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PARTIAL REACTIVATION OF IMPAIRED IMMUNE COMPETENCE IN AGED MICE BY SYNTHETIC THYMUS FACTORS

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SUMMARY. Our results clearly show the profound impairment of the humoral, hemolytic, primary, immune response in aged mice (22 months) as compared with this response in young (10 weeks) mice. A partial but significant reactivation of the age-determined impairment of the immunological responsiveness results from subcutaneous administration of two newly synthesized thymus factors and one analog. These synthetic thymus peptide factors correspond to factors previously isolated and identified in thymus glands or blood.

INTRODUCTION

The modern era of thymus research commenced in the late 1950's and early 1960's when it was established that thymectomy results in retarded growth, poor development of lymphoid tissue, increased susceptibility to infections, increased incidence of tumors and inability to reject foreign skin grafts. It was recognized that these effects were secondary to a decrease in circulatory small lymphocytes, later described as thymus-derived cells or more simply T cells, because they depend on an intact thymus for their development from precursor cells. Recent reviews adequately cover the extensive literature (1–4).

Subsequently, it was demonstrated that the thymus produces a "substance" that has a direct effect on precursor lymphocytes and induces them to mature into functioning T lymphocytes; hence, the nascence of the thymus-hormone concept. This stage led to the development of a number of cell-free extracts that were used to restore, with various degrees of success, the lost thymus activity in thymectomized animals (1–4). The next

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stage in thymus research can be described as identification of the chemical structures and the first syntheses of individual peptides which had been sequenced and which appeared to show biological effects.

The purpose of this communication is to describe the restoration of an age-dependent suppression of the immunological responsiveness by two newly synthesized thymus factors and one analog. This is a useful experimental model for assay of potential immunopotentiating agents (6). Studies carried out during the last decade have established that the thymus is subject to age-related involution, morphological as well as functional, occurring in both animals and man. This involution results in a profound gradual decline of the immunological responsiveness, mainly affecting T cell dependent functions in a manner similar to the post-thymectomy state (reviewed in 6). For this reason, the thymus was declared the "master gland" or "clock" of aging (5).

MATERIALS AND METHODS

BIOLOGICAL. Female CF1 mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA.

SRBC (Baltimore Biological Laboratories, Baltimore, MD.) were used as antigen for primary immunization and were administered via the tail vein (4×10^7 cells/mouse). The day of the antigen administration is designated as day 0.

The test materials were dissolved immediately before use in sterile 0.9% sodium chloride solution (saline) in a concentration of 50 μg/ml.

On day 7 and day 1 before, and again on day 4 after the SRBC administration, groups of mice 22 months old (20 mice in each group) were treated with the test material injected subcutaneously with a dose of 10 μg/mouse. Two control groups (20 mice, 22 months old and 20 mice, 10 weeks old) were treated with saline.

At 24-hour intervals after the SRBC administration, blood was collected from each mouse, pooled and the plasma separated and stored at $-40°$ C.

The humoral, hemolytic antibody titer was determined using the 50% end point method (7). The values shown represent the determined values for pooled plasma from 20 mice in each group with standard deviations indicated.

The methods for SRBC preparation, plasma collection and humoral, hemolytic antibody titer determination have been previously described in more detail (6).

Possible contamination with bacterial lipopolysaccharide (a highly toxic agent and a strong activator of immune maturation) was precluded by the exclusive use of only nonpyrogenic materials.

All glassware was heated for five hours at 170°C. Nonpyrogenic, sterile saline (Abbott Laboratories, North Chicago, IL.), syringes, needles and pipets were used throughout.

PEPTIDE SYNTHESES. Thymopoietin II, FTS, which is a nonapeptide and a dodecapeptide analog of FTS (Val-Lys-Arg-Gln-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn-OH) were syn-

Abbreviations: SRBC, sheep red blood cells; FTS, "*facteur thymique serique.*"

thesized by a solid-phase methodology on a Beckman 990 Peptide Synthesizer. Amino acid derivatives were purchased from either Beckman Instruments, Inc., Palo Alto, CA., or Peninsula Laboratories, San Carlos, CA. The peptides were synthesized on a modified Merrifield resin (8). The program used in the course of the automated solid-phase syntheses was similar to that previously described (9). The removal of the protecting groups and the simultaneous cleavage of the peptides from the resin was accomplished in anhydrous (by using CoF_3) liquid hydrogen fluoride in the presence of anisole. Thymopoietin II was purified by gel filtration on Sephadex G-50 followed by a Sephadex LH-20 column. FTS and the dodecapeptide analog of FTS were purified by gel filtration on Sephadex G-25. Details of these syntheses and purifications will be published separately.

RESULTS

The data showing the dependence of the humoral, hemolytic, primary, immune response on the age of the mice are presented in Table 1. The marked suppression of this response in old mice is partially but significantly reversed by the subcutaneous administration of two synthetic thymus peptide factors and one synthetic peptide analog.

