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Contrasting biological activities of thymopoietin and splenin, two closely related polypeptide products of thymus and spleen

(thymopentin/splenopentin/immunoregulation)

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ABSTRACT **Thymopoietin, a 49 amino acid polypeptide hormone of the thymus discovered by its effect on neuromuscular transmission, was later shown to induce T-cell differentiation and to affect immunoregulatory balance. A radioimmunoassay for thymopoietin revealed a crossreaction with a product found in spleen and lymph node but not other tissues. This product, named splenin, differs from thymopoietin only in position 34, aspartic acid for bovine thymopoietin and glutamic acid for bovine splenin. Synthetic pentapeptides corresponding to residues 32–36, called thymopentin and splenopentin, reproduce biological activities of thymopoietin and splenin, respectively. Thus thymopoietin and thymopentin affect neuromuscular transmission and induce the phenotypic differentiation of T precursor cells *in vitro* while inhibiting phenotypic differentiation of B cells. Splenin and splenopentin, in contrast, do not affect neuromuscular transmission, and they induce both T- and B-cell precursors.**

Thymopoietin, a 49 amino acid polypeptide hormone of the thymus discovered by its effect on neuromuscular transmission (1), was later shown to induce T-cell differentiation (2, 3) and to affect immunoregulatory balance (4–7).

Initial studies leading to the discovery of thymopoietin concerned myasthenia gravis, a human disorder in which neuromuscular impairment accompanies disease of the thymus (8). Pathological and immunological evidence suggested that the thymic lesion might be due to autoimmune thymitis, and an animal model of experimental autoimmune thymitis was shown to be associated with neuromuscular impairment by electromyographic and other neurophysiological techniques (9, 10). Results with this model implied that a product of the thymus can impair neuromuscular transmission. Neurophysiological evidence from thymectomized and thymus-grafted rats indicated that this product, now termed thymopoietin, was secreted by the normal thymus (11, 12).

Neuromuscular transmission was impaired 1–5 days after injection of preparations of thymus but not of spleen or other tissues (1). The delay in appearance of this effect implies a regulatory rather than direct influence on neuromuscular transmission. This assay led to the isolation of two closely related polypeptides of linear 49 amino acid sequence, thymopoietin I and thymopoietin II (1). These shared a common pentapeptide at residues 32–36, Arg-Lys-Asp-Val-Tyr (13), named thymopentin or TP-5, which reproduced the biological activity of thymopoietin (3, 14, 15) and thus was considered to represent the active site. Since thymopoietins I and II were isolated from pooled bovine thymus and are not functionally distinguished, they could be products of a single polymorphic gene.

A radioimmunoassay (RIA) for thymopoietin gave identical displacements for thymopoietins I and II (16). The fact that serum levels of thymopoietin, measured by RIA in rats, failed to decline after thymectomy led to the finding that spleen and lymph nodes, but not other tissues, yield a product that reacts in the RIA for thymopoietin, and this product was isolated and its amino acid sequence was determined (17). Since the only structural difference between the splenic product and thymopoietins I and II, both of bovine origin, is the substitution of Glu for Asp at position 34, it was first named thymopoietin III.

Here we report on the biological activities of this splenic product, which because of its characteristic properties and tissue of origin we now call splenin, and on the synthetic peptide splenopentin (SP-5), which was derived in the same way as TP-5 and corresponds similarly to positions 32–36 of the native molecule (Arg-Lys-Glu-Val-Tyr).

MATERIALS AND METHODS

Materials. Thymopoietin and splenin were isolated as described (17). TP-5 (Arg-Lys-Asp-Val-Tyr) and SP-5 (Arg-Lys-Glu-Val-Tyr) were chemically synthesized by using a modification of the solid-phase method (18). Eight- to 10-week-old female CD_1 mice (Charles River Breeding Laboratories) were used in the neuromuscular assay and 6-week-old male C57BL/6 mice (Sloan–Kettering, New York) were used in the induction assay. Bovine serum albumin (Path-O-Cyte 5) was purchased from Miles. Rabbit anti-mouse $F(ab)_2$ was purchased from Cappel Laboratories (West Chester, PA) and RPMI-1640 medium was purchased from GIBCO.

