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THYMIC HORMONES: BIOCHEMISTRY, AND BIOLOGICAL AND CLINICAL ACTIVITIES[1]

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It is now well demonstrated that the thymus plays a central role in the immune system. Thymus-derived cells (or T cells) mediate delayed hypersensitivity reactions and the rejection of allografts and certain tumors. In addition, they regulate antibody production by bone marrow–derived cells (B cells) either positively as helper cells or negatively as suppressor cells. Abnormalities in their function are considered to be at the origin of many pathological situations including some immunodeficiency syndromes, autoimmune diseases, and numerous acute or chronic infections. It is obvious that any means of manipulating T-cell production by the thymus could be of great help to the clinician.

The mode of action of the thymus in the manufacture of T-cells is still incompletely known. There is, however, increasing evidence that the thymus gland produces humoral factors capable of modifying various functions of thymocytes. Many of these factors, which are secreted by the thymic epithelium, are involved in T-cell diffentiation even if they do not represent the exclusive mode of action of the thymus (the role of direct contact of T-cell precursors with the thymic microenvironment cannot be excluded) (1–3).

Several groups have characterized thymic factors (generally from calf thymus glands) and have provided various data on their biochemical nature and their biological activities. Some preliminary clinical trials have been performed. We review these data, on a pharmacological basis, rather than discuss the indirect ap-

[1]The following abbreviations are used in this review: ARFC, autologous rosette-forming cell; ATS, antithymocyte serum; BM, bonemarrow; BSA, bovine serum albumin; B/W, black/white; DNP, dinitrophenol; GVH, graft versus host; MLC, mixed lymphocyte culture; MSV, Moloney sarcoma virus; NZB, New Zealand black; PBS, phosphate balanced solution; SDS, sodium dodecyl sulfate; SLE, systemic lupus erythematosus; SRBC, sheep red blood cells; TF, thymic factor; and THF, thymic humoral factor.

proaches of the thymic hormone concept and the site of thymic hormone action in T-cell differentiation (see 1–3).

PREPARATION, ASSAY, AND BIOCHEMISTRY OF THYMIC FACTORS

As mentioned above, several groups of investigators have reported biological activities of thymic extracts or of circulating thymic factors. It is difficult to compare these data because the original source of material varies and especially because the assays utilized are very different. Also, the data presented in this section do not necessarily apply to products used in the biological assays presented in the next section, since functional studies were often performed with relatively crude extracts even if the same authors have reported on more refined and sophisticated preparations. In other words, there is a possibility that many of the biological effects described with thymic extracts are not due to the purified and well-characterized entity present in the crude extract and identified on the basis of another assay.

A. L. Goldstein and White's "Thymosin"

BIOASSAY The bioassay utilized by Goldstein, Slater & White until 1971 was a "lymphocyte poietic" assay consisting of measuring DNA synthesis by lymph nodes after in vivo injection of the tested extract (4). More recently, these authors have used in vitro rosette assays (5) and an MLC assay (6).

ISOLATION Calf thymic tissue is homogenized in 0.15 M NaCl and centrifuged at 14,000 g. The supernatant is heated at 80°C and precipitated by acetone. The precipitate is removed by centrifugation and the supernatant is added with ammonium sulfate. The precipitate is collected and dissolved in Tris Cl buffer before being desalted on Sephadex® G-25. This material (called *fraction 5*) has been routinely used in most studies dealing with biological thymosin activities. Further purification has been performed in some studies using DEAE cellulose, Sephadex G-50, and polyacrylamide gel electrophoresis (7).

BIOCHEMICAL CHARACTERISTICS The purity of the last *fraction* has been assessed by analytical polyacrylamide gel filtration: one single band is visible at pH 8.3 or pH 2.9. Estimates of molecular weight have been done on polyacrylamide gels containing 0.1% SDS. By comparison with several markers it was assumed that thymosin (*fraction 8*) has a molecular weight of approximately 12,000. Amino acid analysis revealed that the molecule was rich in acidic residues, contained not unusual amino acids, and only one tyrosyl residue. The terminal amino acid is blocked (7).

