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Neuromodulatory loop mediated by nerve growth factor and interleukin 6 in thymic stromal cell cultures

(neurotrophic factors/neurofilaments/synapsin)

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ABSTRACT **Neural crest cell derivatives have been suggested to be involved in thymus development. We established nonlymphoid thymic stromal cell cultures capable of supporting T-cell differentiation. In these nonlymphoid cell cultures, we identified cells with phenotypic and biochemical markers specific for neuronal cells. Neurofilament mRNA and 68- and 160-kDa neurofilament proteins, as well as 74-kDa synapsin I isoform, were expressed in many of the cultured cells. For example, neurofilament immunoreactivity was detected in 20–30% of the cells. To see whether thymic neuronal-like cells were involved in a neural differentiation pathway, we investigated the effect of nerve growth factor (NGF) and interleukin 6 (IL-6), two known neurotrophic factors. The expression of the above-described neural markers was enhanced by NGF and IL-6, which we report to be produced in an autocrine way by thymic stromal cell cultures. Finally, we found that IL-6 gene expression in these cell cultures was enhanced by NGF. Evidence is thus offered of a neuromodulatory loop within the thymic stromal cell population supported by local production of NGF and IL-6 and involving neural cell elements. Interestingly, IL-6, which is known to be implicated in thymocyte differentiation, also displays a neuromodulatory activity on thymic stromal cells, suggesting a multivalent role for this cytokine within the thymus.**

The thymus is the major site of T-cell differentiation. This process requires direct cell–cell contact (1–3), as well as production of soluble factors by both thymic lymphoid and nonlymphoid components (4, 5). The nonlymphoid population is heterogeneous in composition (fibroblasts, macrophages, dendritic and epithelial cells) and ontogeny (endoderm from the third and fourth pharyngeal pouches and elements derived from neural crest) (6, 7). This heterogeneity has been shown to be necessary for the differentiation of T-cell progenitors into the complex spectrum of thymocyte subsets (8). Dependence of thymus development on neural crest derivatives has been suggested by experiments showing that ablation of the chicken embryo cephalic neural crest results in thymic aplasia/hypoplasia (9). The thymus may thus include a neural crest-derived cell population possibly involved in its differentiation. Here we show that a neuronal-like component of the thymic nonlymphoid population can be cultured *in vitro* and is involved in a neuromodulatory loop including soluble factors [i.e., interleukin 6 (IL-6)] implicated in thymocyte differentiation.

MATERIALS AND METHODS

Cell Cultures. Nonlymphoid thymic stromal cell (TC) cultures (TC-1S and TC-2D) were independently established by pooling three thymuses from 4- to 5-week-old male C57BL6 mice. Thymuses were finely minced and transferred to T75 flasks containing RPMI 1640 (Flow) supplemented with 20% fetal bovine serum and 0.1 mg of sodium pyruvate per ml. Four days after plating, the serum was reduced to 10%. The first passage was done 20 days after plating. All experiments reported were performed with TC cultures from the 3rd to the 40th passage.

Immunofluorescence and Electron Microscopy. Indirect immunofluorescent staining was carried out by using a monoclonal anti-medium neurofilament (NF-M) antibody (Amersham) followed by fluorescein-conjugated goat anti-mouse $(Fab')_2$ IgG (Cappel Laboratories) as second antibody. For ultrastructural analysis, cell layers were fixed for 3 hr in 2.5% glutaraldehyde/0.1 M sodium cacodylate buffer, pH 7.2. They were then washed in 0.1 M sodium cacodylate buffer, scraped, pelleted, and postfixed for 1 hr in 1.33% OsO_4/0.1 M cacodylate buffer, pH 7.2. They were then dehydrated through a graded ethanol series and embedded in Epon.

Northern Blot Analysis. RNA was isolated as described (10), electrophoresed in 1% agarose/formaldehyde gels, blotted onto Hybond-N filters (Amersham), and hybridized with appropriate ^{32}P-labeled cDNA probes. Hybridization and washing conditions were as described (11).