Neuromuscular Assay. Peptides were dissolved in normal saline and injected intraperitoneally into groups of 5–10 mice. Neuromuscular trasmission was assessed after 20–24 hr as described previously (10). Briefly, the median nerve was stimulated with a train of 10 supramaximal stimuli at 50 Hz. Ten forelimb sites were sampled with the recording electrode in each animal and the height of the muscle action potential generated by the 10th impulse was expressed as a ratio to the initial muscle action potential; the mean ratio was calculated for each mouse. The statistical significance of differences in ratios between the experimental and control groups was assessed by the least significant difference test (Fisher's pairwise *t* tests) (19).

Induction Assay. Assays for induced expression of Thy-1 and for Lyb-2 were conducted as described (3). In brief, prothymocytes (Thy-1^-) and pro-Lyb-2 cells were coenriched from B6-Lyb-2.1 congenic mouse spleen by bovine serum albumin density gradient centrifugation (Path-O-Cyte 5, lot 35, 1 ml of 35/29/26/23/18/12%). The 26/23 and 23/18 interface layers were combined, and Thy-1^+ and Lyb-2^+ cells were removed by reaction with monoclonal Thy-1.2 and Lyb-2.1 antibodies (20) and adherence to plates coated with affinity-purified rabbit anti-mouse $F(ab)_2$. The washed nonadherent cells were used for both assays. This starting population contains 30–40% prothymocytes and 30–40% pro-Lyb-2 cells (known to represent separate committed pre-

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Abbreviations: TP-5, thymopentin; SP-5, splenopentin.

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cursor populations) (3). Cells (5×10^6 cells per 0.5 ml of RPMI 1640 medium) were incubated in 5-ml plastic tubes with equal volumes of inducer in serial dilution in RPMI 1640 medium in a humidified 5% CO_2 atmosphere for 3 hr. The cells were then assayed separately for Thy-1 and Lyb-2 expression with monoclonal antibodies in optimal concentration by the staphylococcal protein A–sheep erythrocyte method (20) (controls without inducer register <5% induced cells).

RESULTS

Thymopoietin and TP-5 were both active in the neuromuscular assay at threshold doses of 0.1 and 10 μg per mouse, respectively, as shown in Fig. 1; neither splenin nor SP-5 was active at doses up to 100 μg per mouse. The thymic nonapeptide FTS (facteur thymique sérique) (21) and the 74 amino acid polypeptide ubiquitin (22), which can be obtained from all nucleated cells and induces T- and B-cell phenotypes *in vitro*, were also inactive (data not shown). Thus thymopoietin and TP-5 remain the only products tested so far that display the neuromuscular effect.

Thymopoietin and splenin, and their pentapeptide analogues, were then further tested in serological induction assays *in vitro*. These assays monitor the induced expression of T- and B-cell surface components by committed precursor cells of spleen, within 2 hr *in vitro*, in response to various defined inductive agents (3). The salient property of thymopoietin and TP-5, among active molecules so far tested in induction assays, is the ability to induce phenotypic differentiation of T precursor cells while inhibiting phenotypic differentiation of B cells (3). This selective induction of T-cell precursors is illustrated again in Table 1, where the chosen phenotypic markers were Thy-1 for T cells induced from prothymocytes and Lyb-2 (23) for B cells induced from Lyb-2 precursors. Splenin and SP-5, in contrast, did not show this target cell distinction but, as shown in the table, induced both T- and B-cell phenotypes over a full range of concentrations.

DISCUSSION

Clearly, thymopoietin–TP-5 and splenin–SP-5 are decisively distinguished by their properties in regard to both neuromuscular transmission and induction of lymphocyte phenotypes, and these differences can be attributed only to the Glu/Asp substitution at position 34 since there are no other unique differences between splenin and thymopoietins I and II. It may be that thymopoietin and splenin originated in evolution from gene duplication and variation, the two gene products now having distinct properties and sites of production.

