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PURIFICATION TO APPARENT HOMOGENEITY OF A THYMOCYTE SPECIFIC GROWTH FACTOR FROM CALF THYMUS

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SUMMARY: A thymic peptide previously found to recruit thymocytes from G1 into S phase has been purified from a crude thymic extract by subsequent steps of gel exclusion chromatography and reverse phase high performance liquid chromatography (HPLC). The purified material, which appeared homogeneous on thin-layer chromatography and HPLC, stimulated the DNA synthesis of cultured guinea pig thymocytes in a nanomolar concentration range. The amino acid composition revealed a high content of acidic amino acids and no apparent homology to previously defined growth factors and thymus differentiation hormones.

The important role of the thymus in the development of the cell mediated immunity is now well established. However, the precise intrathymic events behind the differentiation of immunocompetent T lymphocytes are still not completely understood, although accumulative evidence suggests that hormonal factors of the thymic microenvironment have important inductive influences (see e.g. 1). One point that remains to be clarified is how the intense proliferation of immature thymocytes in the thymic cortex is initiated and maintained. The cycling thymocytes, which have a remarkably short generation time of 7-10 h (2), give birth to a progeny sufficient in number to replace the whole population of thymocytes every third day (3). We have previously contributed to the understanding of the thymic growth regulation in a series of papers describing a thymic factor which promotes the DNA synthesis of immature thymocytes cultured in vitro (4,5,6). A rapid recruitment of responsive cells from G1 into S phase was found after exposure to the thymic factor, and we have proposed that it acts as a progression factor for rapidly cycling precur-

Abbreviations: TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; α-MEM, α-modified Eagles Minimum Essential Medium; M_r, molecular ratio; pI, isoelectric point; TNBS, trinitrobenzenesulfonic acid; FTS, Facteur Thymique Serique; A_{280}, absorbance at 280 nm; A_{214}, absorbance at 214 nm.

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sor thymocytes (7). Recently, we have presented an improved schedule for preparation of the growth factor (8). Its biological activity was found to reside in pronase-sensitive material with apparent M_r of 1,600 and pI of 5.7 (8). In this communication we describe the purification of the thymic factor to apparent homogeneity by gel exclusion chromatography, semipreparative TLC and reverse phase HPLC. A preliminary amino acid composition of the purified thymic factor is presented.

MATERIALS AND METHODS

Preparation of thymic extract: The preparative procedure has recently been described in detail (8). A formic acid extract of calf thymus was acetone precipitated, extracted with methanol, precipitated with ether and dried. This extract was separated on a Sephadex G 50 column in 10% formic acid, and fractions pooled with vitamin B_{12} as molecular weight marker and lyophilized.
Protein determination: The protein content of the fractionated material was determined on hydrolysed aliquots by the TNBS method of Spadaro et al. (9).
Gel exclusion chromatography: Material from the Sephadex G 50 column was dissolved in 1% formic acid and applied to a 88x1.5 cm Bio-Gel P-4 column. Elution was performed with 1% formic acid, fractions of 1.2 ml collected, and A_{280} recorded. Aliquots of the fractions were lyophilized, redissolved in tissue culture medium and tested for ability to promote the DNA synthesis of cultured thymocytes (see below). The active fractions were lyophilized and dissolved in distilled water and applied to a 85x1.5 cm Bio-Gel P-2 column. Elution was performed with distilled water, fractions of 1.2 ml collected, and A_{280} recorded. Fractions displaying biological activity were pooled and lyophilized.
Semipreparative TLC: Semipreparative TLC and assay of the separated material in cultures of thymocytes was performed as described previously (10). In short, active material from the Bio-Gel P-2 column was applied to a Polygram MN 300 cellulose plastic sheet and development performed to 10 cm with butanol-acetic acid-water (60:15:25). Disks were punched out from the sheet in a continuous sequence in the direction of development, and tested for stimulating effect on the DNA synthesis of thymocytes, cultured as described below. Cellulose areas corresponding to disks which were active in the assay were scraped off from the sheet and the separated material eluted. A strip from the sheet was stained with ninhydrin-cadmium reagent (11), with increased sensitivity by omission of heating and letting the colour develope during 24 h in the dark.
Reverse phase HPLC: A 300x3.9 mm μBondapak C18 column (Waters Ass.) was used for separation, a SP 8700 system (Spectra Physics) for solvent delivery and a UV Absorbance Detector Mod. 4411 (Waters Ass.) for monitoring A_{214}. Gradient elution was performed with a 20 min linear gradient from 2 to 40% acetonitrile in 0.1% aqueous TFA. The flow rate was 1.5 ml per min. Methanol-water-TFA (40:60:0.05) and a flow rate of 1 ml per min was used for isocratic elution. In preparative runs, the peaks were manually collected, and aliquots tested for biological activity in thymocyte cultures after evaporation of the solvent and reconstitution in water.
Amino acid analysis: Acid hydrolysis was performed at 110°C for 20 h in 6 M hydrochloric acid in evacuated and sealed glass tubes. Two-dimensional TLC of dansylated amino acids on 5x5 cm polyamide sheets (Schleicher & Shuell) as