Western Blot Analysis. For immunoblot analysis of synapsin I, cells or tissues were homogenized in 100 mM Tris·HCl, pH 6.8/4% (wt/vol) SDS/20% (vol/vol) glycerol/10% (vol/vol) 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride. Cell lysate proteins were separated by 0.1% SDS/7.5% polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and probed with a rabbit polyclonal anti-synapsin I antiserum (12). Synapsin I–antibody complex was revealed by ^{125}I-labeled donkey anti-rabbit $F(ab')_2$ immunoglobulin (Amersham).

For analysis of NF protein, cells or tissues were homogenized in 10 mM Tris·HCl, pH 7.6/140 mM NaCl/5 mM $MgCl_2$/5 mM EDTA/2 mM phenylmethylsulfonyl fluoride containing aprotinin (2 μg/ml) and trypsin inhibitor (20 μg/ml). Cell lysate proteins were then separated by SDS/PAGE. After transfer, nitrocellulose was incubated with a mixture of monoclonal anti-68 kDa and anti-160 kDa NF antibodies (Amersham), followed by biotin-conjugated goat anti-mouse IgG (Zymed Laboratories). Bound antibodies were detected with streptavidin–horseradish peroxidase conjugate (Zymed) and diaminobenzidine (Sigma).

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Abbreviations: IL-6, interleukin 6; NGF, nerve growth factor; NF, neurofilament; TC, nonlymphoid thymic stromal cell.

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RESULTS AND DISCUSSION

We established several TC cultures that were functionally competent in supporting T-cell differentiation. Coculturing a suspension of 99% pure $CD4^-$ $CD8^-$ ("double-negative") thymocytes on a monolayer of TC cultures resulted in the generation of 28.4% $CD4^+$ $CD8^+$ cells and 13.2% $CD4^+$ $CD8^-$ after 48 hr (data not shown). These cultures presented two morphologically defined histotypes: large cells with a typical epithelial ultrastructure (Fig. 1 *a* and *b*) and cells with elongated cellular processes and ultrastructural neuroepithelial-like features (Fig. 1 *a* and *c*). Phenotypic characterization of TC-1S and TC-2D showed that 85–90% of the cells expressed cytokeratin, whereas the macrophage-specific Mac-1, the fibroblast-specific vimentin, and the myoid-specific desmin markers were not expressed. Twenty to 30% of the cells expressed the neural crest-derived cell-related A2B5 and Leu-7 surface antigens (13, 14). Immunohistochemical evidence of A2B5 and of several neuropeptides has been reported for thymic tissue *in vivo* and related to the organ's neuroendocrine function (15). We also found that 20–30% of the cells expressed immunoreactivity for light and medium NFs (NF-L and NF-M) (see Fig. 3*c*), the intermediate filaments specific for neuronal cells (16). A 3.5-kilobase mRNA band was observed after hybridization with a NF-M probe (Fig. 2*a*). The presence of 68-kDa (NF-L) and 160-kDa (NF-M) cytoskeletal components was also confirmed by Western blot analysis (Fig. 3*b*). The molecular and phenotypic neuronal-like features shown by some cells (Figs. 2*a* and 1*a*) led us to search for proteins specifically associated with neuronal organelles. We found that TC-1S cells expressed immunoreactivity for synapsin I, which belongs to a family of proteins found in neurons and is highly specific to nerve terminal, where it associates with the synaptic vesicles (17). Western blot analysis showed a 74-kDa synapsin I-immunoreactive protein comigrating with the higher molecular weight synapsin I (synapsin Ia) of the doublet observed in the brain (Fig. 2*b*). The presence of only one synapsin I isoform is in keeping with observations reported in a few neuroendocrine cells or in regionally distinct synaptic structures (18–20).