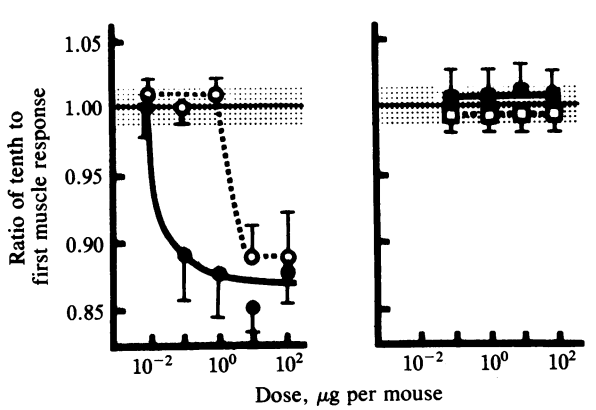

FIG. 1. Dose–response effects of thymopoietin and TP-5 on neuromuscular transmission, and absence of demonstrable effect with splenin and SP-5. ●, Thymopoietin; ○, TP-5; ■, splenin; □, SP-5. Results are given as mean ± SEM; the stippled area indicates the control mean ± SEM.

Table 1. Comparative T- and B-cell inductivity by thymopoietin, splenin, and the synthetic pentapeptides TP-5 and SP-5

Inducer	Conc., μg/ml	% marker-positive cells induced* T-cell assay (Thy-1$^+$)	B-cell assay (Lyb-2.1$^+$)
Thymopoietin	10^1	100 (6)	<2 (6)
	10^0	90 ± 0 (6)	<2 (6)
	10^{-1}	86 ± 10 (6)	<2 (6)
	10^{-2}	70 ± 2 (6)	<2 (6)
	10^{-3}	50 ± 9 (6)	<2 (6)
	10^{-4}	16 ± 16 (6)	<2 (6)
TP-5 (Arg-Lys-Asp-Val-Tyr)	10^1	96 ± 5 (6)	<2 (6)
	10^0	85 ± 6 (6)	<2 (6)
	10^{-1}	73 ± 8 (6)	<2 (6)
	10^{-2}	44 ± 3 (6)	<2 (6)
	10^{-3}	21 ± 10 (6)	<2 (6)
	10^{-4}	5 ± 9 (6)	<2 (6)
Splenin	10^1	100 (1)	100 (4)
	10^0	96 ± 6 (3)	91 ± 10 (4)
	10^{-1}	88 (2)	84 (2)
	10^{-2}	83 ± 4 (3)	67 ± 5 (3)
	10^{-3}	80 ± 6 (3)	47 ± 3 (3)
	10^{-4}	69 (2)	43 ± 4 (3)
	10^{-5}	53 (2)	38 ± 6 (3)
	10^{-6}	25 (2)	39 (2)
	10^{-7}	42 (2)	27 (2)
	10^{-8}	19 (2)	16 (2)
	10^{-9}	5 (2)	2 (2)
SP-5 (Arg-Lys-Glu-Val-Tyr)	10^1	97 ± 4 (4)	93 ± 0 (4)
	10^0	78 ± 4 (4)	75 ± 5 (4)
	10^{-1}	55 ± 4 (4)	55 ± 3 (4)
	10^{-2}	65 (2)	44 (2)
	10^{-3}	9 (2)	15 (2)
	10^{-4}	<2 (2)	<2 (2)
Control: Synthetic rearranged pentapeptide Tyr-Arg-Lys-Asp-Val	10^1	<2 (3)	<2 (3)
	10^0	<2 (3)	<2 (3)
	10^{-1}	<2 (3)	<2 (3)
	10^{-2}	<2 (3)	<2 (3)
	10^{-3}	<2 (3)	<2 (3)

*The data are expressed as percentage of maximal induction observed with thymopoietin (T-cell assay) or splenin (B-cell assay), respectively, at 10 μg/ml in each experiment. Mean ± SEM for the number of experiments shown in parentheses is tabulated.

Thymopoietin and TP-5 have immunoregulatory effects in a number of animal model systems (4–6, 14, 15) and it will be important to know the comparative properties of splenin and SP-5 in these same systems. For instance, in a model system based on the ability of C3H females, intact or thymectomized, to reject male C3H skin grafts, TP-5 and SP-5 affected this response *in vivo* in different ways (24). Thus the biological distinctions reported in the present paper, related to a substitution of only 1 of 49 amino acid residues, appears to have a counterpart in immunoregulatory effects of these polypeptides.

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