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Induction of Thymocyte Proliferation by Supernatants from a Mouse Thymic Epithelial Cell Line[1]

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The thymic stroma plays a critical role in the generation of T lymphocytes by direct cell-to-cell contacts as well as by secreting growth factors or hormones. The thymic epithelial cells, responsible for thymic hormone secretion, include morphologically and antigenically distinct subpopulations that may exert different roles in thymocyte maturation. The recent development of thymic epithelial cell lines provided an interesting model for studying thymic epithelial influences on T cell differentiation. Treating mouse thymocytes by supernatants from one of TEC line (IT-76M1), we observed an induction of thymocyte proliferation and an increase in the percentages of $CD4^-/CD8^-$ thymocytes. This proliferation was largely inhibited when thymocytes were incubated with IT-76M1 supernatants together with an anti-thymulin monoclonal antibody, but could be enhanced by pretreating growing epithelial cells by triiodothyronine. We suggest that among the target cells for thymulin within the thymus, some putative precursors of early phenotype might be included.

INTRODUCTION

The thymus plays a critical role in the generation of immunocompetent T lymphocytes (1, 2). It is accepted that this process is largely mediated by the thymic microenvironment, which is composed of distinct nonlymphoid cells such as macrophages, dendritic cells, fibroblasts, and the major component corresponding to the epithelial cells. The precise influences of each of these cells on distinct events of intrathymic T cell maturation are not well established. Thymic epithelial cells (TEC) participate in this process in at least two distinct ways: (a) secretion of polypeptides as thymic hormones (see Ref. (3) for a review), interleukin-1, interleukin-3, interleukin-6, and granulocyte–macrophage colony-stimulating factor (4–7), and (b) cell-to-cell contacts through classical adhesion molecules (8, 9) and also the interactions mediated by the major histocompatibility complex products, highly expressed on TEC membranes (10, 11). Interestingly, TEC physiology is under neuroendocrine control. Par-

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ticularly thyroid hormones were able to modulate thymulin production both *in vivo* and *in vitro* (reviewed in Ref. (12)).

Cumulative data demonstrated the existence of a heterogeneity of the thymic epithelium. In this respect, TEC subsets were evidenced by morphological criteria (13, 14), and by their antigenic differences defined by anti-TEC or anti-cytokeratin monoclonal antibodies (15–17). However, whether these TEC subsets play differential roles on intrathymic T cell maturation events is to be determined. In this vein, the use of homogeneous TEC models *in vitro* might be useful. One of these so-called TEC subsets, initially isolated *in vitro,* has been described by Wekerle *et al.*: the thymic nurse cells (TNC) that were able to make complexes with a large number of immature thymocytes (18, 19). From a functional point of view, TNC were shown to secrete thymic hormones (20, 21), and promote phenotypic changes on maturing thymocytes (22, 23).

In addition to TNC, some TEC lines have been obtained, most of which are able to modulate differentiation of prothymocytes and/or thymocytes (24–27). One of them was described to secrete a polypeptide able to attract precursors into the thymus (28, 29). This prompted us to investigate if supernatants from a mouse TEC line, hereafter named IT-76M1 (30), could modulate thymocyte behavior, as concerns proliferation and surface phenotype. Herein we present evidence that this TEC line stimulates thymocyte proliferation and modulates the percentages of CD4/CD8-defined thymocyte subsets. Moreover, we show that this proliferative activity is inhibited by the addition of an anti-thymulin monoclonal antibody. Conversely, this function can be enhanced by pretreating growing TEC with triiodothyronine.

MATERIALS AND METHODS

Animals. Male C57BL/Ka mice, aged 4–5 weeks, were obtained from the animal house of Liège University.

Cell cultures. The TEC line IT-76M1, was initially developed by Dr. T. Itoh (Japan) from primary cultures of thymic stromal cells from a Balb/c mouse (30). The epithelial nature of this line was demonstrated by the expression of different cytokeratins (31) and presence of tonofilaments and desmosomes (30). It was cultured in RPMI 1640 medium, with 10% heat-inactivated FCS, 2×10^{-3} *M* L-glutamine, 10^{-3} *M* sodium pyruvate, 1% nonessential amino acids, 5×10^{-5} *M* 2-mercaptoethanol, 100 UI/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Ltd., Paisley, Scotland). The cultures were kept at 37°C in humidified atmosphere with 5% CO_2.