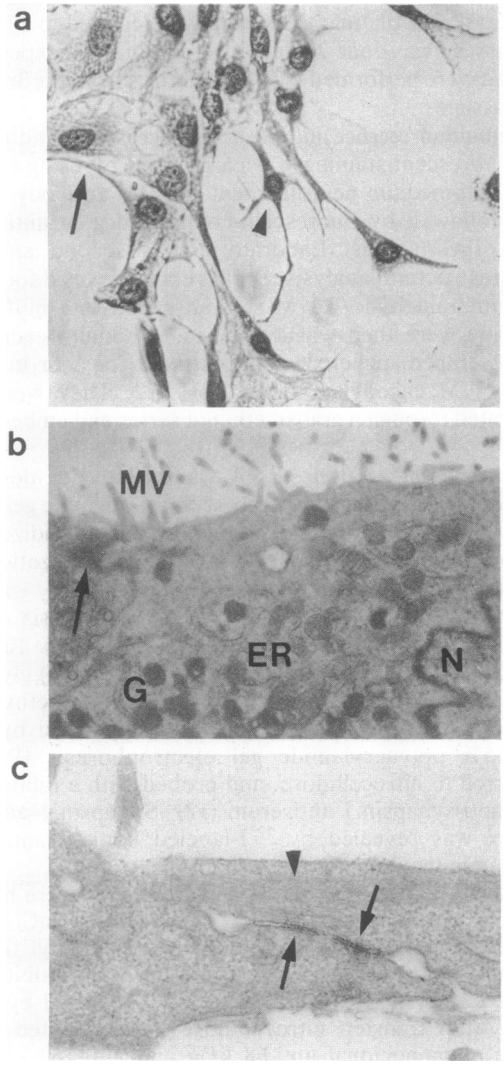

FIG. 1. (*a*) Micrograph of TC-1S culture showing two histotypes: large epithelial cells (arrow) and cells with many elongated cellular processes (arrowhead). (×1000.) (*b*) High magnification of contact between two large cells with typical epithelial ultrastructural markers: microvillae (MV), secretory granules (G), abundant rough endoplasmic reticulum (ER), cell–cell adhesion plaque with a desmosome-like structure (arrow). N, nucleus. (×12,000.) (*c*) High magnification of neuroepithelial-like cellular junction between two cells (arrows). Cytoskeletal filaments are evident in the terminal part of cellular processes (arrowhead). (×27,500.)

To see whether thymic neuronal-like cells were involved in a neural differentiation pathway, we investigated the effect of treatment with nerve growth factor (NGF), a specific neurotrophic factor responsible for the differentiation and survival of neurons (21). NGF treatment of both TC-1S and TC-2D cultures induced a significant increase of NF-M mRNA expression, as well as an increase in 68-kDa and 160-kDa NF protein levels (Fig. 3 *a* and *b*), in keeping with data observed during NGF-induced neural differentiation of pheochromocytoma-derived PC12 cells (22). NGF-treated cells also became intensely immunostained for NF and displayed thin and long cytoplasmic processes (Fig. 3 *d* and *e*).

The presence of a cell population with a neuronal-like phenotype suggests that its survival in culture may be sustained by a specific neurotrophic microenvironment. Fig. 4*a* shows that the NGF gene is indeed expressed in TC-1S and TC-2D, in keeping with the observation of *in vivo* NGF mRNA expression in rodent thymus (24). Moreover, of the several soluble factors produced by thymic nonlymphoid cells, IL-6 could also sustain a neurotrophic microenvironment, since it is produced by thymic stroma-derived cell lines (25) and induces NGF-like neuronal differentiation (26). We found that the IL-6 gene is indeed expressed by TC-1S and TC-2D cells (Fig. 4*a*). Interestingly, IL-6 plays a neuromodulatory role in