N. Trainin's Thymic Humoral Factor

BIOASSAY Bioassay is an in vitro model of graft versus host reaction primitively developed to study the immunocompetence of isolated lymphoid cell populations: competent T cells normally induce in vitro an increase in an allogeneic spleen

explant whereas spleen cells from neonatally thymectomized mice do not achieve it except if they are previously incubated with thymic extracts (8, 26).

ISOLATION Thymus glands are cleaned of blood vessels and connective tissues, homogenized in a Virtis® blender in PBS, and centrifuged for 20 min at 2,500 g. The supernatant is strained through gauze and centrifuged again at 100,000 g for 5 hr. The supernatant collected after this ultracentifugation step is then passed through Millipore® filters of 0.45 μm porosity. The last dialysis purification step is based upon the fact that the active material is of low molecular weight and therefore passes through cellophane dialysis bags. Further purification has been achieved for THF biochemical characterization, using Sephadex G-25 and DEAE-Sephadex A-25 chromatography (9, 10, 26).

BIOCHEMICAL CHARACTERISTICS Purity of the material obtained has been analyzed by isoelectric focusing on polyacrilamide gel. It is probably a polypeptide since it is destroyed by proteolytic enzymes and not by ribonuclease or deoxyribonuclease. The amino acid analysis reveals the presence of Asp, Ser, Glu, and Gly in large amounts. The pH is acidic (between 5.7 and 5.9). Its molecular weight is certainly less than 10,000 from dialysis data and close to 3,000 from G-25 data. However Sephadex G-25 does not provide precise evaluation of molecular weight and it is not possible from present data to differentiate between THF with a reported molecular weight of close to 3000 and a circulating thymic factor (see section on the circulating thymic factor) (9).

G. Goldstein's Thymopoietins I and II

BIOASSAY Patients with myasthenia gravis show symptoms of motor weakness as a result of a partial failure of neuromuscular transmission. The association with thymic pathological abnormalities (germinal centers, thymomas, or thymic hyperplasia) has long been known, and thymectomy has been found empirically to improve the status of a large proportion of patients. Hence the idea of characterizing thymic extracts by injecting them into test mice and studying neuromuscular transmission electromyographically (11).

ISOLATION Calf thymus is homogenized and heated at 70°C for 30 min. The material is then centrifuged and the supernatant is concentrated on Amicon® membranes before being chromatographed on Sephadex G-50 columns and on hydroxyapatite (11, 12).

BIOCHEMICAL CHARACTERISTICS The purity of the two thymopoietins has been assessed by polyacrylamide disc electrophoresis at pH 8.9. With 200 μg of thymopoietin I and II per gel, there was a single major band stained with Coomassie blue ingels run at pH 8.9 and 4.3. Analysis of the terminal amino acids provided further evidence for purity since no residue could be identified. Thymopoietins I and II are related entities, as shown by peptide mapping and by the existence of immunological cross-reactions (12).

The amino acid sequence of thymopoietin II was established using an automated sequenator (13). The peptide contains 49 amino acids. The precise molecular weight is 5562. There is microheterogeneity at the C terminal with approximately two thirds of the molecules lacking the C terminal arginine found on the remaining molecules. It is interesting to note that a randomly selected peptide corresponding to residues 29–41 was synthesized and shown to have a selectivity of action comparable to thymopoietin (14).

The Circulating Thymic Factor

BIOASSAY The principle of the assay consists in the induction of θ antigen on θ negative T-cell precursors after incubation with the thymic hormone. θ conversion is studied on θ negative rosette-forming cells (toward sheep erythrocytes) in normal bone marrow or adult thymectomized mouse spleen (15, 16).

ISOLATION Isolation of the polypeptide has been achieved by six successive operations: (*a*) defibrination, (*b*) dialysis, (*c*) concentration on UM2 Amicon membrane, (*d*) Sephadex G-25 filtration, (*e*) CM cellulose chromatography, and (*f*) Sephadex G-25 filtration in acetic acid (15).

BIOCHEMICAL CHARACTERISTICS The purity of G-25 acetic acid–eluted fractions is assessed (*a*) by the observation of a single spot in five thin layer chromatographies using different solvents, (*b*) by the absence of detectable amino acid by dansylation after treatment of protein quantities (1 nM) largely superior to the threshold of the dansyl technique, and (*c*) by the reproducibility of amino acid analysis with a satisfactory stoichiometry (15).