Primary fibroblast cultures were obtained from 16- to 17-day C57BL/Ka fetuses. Animals were sliced with sterile blades; fragments were washed in complete RPMI and plated in 25-ml culture flasks (Falcon-Becton Dickinson & Co., California) at the ratio of 8–10 explants/culture flask. When confluence of growing fibroblasts was achieved, cells were trypsinized and replated.

Supernatants of cell cultures. IT-76M1 cells (2×10^5 cells) were plated in 25-ml culture flasks and allowed to grow for 72 hr in complete RPMI medium. When cultures were 70–90% confluent, this medium was replaced by a serum-free medium (HB-101, DuPont, Boston) for 24 hr. Culture supernatants were collected, pooled, centrifuged at 2500 RPM for 10 min, and stored at −70°C until use. In some experiments, IT-76M1 cells were cultured in the presence of triiodothyronine (T_3) (Sigma Chemical Company, St. Louis), added in a concentration of 10^{-7} *M* on Days 2 and 3 of the culture. The protocol for generating supernatants was the same as described before.

Control supernatants were obtained from fibroblastic primary cultures in the same way as for epithelial cultures.

Thymocyte proliferation assay. Cell suspensions were obtained by gently teasing 4–5 thymuses with fine scissors. In some experiments, thymocytes were passed through a nylon–wool column, to eliminate adherent cells (32). A suspension of 10^6 thymocytes in 100 μl of complete RPMI was plated on each well of a 96-well flat-bottom dish (Falcon–Becton–Dickinson & Co., CA). One hundred microliters of epithelial culture supernatants (either undiluted or diluted up to 10^{-6}) was then added. After a 48-hr incubation period, cultures were pulsed with 1 μCi/well of [^{3}H]thymidine (sp act: 5 Ci/mmol, Amersham International, UK) and reincubated for an 18-hr period. Cells were then harvested and counted for β-emission. As controls, we included primary fibroblast culture supernatants used in equivalent dilutions as those of TEC supernatants, as well as the incubation of thymocytes in complete RPMI alone.

Thymocytes were alternatively treated by synthetic thymulin (Choay, France) in doses ranging from 10^{-5} to 10^{-12} *M*.

Inhibition of proliferation with anti-thymulin monoclonal antibody. Anti-thymulin monoclonal antibody (mAb) was produced as previously described (33), after immunizing a mouse with synthetic thymulin coupled to bovine serum albumin (BSA). This mAb specifically binds to murine (34) and human (35) TEC. It was added, at final dilutions of 1/25 to 1/250 (IgG1 concentrations of 10^{-2} to 10^{-3} mg/ml), to thymocyte cultures alone or together with TEC supernatants. Control cultures were represented by thymocytes treated by different dilutions of a mouse unrelated ascitic fluid alone (at equivalent Ig concentrations) or together with epithelial supernatants. Thymocytes were incubated as mentioned above for proliferation assay.

Phenotypic study of thymocytes submitted to different culture conditions. Thymocytes (10^6 cells/well) were cultured for 24 or 48 hr, in the same experimental conditions as used for proliferation studies, in order to be phenotypically evaluated by immunofluorescence. Fresh thymocytes were always analyzed in parallel.

The anti-CD4 mAb (H 129.19) (36) was used as phycoerythrin conjugate (Becton–Dickinson & Co., CA), whereas the anti-CD8 mAb, 53.6.72 (37) was coupled to fluorescein. The anti-IL-2 receptor mAb (7D4) was purchased from ATCC (38), and the mAb recognizing the β chain of T cell receptor V 8 chain (KJ16) was generously donated by Dr. Paola Ricciardi-Castagnoli (39). These mAb were used together with fluorescein-conjugated F(ab')$_2$ fragments of goat anti-rat Ig (Nordic, Leuven, Belgium).

Cultured thymocytes were washed in RPMI containing 10% FCS and incubated with specific mAb for 30 min at 4°C. Cells were then washed in FCS and, for indirect labeling, subsequently incubated with the second fluorescein-coupled antibody. Analysis was performed by flow cytometry using a FACS IV (Sunnyvale, CA). Only viable cells were considered, dead cells being excluded on the basis of propidium iodide labeling (1 μg/ml), added just before analysis.

