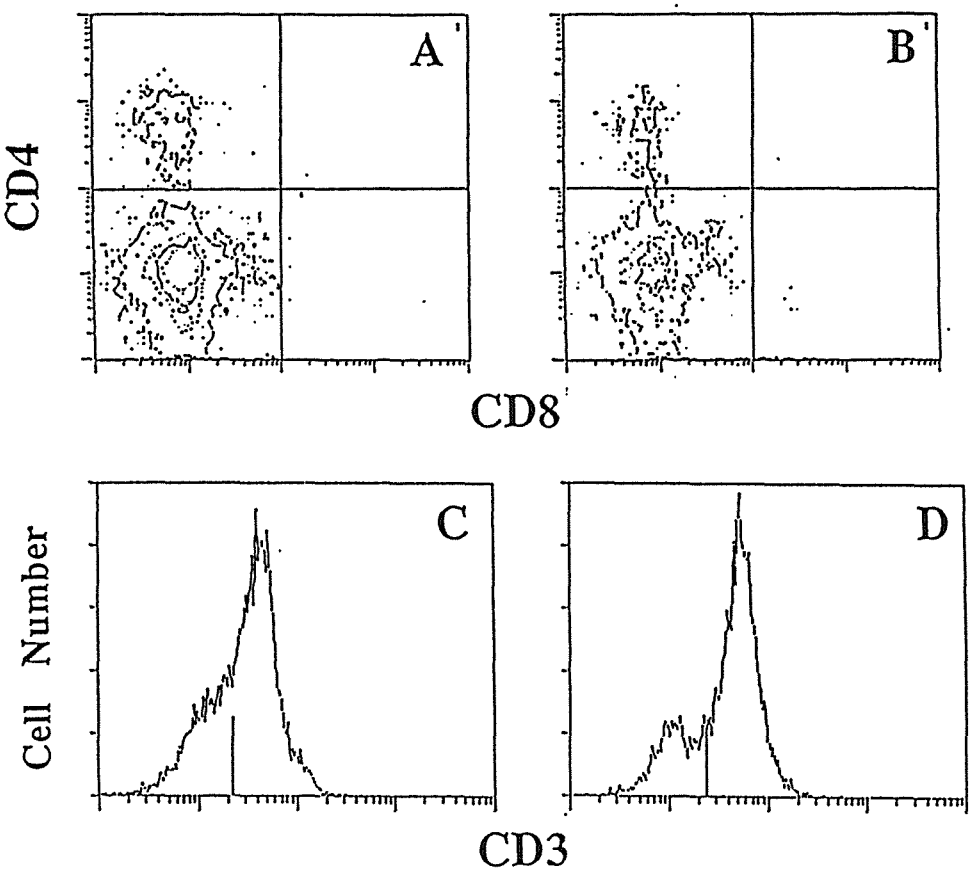

Fig. 8. Flowcytometric analysis of cells cultured with TSt-4 plus IL-7 or with IL-7 alone. DN cells were cultured on TSt-4 layer in the presence of IL-7 (100 units/ml) (A, C) or with IL-7 alone (B, D) for 7 days. Cultured cells were stained with anti-CD4 and anti-CD8 (A, B) or anti-CD3 (C, D).

DISCUSSION

TSt-4 cells are morphologically fibroblastoid, express neither class I nor class II MHC antigens, but express class II antigens after exposure to γ-interferon. Cultivation of DN cells, on TSt-4 monolayers resulted in the generation of $CD4^+8^-$ helper active cells, strongly suggesting that TSt-4 supported the differentiation of $CD4^+8^-$ T cells from a late stage of DN cells.

Normal T cell development can easily be induced from thymic (Ready et al., 1984; Watanabe et al., 1989) as well as prethymic precursors (Watanabe et al., 1989; Jenkinson et al., 1982) in deoxyguanosine-treated fetal thymus organ cultures that are mainly composed of epithelial cells and a relatively small number of fibroblasts. Thus it is strongly suggested that epithelial cells and fibroblasts in the thymus play key roles in the induction of T cell development. To clarify the roles of these cells, thymic epithelial (Denning et al., 1988; Ranson et al., 1987; Eshel et al., 1990) and fibroblastoid (Eshel et al., 1990; Tatsumi et al., 1990) cell lines have been established. None of these lines, however, are found to induce mature $CD4^+8^-$ cells from DN cells.

In the present work, we showed that a fibroblastoid stromal cell line TSt-4 supported the generation of mature $CD4^+8^-$ cells from DN cells. The results may indicated that thymic fibroblasts play a part in inducing the differentiation or maturation of T cells from a late stage of DN cells. The possibility that TSt-4 dose not induce the differentiation of T cells but supports the proliferation of a very small number of contaminating mature $CD4^+8^-$ cells can not be completely excluded before the differentiation of mature T cells from a cloned pre T line on TSt-4 is shown. However, it seems unlikely, because when 10^4 or 10^5 purified $CD4^+8^-$ thymus cells were cultured for 7 days on a TSt-4 layer, the cell number did not increase but decreased by 70-80% (data not shown).

Differentiation inducing activity of non-epithelial cells is also reported by several other groups. Tatsumi et al. (1990) showed that $CD4^-8^+$ cells were generated from DN cells by culturing them on their fibroblastoid cell line. Nishimura et al. (1990) showed that cultivation of DP cells on a thymic nurse cell-like line in the presence of IL-2 and anti-CD3 mAb resulted in the generation of $CD4^-8^+$ $\alpha\beta$ T cells . These cell lines consistently express class I antigen, whereas TSt-4 expresses neither class I nor class II antigen without stimulation. TSt-4 is unique in that it supports the differentiation of DN cells into $CD4^+8^-$ cells. Since TSt-4 expresses class II antigen by culturing them with γ-interferon, the skewing to $CD4^+8^-$ lineage could be ascribed to the selective growth or survival of $CD4^+8^-$ cells capable of interacting with such class II molecules, as has been suggested in the case of positive selection mediated by cortical epithelial cells (Berg et al., 1989).

We found that TSt-4 expressed mRNA for M-CSF (Figure 7) and IL-7 (Figure 6), and the activity of M-CSF was detectable in the culture supernatant (Fig.5). On the other hand, TSt-4 sup. alone have not maintained DN cells. Nevertheless, it seemed probable that a small amount of IL-7 produced by TSt-4 supported the growth or differentiation of $CD4^+8^-$ cells, because the addition of recombinant IL-7 in cultures of DN cells resulted in marked increase of cell recovery (Table 3) and the development of $CD4^+8^-$ cells (Figure 8). This suggests that enough IL-7 for induction of $CD4^+8^-$ cells is produced by TSt-4 through interaction with DN cells, or that IL-7 exists mainly bound on TSt-4, but very few in TSt-4 sup..

We demonstrated that stromal cell line TSt-4 supports the differentiation of DN cells

into $CD4^+8^-$ helper T cells in which generation or proliferation IL-7 is involved. Studies are in progress to examine the effect of IL-7 on the development of fetal thymocytes which contain T cell progenitors as well as DN cells.

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