The molecular weight of the circulating thymic factor has been evaluated by G-25 chromatography in phosphate buffer 0.2 M, pH 7.3, by comparison to several markers, as well as by calibrated dialysis (in a standardized system with 1 m^2 area). Both evaluations provided a molecular weight which is compatible with the amino acid stoichiometry (15).

The circulating thymic factor loses its biological activity after trypsin, chymotrypsin, or pronase treatment. Its pH is close to 7.5. It contains a lysine but no aromatic amino acids. Absence of detectable amino-terminal, as shown by dansylation studies indicate that the amino-terminal is blocked (likely under the form of pyrroglutamic acid) (our unpublished results). The amino acid sequence of the circulating thymic factor has recently been determined in the pig (52): Gln-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn.

Other Preparations

Factors other than the four just described have also been reported. They have been isolated on the basis of various and sometimes unexpected bioassays. The most important are: (*a*) Luckey's lymphocyte-stimulating hormones, two proteins with molecular weights of 17,000 and 80,000, respectively, evaluated by their action on lymphocytosis and increase in antibody synthesis of newborn mice (17); (*b*) Comsa's homeostatic thymus hormone, a heat-labile glycopeptide of 1800–2500 mol. wt.

showing antagonistic effects against ACTH, TSH, thyroxine, gonadotropins, and synergy with growth hormone as well as a chemotactic influence on lymphocytes (18); (*c*) and Mizutani's thymic hypocalcemic factors (T1 and T2) inducing hypocalcemia and lymphocytosis (19).

It should be made clear that the factors mentioned here have often been isolated on the basis of assays not directly related to immunology. This is unfortunate since several of these thymic factors have been fairly well defined biochemically.

Conclusions: One or Several Thymic Hormones?

It is difficult to draw conclusions from the present state of knowledge. The main question is to determine whether the various thymic polypeptidic factors isolated by the groups quoted in the preceding pages are related. There are differences in the molecular weights but this cannot be considered a conclusive argument since the same product could exist under different molecular forms. It could thus be possible that thymosin is a precursor or contains precursors of the circulating TF. A relationship is made unlikely between thymopoietins and circulating TF after examination of the thymopoietin sequence and TF amino acid analysis. In contrast, there are very few differences between THF and circulating TF; it is very possible that they are identical.

BIOLOGICAL ACTIVITIES

Several in vitro and in vivo biological activities of thymic extracts have been demonstrated in the mouse. It should, however, be stressed that none of the biological effects detected in these tests are specific to thymic products. Indeed several pharmacological substances, in particular cyclic AMP, can induce similar or sometimes more dramatic effects. This underlines the need for critical interpretation of the biological significance of these in vitro assays and indicates that in vivo restoration experiments are still highly relevant, all the more so since purified extracts are now available which prevent heavy protein overloading. Finally, the convergence of data from both in vivo and in vitro experiments provides the most convincing arguments for the role of thymic hormones.

Induction of T-Cell Markers on T-Cell Precursors

We reported in 1971 that thymic extracts induce the appearance of the Θ antigen on Θ-negative normal bone marrow rosette forming cells (RFC) or on spleen RFC from adult thymectomized (ATx) mice (5). This induction, which occurs within 60 min at 37°C, was confirmed in 1973 by Komuro & Boyse, who showed that incubation of thymic extracts with normal spleen cells previously fractionated on a BSA gradient rendered a significant proportion of Θ-negative cells Θ-positive (20). In both experiments the minority of precursor cells were separated from the whole population by rosette formation or BSA fractionation. Similar results were also obtained with unfractionated nude mouse spleen cells (21).