RESULTS

Effects of IT-76M1 Cell Line Supernatant on Thymocyte Proliferation

Thymocytes treated by IT-76M1 supernatants exhibited a dose-dependent enhancement in their proliferation rate. Maximal response was obtained with $\frac{1}{2} \times 10^2$ final dilution of TEC supernatants, and corresponded to a [^{3}H]thymidine incorporation about 40 times greater than in controls, namely, thymocytes cultured in RPMI alone

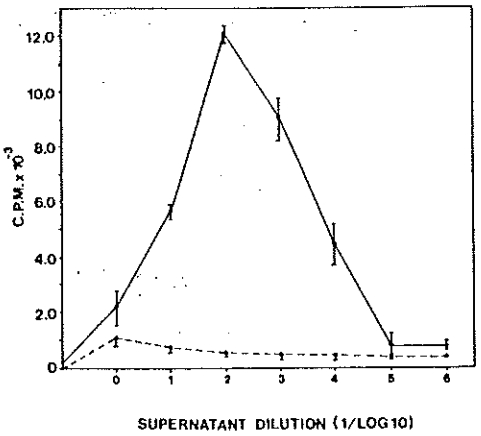

FIG. 1. Dose-dependent induction of young adult thymocyte proliferation by IT-76M1 culture supernatant (—). Thymocytes (10^6/well) were cultured in triplicate for 48 hr with different supernatant dilutions. Cultures were then pulsed with [^{3}H]thymidine for 18 hr. (---) Primary fibroblastic culture supernatant.

or treated by fibroblastic culture supernatants (Fig. 1). Importantly, the stimulatory effect of IT-76M1 supernatant was the same when thymocytes were previously passed through a nylon–wool column to eliminate adherent cells, such as macrophages.

Effect of Anti-Thymulin Monoclonal Antibody on Thymocyte Proliferative Response to IT-76M1 Cell Line Culture Supernatant

To determine whether the proliferative effect was due to the presence of thymulin within the epithelial supernatants, we tested the effect of the anti-thymulin mAb upon thymocyte proliferative response. Thymocytes incubated with the TEC supernatant together with anti-thymulin mAb showed a [^{3}H]thymidine incorporation lower than that of thymocytes treated by epithelial supernatants alone (Table 1). Control thy-

TABLE 1

Inhibition of IT-76M1 Culture Supernatant-Induced Thymocyte Proliferation by Anti-Thymulin mAb

Condition	cpm × 10^{-3}
RPMI	398 ± 70
Control supernatant (CSN)	12,503 ± 266
CSN + anti-thymulin 1/25	3,520 ± 203
CSN + anti-thymulin 1/50	5,118 ± 354
CSN + anti-thymulin 1/100	5,565 ± 365
CSN + anti-thymulin 1/250	8,795 ± 610
CSN + unrelated ascites 1/25	12,225 ± 302
CSN + unrelated ascites 1/50	12,352 ± 258
Synthetic thymulin	329 ± 17

Note. Thymocytes were incubated with IT-76M1 cell culture supernatants (CSN) alone ($1/2 \times 10^2$ final dilution) or together with anti-thymulin mAb. Synthetic thymulin data are representative for hormone concentrations from 10^{-5} to 10^{-12} *M*.

thymocytes treated by epithelial supernatants plus an unrelated mouse ascitic fluid had no diminished proliferation compared to those treated by TEC supernatants alone.

Considering the results found after adding anti-thymulin mAb, we attempted to obtain a reverse effect by the addition of synthetic thymulin to thymocytes. Nonetheless, none of the concentrations of the thymic hormone applied to thymocytes stimulated [^{3}H]thymidine uptake (Table 1).

Modulation of Supernatant Effect by Treatment of IT-76M1 Cell Line with Triiodothyronine (T_3).

In order to evaluate the influence of T_3 on IT-76M1 secretion, we compared the capacity of different supernatants (obtained from cells either treated or not by T_3) in stimulating [^{3}H]thymidine incorporation by thymocytes. We observed that supernatants from T_3-treated cells exhibited a greater stimulatory capacity (Table 2). It should be pointed out that we could never see stimulation of proliferation when thymocytes were directly treated by T_3, in concentrations ranging from 10^{-6} to 10^{-9} *M*.