described by Hartley (12), was used for qualitative amino acid analysis. An automated amino acid analysis system (Waters Ass.) with a Resolve-RP^R column and a fluorescence detector (Mod. 420-AC) was used for quantitative estimation of precolumn o-phtaldialdehyde-derivatized amino acids.

Cell culture: The cell culture system has been described in detail previously (5). Briefly, freshly isolated guinea pig thymocytes at a concentration of $5x10^6$ cells per ml, were cultured for 5 h in 100 µl portions of serum-free α-MEM in flat-bottomed microwells in a Linbro multiwell plate. Material to be tested or control solvent was added in 5 µl at the onset of culture. One hour prior to harvest in a Skatron manifold harvester, the cultures received {^{3}H}thymidine (0.5µCi, 5Ci/mmol). Incorporated radioactivity was measured in a liquid scintillation spectrometer and expressed as mean cpm of replicate cultures. The results of this assay reflects the high proliferation rate of thymocytes in vivo and are compatible with those obtained from autoradiographic estimations of {^{3}H}thymidine labelling indices (6,7) and mitotic counts (13) in cultures of thymocytes.

RESULTS AND DISCUSSION

Fractions from a Sephadex G 50 column corresponding to an apparent M_r of 1,000 to 2,000 were used as starting material in this study. With more purified material it has previously been shown that the biological activity could be recovered in this molecular weight range (8). Only a minor effect, 20-25% stimulation of thymocyte DNA synthesis, was obtained with the Sephadex G 50 material, probably due to contamination with inhibitory components (data not shown). The elution profile after a separation of Sephadex G 50 material on a Bio-Gel P-4 gel exclusion column is shown in Fig. 1. Consistent with previous

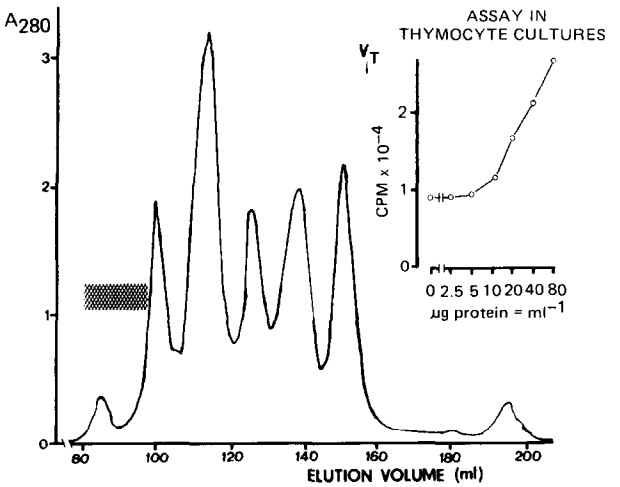
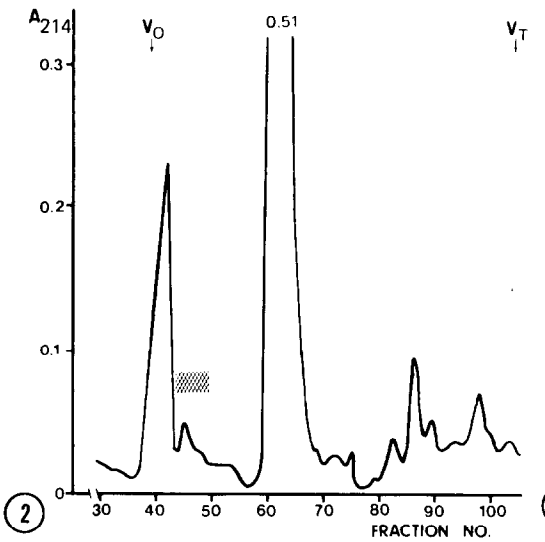