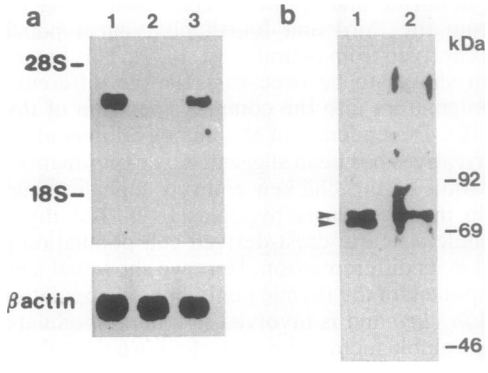

FIG. 2. (*a*) Northern blot analysis of NF-M mRNA. Total RNA extracted from mouse brain (lane 1) or poly(A)$^+$ RNA from NIH 3T3 cells (lane 2) and TC-1S cells (6th passage) (lane 3) were electrophoretically resolved in 1% agarose/formaldehyde gels, blotted, and hybridized with a NF-M-specific ^{32}P-labeled probe (human pNf36, American Type Culture Collection) (*Upper*) and, after stripping, with a β-actin probe (*Lower*). Positions of 28S and 18S rRNA are indicated. (*b*) Western blot analysis of synapsin I extracted from mouse brain (0.8 μg of cell lysate, lane 1) or TC-1S at the 40th passage (80 μg of cell lysate, lane 2).

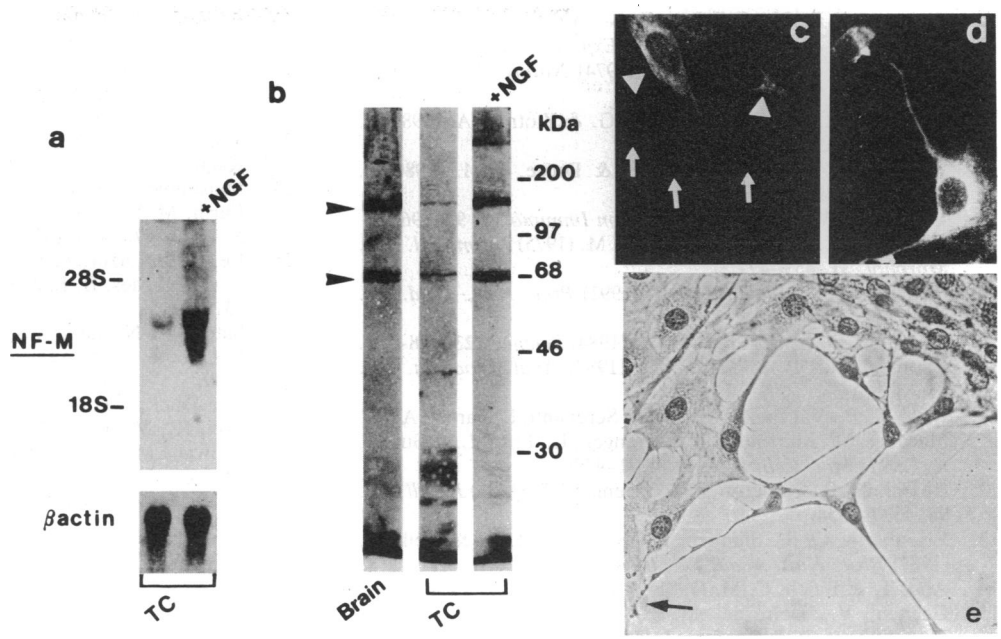

FIG. 3. (*a*) Northern blot analysis of NF-M mRNA isolated from untreated and NGF-treated TC-2D cells. TC-2D cells (10th passage) were cultured for 48 hr in the absence or presence of murine 7S NGF (50 ng/ml, Collaborative Research). (*b*) Western blot analysis of NF extracted from mouse brain or TC-1S cells (32nd passage) cultured for 48 hr in the absence or presence of NGF (50 ng/ml). (*c* and *d*) Indirect immunofluorescent staining of untreated (*c*) and NGF-treated (*d*) TC-1S cells (6th passage) with a monoclonal anti-NF-M antibody and fluorescein-conjugated goat anti-mouse $(Fab')_2$ IgG as second antibody. In *c*, NF-positive (arrowheads) and -negative (arrows) cells are shown. Staining is distributed over the whole cytoplasm and along the cytoplasmic extensions. In *d*, after NGF treatment neurofilament staining becomes extremely intense in the whole cytoplasm and along the cytoplasmic processes. The cytoplasmic elongations become very thin and neurite-like. (*e*) Morphological aspect of TC-1S cells treated with NGF for 24 hr. Several cells with thin, elongated processes with terminal varicosities are evident. Some processes are in close contact with cells of epithelial appearance (arrow). (×850.)