Phenotypic Analysis of Thymocytes Treated by Thymic Epithelial Cell Line Supernatants

Thymocytes treated by IT-76M1 supernatants showed no differences in cell viability compared to those cultured in complete RPMI alone, as assessed by propidium iodide staining. In all cases 45–50% of thymocytes died after a 48-hr period of culture. When CD4 and CD8 antigens were examined, we could see that the percentages of $CD4^+/CD8^+$ (double-positive) cells were lower for IT-76M1 supernatant-treated thymocytes, when compared to controls, whereas $CD4^-/CD8^-$ (double-negative) cells increased. In contrast, $CD4^+/CD8^-$ or $CD4^-/CD8^+$ populations showed no significant changes (Fig. 2). Importantly, when anti-thymulin mAb was added to the TEC supernatants, the increase in $CD4^-/CD8^-$ cells was no longer significant, suggesting a partial suppression of the supernatant-derived effects (Fig. 3).

Despite the alterations in the CD4/CD8-defined phenotypes, no significant changes were detected, as regards the percentages of labeling obtained with 7D4 or KJ16 mAb (respectively recognizing IL-2 and T-cell B chain receptors), as depicted in Fig. 4.

TABLE 2

Effect of T_3 on IT-76M1 Supernatant Capacity to Stimulate [^{3}H]Thymidine Uptake by Thymocytes (cpm $\times 10^{-3}$)

Dilution	CSN	SN/T_3 (10^{-7} *M*)
$1/2 \times 10^3$	8708 ± 362	10,497 ± 487
$1/2 \times 10^4$	4529 ± 301	9,118 ± 447
$1/2 \times 10^5$	895 ± 93	5,387 ± 345
$1/2 \times 10^6$	890 ± 78	2,893 ± 401

Note. Thymocytes were incubated with supernatants from control (CSN) or T_3-treated cells (SN/T_3). Thymocytes incubated directly with T_3 (10^{-6} to 10^{-9} *M*) showed values comparable to those when cultured in complete RPMI alone.

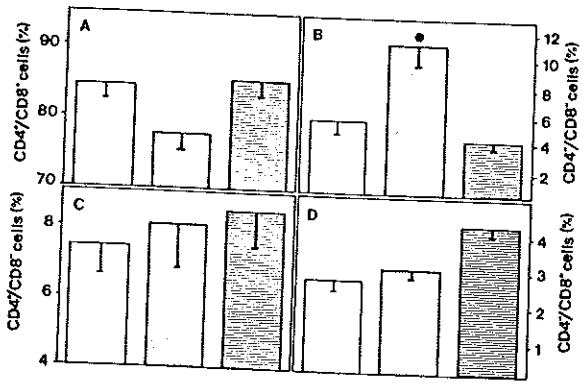

FIG. 2. Phenotypic analysis of thymocytes cultured in complete RPMI (□) or in the presence of IT-76M1 culture supernatant in $\frac{1}{2} \times 10^2$ dilution (□) for 48 hr. Fresh thymocytes (□) were analyzed in parallel as controls. Bars are representative of four different experiments. $^*P < 0.05$.

DISCUSSION

In the present report we showed that supernatants from IT-76M1 thymic epithelial cell line were able to stimulate thymocyte proliferation. This effect was not MHC class II restricted, as ascertained by (a) target thymocytes were from C57BL/Ka mice whereas the TEC line derived from a Balb/c animal, and (b) this TEC line does not express Ia antigens unless stimulated with interferon-γ (40).

Previous work concerning the effects of supernatants from TEC lines or primary cultures on thymocyte activity presented variable results, such as modulation of mitogen response (4, 6, 7, 41–46) together with induction of immunocompetence and differentiation (26, 42, 47, 48). However, spontaneous proliferation is not commonly observed. Some authors obtained proliferative effects by coculturing thymocytes and TEC lines, but not with TEC supernatants alone (49). Yet, very recently Eshel *et al.* described an enhancement in spontaneous [^{3}H]thymidine uptake by thymocytes submitted to supernatants from TEC primary cultures grown on extracellular matrix-coated dishes (7).

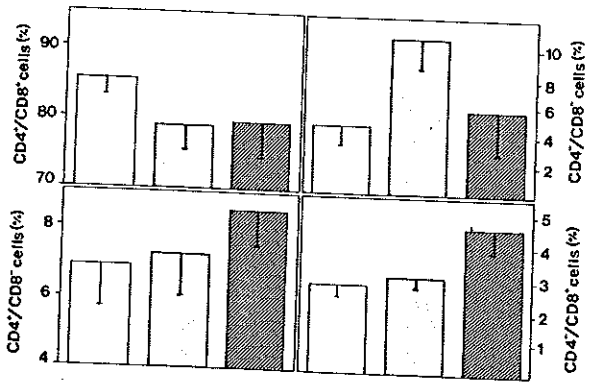

FIG. 3. Phenotypic analysis of thymocytes cultured in complete RPMI alone (□), in the presence of IT-76M1 culture supernatant in $\frac{1}{2} \times 10^2$ alone (□), or together with an anti-thymulin mAb in $\frac{1}{20}$ dilution (□) for 48 hr. Bars are representative of three different experiments.