Fig. 1. Gel exclusion chromatography of thymic material (30 mg protein) on Bio-Gel P-4. Lyophilized aliquots of the fractions were tested for ability to promote DNA synthesis of cultured thymocytes and fractions corresponding to the dashed area found active. The inset figure shows the result of an assay of pooled active fractions. V_T indicates total volume of the column.

findings (10), the biological activity eluted approximately corresponding to the first peak. An assay of the pooled active fractions with cultured thymocytes demonstrated a dose-dependent effect (Fig.1). Because the starting material was more crude, and the protein content estimated with hydrolysed aliquots to include also N-terminal blocked peptides, the apparent protein concentration needed for stimulatory effect was higher here than previously reported (7).

Further separation was performed on a Bio-Gel P-2 gel exclusion column. The stimulating activity eluted adjacent to the void volume (Fig.2). The active fractions were assayed in a combined TLC and cell culture system described previously (10). The stimulating activity was ascribed to a faint ninhydrin-cadmium positive spot with an R_f value of 0.1 (Fig. 3). This spot was clearly detected only after prolonged ninhydrin reaction with increased sensitivity, indicating the presence of an N-terminal blocked peptide, apparently visual-



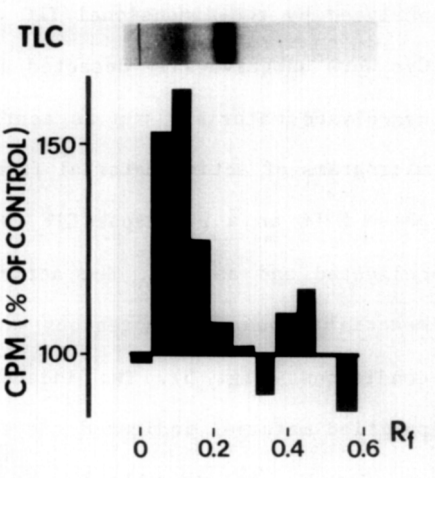


Fig. 2 (left). Gel exclusion chromatography on Bio-Gel P-2 of active material (530 µg protein) from the Bio-Gel P-4 column. The dashed area indicates fractions which were found to stimulate the {^{3}H}thymidine incorporation of cultured thymocytes. V_0 = void volume of the column as indicated by the elution volume of bovine serum albumin. V_T = total volume of the column.
Fig. 3 (right). TLC of active material from the Bio-Gel P-2 column and assay with cultured thymocytes. Disks were punched out from the TLC sheet, put into thymocyte cultures and the incorporation of {^{3}H}thymidine measured. Each column represents the mean cpm of duplicate cultures. The peak activity corresponds to a faint ninhydrin-cadmium positive band.