The significance of the changes induced by thymic extracts is still open to speculation, particularly with regard to its relevance to T-cell differentiation. One may think

that the appearance of T-cell markers is the first step of the irreversible differentiation of precursor cells in the direction of mature T cells. On the other hand, one may speculate that the changes in question are in fact associated with reversible membrane changes. The fact that in vivo Θ induction in ATx mice by thymic extracts (22) or cyclic AMP (23, 24) is reversible within 24–48 hr is compatible with this hypothesis. Such reversible changes could probably better fit with membrane rearrangement than with gene activation and would imply that Θ induction is not equivalent to true T-cell differentiation. However, it might very well be that these changes put the precursor cells in a particular metabolic state, which is a prerequisite for the cell to proceed in the differentiation process (according to its own program, independently of any further thymic stimulus). Consequently, one should not accept definitively the concept of thymic hormone (considered as a thymus substitute) for a given product before other in vitro and especially in vivo activities of the factor have been demonstrated as has been the case for several products (15, 25, 26).

Mitogen and MLC Responses

Several groups of authors have reported thymic factor–induced increase in mitogen responsiveness in thymectomized mice (27) and unexpectedly in normal spleen cells (28, 29, 30) either in vivo or in vitro. The increment in responses, however, remained generally modest and the overall interpretation of these data is not totally convincing. The same comments apply to MLC assays (6, 31).

Homograft and GVH Responses

One of the most salient manifestations of newborn thymectomy is a severe impairment of cell-mediated responses. Many studies have been undertaken in order to try to restore such responses by the administration of thymic extracts. Two models mainly have been investigated: the homograft response directed against either skin or tumor H-2 incompatible grafts and the capacity of inducing GVH reaction in an allogeneic host.

Cytotoxic T lymphocytes are generated and can be easily detected in the course of an immune reaction against histocompatibility antigens. However, the activity of such effector T cells is almost nonexistent in newborn Tx mice. Ikhehara et al have recently reported that thymus-deprived mice treated with a thymosin-like extract developed the capacity to reject an allogeneic sarcoma (27). The repair of T-cell function was also manifested by the presence in the spleen of these animals of specifically committed killer T cells, which could be inhibited by an anti-Θ serum. It had been previously shown by Trainin's group that THF restored the capacity of T cells from Tx donors to become educated after in vivo transfer into an H-2 incompatible irradiated host and to differentiate into cytotoxic cells (32). Lastly, M. A. Bach has recently shown that the decreased capacity of ATx C57BI/6 mice to generate cytotoxic cells against DBA/2 mastocytoma cells could be returned to normal levels by in vivo treatment with purified circulating TF (33).

In contrast with the above experiments which required in vivo thymic factor administration during several weeks, a series of experiments was reported indicating

that a short in vitro incubation (1 to 2 hr) with a thymic extract rendered bone marrow cells immunocompetent in a GVH assay. Goldstein et al showed that bone marrow cells incubated in vitro with thymosin induced splenomegaly after injection into lethally irradiated hosts (34). Trainin et al were unable to obtain with THF such direct conferment of immunocompetence upon BM cells. The THF-treated cells required an additional in vivo or in vitro contact with splenic tissue before acquiring the capacity to induce an in vitro GVH reaction (35).

Anti-Tumor Responses

Zissblatt et al have shown that the administration of thymosin to newborn mice accelerated their ability to reject an MSV-induced sarcoma (36). Thymosin could also delay to a certain extent the death by tumor growth of adult mice immunologically impaired by ATS or thymectomy plus irradiation (37). We have reported similar findings with purified circulating TF: whereas Tx-irradiated mice had no regression of tumor growth, similar mice treated with TF showed a 85% incidence of regression. However, in contrast with normal animals, regression was only transient and tumors reappeared after the treatment was stopped (15).

Antibody Responses

Most data presented above deal with T-cell–mediated response. One may wonder whether thymic hormones can as well influence antibody responses, either positively (by acting on helper T cells) or negatively (by acting on suppressor T cells). Trainin has shown that thymus-deprived mice treated repeatedly with THF had an improved response towards SRBC as compared with nontreated Tx controls (38). However, the degree of restoration was rather modest, especially when compared with that achieved in cell-mediated responses with the same thymic extract. Experiments with nude mice have been recently reported by two groups (27, 39), suggesting that thymic factors can induce the differentiation of prethymic cells into T helper cells. Ikhehara et al injected a thymosin-like extract into nude mice for more than 60 days, and then challenged the animals with SRBC. A low but significant number of plaque-forming cells were found in the spleen of these mice after stimulation with SRBC (27). Katz & Armerding tested the effect of a thymic preparation in Mishell & Dutton's in vitro system of primary antibody response (39). Spleen cells were primed against SRBC or against a DNP protein conjugate with or without thymosin. The presence of thymosin increased the amount of plaque-forming cells. Recently the action of circulating TF on the effect of suppressor T cells on antipolyvinylpyrrolidone antibody production has been described (40).