TC cultures, since addition of the recombinant IL-6 to TC-1S cells significantly increased NF-M mRNA levels (Fig. 4*a*).

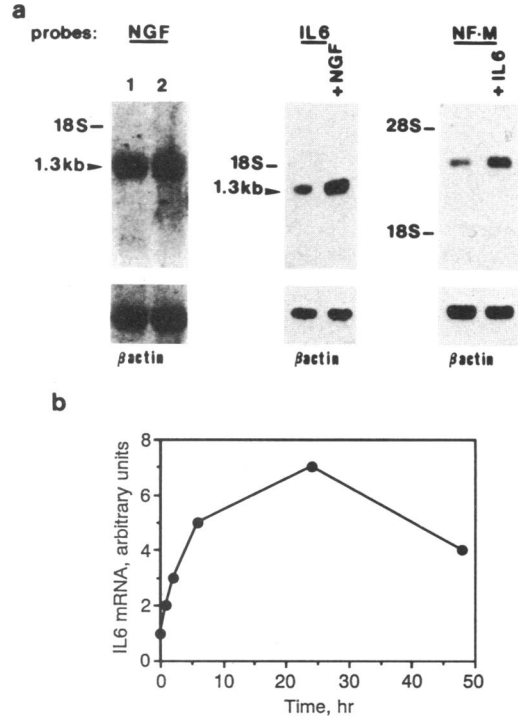

FIG. 4. (*a*) Northern blot analysis of 1 μg of poly(A)$^+$ RNA (*Left*) and 20 μg of total RNA (*Center* and *Right*) isolated from TC-1S and TC-2D (30th passage) (*Left*, lanes 1 and 2, respectively) or TC-1S cells cultured for 24 hr in the absence or presence of NGF (50 ng/ml) (*Center*) or recombinant IL-6 (6 ng/ml, British Biotechnology, Oxford, U.K.) (*Right*). Filters were hybridized with human NGFβ (clone phβN8B9, American Type Culture Collection) (*Left*), murine IL-6 cDNA (23) (*Center*), and NF-M (*Right*) probes and, after stripping, with a β-actin probe. kb, kilobases. (*b*) Time course of the IL-6 mRNA levels in TC-1S cells (24th passage) cultured for the indicated times with NGF (50 ng/ml). IL-6 mRNA was quantified by scanning of autoradiographic films after normalization of the IL-6 mRNA signal against the β-actin signal at each time.

Besides the variety of stimuli related to inflammatory and immune networks (27), a neural microenvironment could be involved in the control of IL-6 production: it is present in the central nervous system (28) and its production is increased by neuropeptides (29). We report here that IL-6 gene expression is enhanced by NGF: IL-6 mRNA levels increased significantly 1 hr after NGF addition to each of the TC cultures and reached a maximum after 24 hr (Fig. 4*b*). Autocrine/paracrine IL-6 production thus appears to be sustained by local production of NGF and to either mediate or amplify the neurotrophic effect of NGF on TC cultures.

Evidence is thus offered of a neuromodulatory loop within the thymic nonlymphoid population. The presence of neuronal-like cells and their relationships with IL-6, which is known to directly affect the T-cell proliferative/differentiative program (30), suggests the role of more complex and indirect pathways in thymocyte development that may involve neural-related components. The ability of thymocytes and T cells to respond to neuropeptide actions (31, 32) and the dependence of thymus development on neural crest derivatives (9) are in keeping with this hypothesis.

The thymic nonlymphoid population includes endocrine and neuroendocrine cells (15, 33). The previously reported (34) functional and structural relationships between endocrine and neural cells suggest that they may have common progenitors. The fate of the endocrine versus neural or intermediate stages may thus be determined by specific soluble factors, as described in embryonic adrenal medulla and sympathetic ganglia, in which NGF or glucocorticoids determine either neural or endocrine differentiation (35). Our data suggest that IL-6 may determine such a choice in thymic stroma and that IL-6 thus may be a multivalent regulator in the thymus.

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