Our preliminary data show that the administration of the synthetic thymus factors is not accompanied by hyperplastic alterations in the reticuloendothelial system organs or any other toxic effects.

DISCUSSION

Recognition of the thymus involvement in immune competence and evidence of an endocrine function have stimulated increasing efforts to isolate and identify individual factors from thymus glands or blood (1–4).

"Thymosin" is a fraction which was isolated by a five-stage procedure from bovine thymus tissue (10) and appears to consist of numerous polypeptide components. This fraction induces an immunological restoration in thymectomized and athymic nude mice (11). Further purification yielded first, fraction V, containing polypeptides with molecular weight from 1,200–14,000, and later a single acidic polypeptide, fraction VIII, with 108 amino acids and a molecular weight of 12,800.

TABLE 1

Hemolytic, primary, immune response (4×10^7 cells/mouse) in 10 weeks and 22 months old CF1 female mice, and partial compensation of the age-dependent suppression of this response by subcutaneous administration of synthetic thymus factors (10 μg/mouse/injection). The values shown represent the determined values for pooled plasma from 20 mice in each group with standard deviations indicated.

TREATMENT	HEMOLYSIN LEVEL ON DAY AFTER SRBC ADMINISTRATION:				
	4	5	6	7	8
A. *Young Adult Mice* (10 weeks)					
Saline (Control)	396.4 (385.3–408.0)	570.5 (537.5–605.4)	419.1 (406.5–431.9)	302.1 (293.8–310.6)	245.7 (234.6–257.5)
B. *Old Mice* (22 months)					
Saline (Control)	4.9 (2.8–6.9)	60.9 (58.5–63.4)	55.7 (53.7–57.9)	37.4 (35.9–39.0)	31.6 (28.5–35.2)
Thymopoietin II	3.4 (2.8–4.1)	57.2 (54.9–59.6)	70.4 (72.9–77.9)	56.8 (54.8–58.9)	44.0 (39.9–48.5)
Nonapeptide	6.2 (3.6–8.6)	89.0 (85.0–93.2)	95.8 (92.4–99.3)	65.6 (63.2–68.2)	55.6 (53.2–58.1)
Dodecapeptide	4.2 (2.6–5.7)	64.3 (60.2–68.7)	87.2 (82.7–91.9)	59.4 (55.3–63.9)	49.5 (45.4–53.8)

In another approach, two closely related polypeptides, thymopoietin I and II, have been isolated from bovine thymus. They induce differentiation of T cell precursors, and their sequences of 49 amino acid residues have been determined (12, 13).

Another factor isolated from calf thymus has a molecular weight of about 3,000 and appears to be an acidic polypeptide of 31 amino acids (14).

"Facteur thymique serique" has been isolated from porcine serum. It is an acidic polypeptide of about 1,000 molecular weight and is biologically active in 10^{-11} molar concentration. The amino acid sequence has been determined and based on this determination, a peptide has been synthesized with biological activity similar to the effect produced by natural peptide (15).

Recently, the entire amino acid sequence of bovine thymopoietin II was determined (13) and a classical synthesis of a nonatetracontapeptide, corresponding to its entire

amino acid sequence was accomplished (16). Biological evaluation and activity confirmation *in vivo* has not been reported.

The mode of biological action of the various thymus factors is still not fully understood, but the general concensus is that they induce T cell maturation probably through the adenyl cyclase–cyclic AMP pathway (17), although the specific steps remain to be elucidated. Based on studies with "thymosin," a two-step T cell maturation sequence has been proposed (18).

In our present and previous studies, we clearly demonstrated the profound suppression of the primary immune response in aged mice* to SRBC, a thymus dependent antigen (6). We have shown that this suppression is accompanied by a reduced thymus weight-body weight ratio (6). Goldstein *et al* (19) and Bach (20) reported that blood levels of circulating thymus hormones decrease with advancing age, thus the thymus involution is also associated with a functional deficiency.

Only recently have the complexities of cell interactions in the immune response been realized and it is safe to assume that in our present study, administration of exogenous thymus factors compensates the declining function of the thymus during senescence. This apparently restores the functional balance between T cells and B cells which is required for an optimal immunological response to SRBC.

Our work experimentally demonstrates control *in vivo* of depressed thymus function with synthetic thymus hormones.

Further tests are in progress in order to optimize the experimental conditions. Because of the chemical nature of the synthetic thymus factors, their half-life in the circulation will be very short (19). This necessitates daily administration in future experiments.

Our study guides us closer to the possibility of restoring youthful "immunological vigor" in aging organisms.

*During the past year, many investigators active in the field of aging were strongly criticized for using "old" animals when they were not, or for using mice of various ages as controls ("young," "adult" or "young-adult"). In our experiments, this error has been avoided (6).

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