Control of Autoimmunity

INTRODUCTION Increasing evidence indicates that a T-cell deficiency could be a major etiological factor in the development of several autoimmune conditions; also, the question of the origin of such T-cell deficiency has been raised. The serum level of the circulating thymus factor (assessed by the rosette assay mentioned above) has been tested in several experimental autoimmune conditions (41, 42).

NZB and B/W mice have a normal T-cell level at birth, but it decreases prematurely between the third and sixth week of life. At two months NZB and B/W mice have no significant thymus factor (TF), whereas TF is still at birth level in control mouse strains and remains at this level until the fourth to the sixth month. The influence of thymic factors on autoreactivity has been demonstrated in two experimental models.

IN VITRO AUTOSENSITIZATION Cohen & Wekerle have shown that normal lymphoid cells cultured for 5 days on syngeneic fibroblast monolayers differentiate into specifically sensitized T cells able to mediate specific cytotoxicity against syngeneic (43) or H-2-compatible target cells (44) and to induce a GVH-like reaction of splenomegaly or of lymph node enlargement after in vivo transfer into a syngeneic host (45).

This experimental system has been used by Trainin's group to investigate the effects of a calf thymus extract on autoreactivity (46). It has been found that addition of such an extract to the culture medium during the sensitization phase inhibited the generation of effector cells as measured by cell-mediated cytotoxicity or by an in vitro GVH-like assay on syngeneic spleen fragments (47). Normal syngeneic serum also blocked the reaction. However, syngeneic serum from newborn Tx donors did not show any blocking activity. It was therefore assumed that the blocking factor present in normal serum was a humoral substance secreted by the thymus and disappearing from the blood stream after Tx.

AUTOLOGOUS ROSETTES Further evidence for the role of the thymus in the control of autoreactivity is derived from the study of autologous rosettes (ARFC) formed between lymphocytes and autologous or syngeneic erythrocytes. Thymectomy in adult mice increases more than 10 times the incidence of ARFC in the spleen (48). A high number of RFC are also found in the spleen of athymic nude mice and aging mice. It is likely that ARFC formation expresses a true recognition event of self-antigenic determinants (J. F. Bach, unpublished results). The high number of rosettes found in the spleen of adult Tx animals is reduced to normal values after a single injection of purified TF, which adds support to the concept of a control exerted by the thymus upon ARFC which are probably immature T cells.

CLINICAL ACTIVITIES

All the above data argue in favor of the indication of thymic factors in the treatment of patients with immune deficiency, SLE, and related diseases. A few prelimary clinical trials, mainly in immunodeficiency syndromes, have been made recently in various centers, using A. L. Goldstein's thymosin (49, 50) and Trainin's THF (51). It is too early to interpret the preliminary clinical results. The most clear-cut effects deal with in vivo correction of low E rosette values, reminiscent of what has been shown in in vitro experiments, especially in immunodeficiency and SLE. It will probably take some time before conclusive results are obtained with randomized trials using standardized preparations with well-defined half-lives (delay preparations will

be necessary as for most peptidic hormones). In addition, it will perhaps be necessary to select patients who do not have too advanced a disease since, for example in the NZB model, old mice appear to be no longer responsive. Potential clinical applications of thymic hormones raise the problem of their relationship to transfer factor. Transfer factor is a dialyzable leukocyte extract which confers specific delayed hypersensitivity to anergic subjects. Little is known in fact of its biochemical nature because of the lack, until recently, of available in vitro assays. The fact that transfer factor is antigen specific suggests that it is different in nature from thymic factors. However, this specificity is not absolute; thus, the possibility that transfer factor preparations contain thymic hormone-like products especially with small molecular weights cannot be ruled out.

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