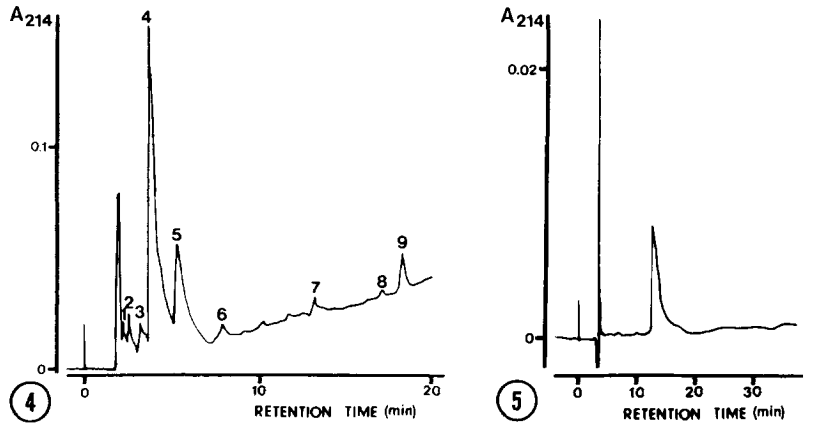


Fig. 4 (left). Reverse phase HPLC on μBondapak C18 of active material from a Bio-Gel P-2 column. Elution was performed with a linear gradient from 2 to 40% acetonitrile in 0.1% TFA. The numbered peaks were collected and assayed. The stimulating activity was recovered in peak 5.
Fig. 5 (right). Reverse phase HPLC on μBondapak C18 of active material from peak 5 in the gradient run (Fig. 4). Elution was performed with methanol-water-TFA (40:60:0.05). Only one peak was detected.

ised due to partial decomposition in situ on the TLC sheet. The active moiety was eluted and found homogeneous in two separate two-dimensional TLC systems (data not shown). A small amount was hydrolysed, dansylated and amino acids analysed by two-dimensional TLC on polyamide sheets. Glu, Ala, Gly, Val and Cys were unequivocally detected and also a small amount of Asp was found. Unhydrolysed material, run as control, was devoid of free amino acids. A few micrograms of active material from a Bio-Gel P-2 run was subjected to reverse phase HPLC on a μBondapak C18 column with gradient elution. All peaks were collected and assayed. The activity was recovered in peak 5 (Fig. 4). This material appeared homogeneous when run on the same column under isocratic conditions (Fig. 5). Two independent determinations with small amounts of purified material indicated the following amino acid composition (mole%): Glu 44, Gly 19, Asp 18, Ala 12, Val 7; Cys not assayed. This result is fairly consistent with our TLC data. The hydrophilic nature of our thymic growth factor, which has been discussed previously (5), was evident from the HPLC elution profiles and confirmed by the amino acid composition presented here. From the high content of acidic amino acids a lower pI can be predicted than that previously reported (8). However, it remains to be determined whether

some of the acidic amino acids exist in their amidated forms in the native peptide. Previous results have indicated a blocked N-terminus of the active moiety (8,10), and this is also supported by the above data on ninhydrin reactivity. In preliminary experiments, a reduced activity was found after treatment with pyroglutamate aminopeptidase indicating the presence of a functionally important pyroglutamyl residue at the N-terminus. Currently, work is in progress to further elucidate the structure of the thymic growth factor. The data presented here indicate no biochemical relationship to defined thymic differentiation hormones (14) and growth factors for other cell types (15).

The amount of thymic factor needed to obtain half-maximal biological response has previously been used to follow the degree of purification (5,8). In the present study, this figure was 250 ng per ml for the Bio-Gel P-2 material and 20 ng for material from TLC. The corresponding figure for the homogeneous HPLC peak was 14 ng and only a few nanograms were needed to get a detectable response in the assay. Growth factors and thymic hormones are active in a similar dose range (15,16), supporting the concept that our thymic growth factor is of physiological significance.

We have previously isolated material from the guinea pig thymus with similar biochemical and functional properties as our growth factor from calf thymus (17). However, we have not been able to detect any similar activity in an identically fractionated extract of human thymus, when assayed with guinea pig thymocytes. Furthermore, no effect of our calf thymus material was obtained with human (3 exp.) and rabbit (2 exp.) thymocytes, while mouse and rat thymocytes responded, although to a lower extent than guinea pig thymocytes (unpubl. results). These findings may be compared to the species restriction reported for lymphokines (18, 19), growth hormone (see e.g. 20) and mitogenic lectins (21). The question whether a corresponding activity exists in the human must await assay of fractionated human thymic material with human thymocytes, and such studies are under way.

An effect similar to that of our material has recently been demonstrated for the thymic hormone FTS in mice (22). However, synthetic FTS lacked such

growth-stimulating activity when tested with guinea pig thymocytes (17). Furthermore, FTS failed to induce responsiveness to mitogenic lectins in guinea pig thymocytes. Such induction has been interpreted as a differentiation effect of thymic hormones (23) and was confirmed for FTS with human thymocytes (own unpubl. results), indicating a species restricted effect also for FTS.

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