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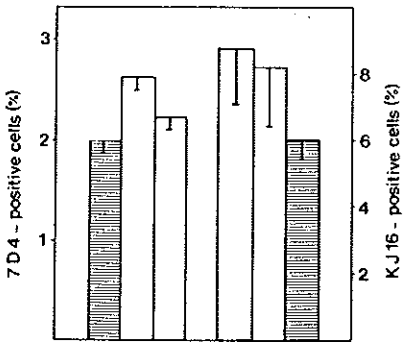

FIG. 4. Analysis of 7D4 (anti-IL-2 receptor) and KJ16 (anti-T cell receptor) mAb positivity in thymocytes cultured for 48 hr in complete RPMI (□) or IT-76M1 supernatant ($\frac{1}{2} \times 10^2$ dilution) (□). Fresh thymocytes (□) were analyzed in parallel. Bars are representative of four different experiments.

Taking into account our previous data showing that IT-76M1 cell line was able to produce thymulin (50), we asked the question whether the hormone present in the supernatants was involved in thymocyte proliferative effect. For that, we assayed the effect of an anti-thymulin mAb in supernatant-treated thymocyte cultures. This mAb decreased thymocyte proliferative response to TEC supernatants, although the abrogation was not complete. Interestingly, synthetic thymulin alone was not able to stimulate thymocyte proliferation, as previously shown by Andrews *et al.* (51). This seems not to be due to a binding of thymulin to the carrier protein described in the serum (52), since the same data were also obtained in the absence of FCS.

Facing all these results, it is likely that the thymocyte-stimulating capacity of IT-76M1 culture supernatants would be largely dependent on TEC production of thymulin, whose biological activity (in terms of thymocyte proliferation) probably requires a comitogenic factor (whose nature is yet undetermined) to be triggered.

Despite the unchanged thymocyte viability, phenotypic changes were evidenced upon TEC supernatants. The percentage of $CD4^-/CD8^-$ cells was significantly enhanced, whereas double-positive thymocytes decreased. These effects were partially blocked by the addition of the anti-thymulin mAb in the culture supernatants, suggesting that thymulin may be involved, not only in the control of thymocyte proliferation, but also in the modulation of CD4 and CD8 molecules. Considering that cell viability did not change, it is possible that the increase of double-negative thymocytes reflects their preferential stimulation for proliferation.

In contrast to the effects on CD4/CD8 expression, the staining patterns obtained with 7D4 or KJ16 mAb were not different between control and IT-76M1 supernatant-treated thymocytes after 24 or 48 hours. Taken together, these findings suggest that among the target cells for factors (thymulin and others) produced by TEC within the thymus might be a putative early precursor with the phenotype $CD4^-/CD8^-/IL\text{-}2R^-$, whose proliferation would yield thymocytes of the same phenotype as parental cells, at least during the experimental period analyzed. This is in keeping with the data reported by Denning *et al.* (6), who showed that cocultures of double-negative human thymocytes with autologous TEC cultures (or their supernatants) stimulated proliferation of thymocytes, which remained double-negative.

A last aspect of discussion regards the control mechanisms acting directly on TEC and modulating their ability to stimulate thymocytes. We showed that thymocyte

proliferation induced by T_3-treated TEC supernatants was greater than respective controls. Interestingly, we previously demonstrated that T_3 was able to increase *in vivo* and *in vitro* thymulin secretion by TEC (53, 54). In contrast, treatment of thymocytes with T_3 did not promote any change in their proliferation rate or phenotype. These findings raise the concept that, at least in terms of cell cycle and CD4/CD8 expression, the effects of thyroid hormones on thymocytes occur via the thymic epithelium, and that one of the putative mediators is thymulin. This is in keeping with the recent demonstration of nuclear T_3 receptors in murine TEC, including the IT-76M1 cell line (for review, see Ref. (55)).

In conclusion, we believe that the use of established TEC lines represents a useful tool for a better understanding on the thymulin-mediated mechanisms by which TEC influence thymocyte differentiation and proliferation.

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