

CHAPTER I: Introduction - The Role of the Thymic Hormone Thymulin as an Interface Between the Neuroendocrine and the Immune Systems in Mammals

1 The Neuroendocrine System

The earliest allusions to an endocrine system came from Aristotle who had described the effects of castration on the songbird around 2400 years ago in his 'historia animalium'. From then on, scientists continued to contribute to our present understanding of endocrinology: Galen was the first one to describe and name the thyroid gland in dissections (Sarton 1954). The concept of internal or endocrine secretion, in which an organ or tissue discharges its secretion directly into the bloodstream to influence other parts of the body, was first proposed by Théophile de Bordeu (1722-1776) in 1775. A few years later, in 1849, Arnold Adolph Berthold (1803-1861) from Göttingen, Germany, proved that a humoral substance could produce an effect on a site or organ remote from its origin (Sebastian 1999). He transplanted the testis to a rooster which previously had its testis removed and showed normal resumption of male sexual characteristics. Bayliss and Starling participated with a series of experimental studies (Bayliss 1902) and innumerable great physicians such as Addison, Basedow, and Cushing helped to clarify the pathophysiology of endocrine diseases.

Endocrinology is the study of cell-to-cell communication by messenger molecules traversing an extracellular space, allowing the cells to communicate with themselves, nearest neighbors or distant cells via the circulatory system. These types of intercellular communication are designated as autocrine, paracrine and endocrine, respectively. Messenger molecules were first referred to as hormones, derived from the Greek word ὁρμάω [*hormaô*] (= set in motion, urge on (Liddell 1996)) by Professor Starling in 1905. The corresponding 'receptor'-concept was advanced primarily by the example of the syndrome of pseudohypoparathyroidism, a receptor-mediated resistance to the effects of parathormone described by Fuller Albright et al. in 1942 (Albright F 1942), leading nowadays to modern areas of investigation in endocrinology, like cell biology of hormone action, receptor structure and function, signal transduction, gene regulation, peptide processing and mechanisms of hormone secretion.

In regard to evolution, rapid development of endocrine systems probably occurred as early as the transition from unicellular to multi-cellular organisms took place, the growing size of those

organisms quickly prohibiting direct communication between all constituent cells. Because of this early occurrence in the process of evolution, endocrine systems play fundamental roles in many of the most basic biologic activities of complex organisms: food seeking and satiety, metabolism and caloric economy, growth and differentiation, reproduction, homeostasis, response to environmental change such as arousal, defense, flight, and so on.

The hypothalamus is the part of the brain where the activity of the autonomic nervous system and endocrine glands is integrated with input from higher centers that give rise to emotions and behavior. The hypothalamus thus ensures appropriate response of the organism to deviations from different internal set points. It can be seen as the interface between internal and external environment. On the 'body-side' of this mind-body interface, the pituitary gland acts as the partner of the hypothalamus. Once considered the 'master gland' in the regulation of neuroendocrine systems, the pituitary is now consensually and accurately seen as a 'middle manager' responding to input from both the brain (via the hypothalamus) and the body (via the various peripheral endocrine glands). The pituitary gland lies in the sella turcica at the base of the brain, and is connected to the hypothalamus through the hypophyseal stalk. The anterior pituitary comprises at least five different types of secretory cells, each of which produces and contains a particular hormone whose release is directed by one or more hypothalamic peptides called releasing (or release-inhibiting) factors or hormones. After release, the pituitary hormone, in turn, stimulates a particular target organ whose products provide feedback inhibition for the pituitary hormone and/or hypothalamic peptide. This cascade of interacting products reaching from various regions of the central nervous system down to peripheral target tissues is called the *Neuroendocrine Axis*.

2 The Immune System

2.1 The Innate and Acquired Immunity

The vertebrate immune response is a highly specific defense system, which distinguishes self from non-self and thereby protects the organism from external aggressive factors such as infectious agents (bacteria, viruses, fungi and parasites) and toxins. The immune system consists of two main compartments, the *humoral immunity* that leads to the synthesis of circulating antibody molecules by B-cells, and the *cellular immunity* that culminates in activated T-cells. This differentiation has first been clearly demonstrated by Cooper et al. in 1965 (Cooper, Peterson et al. 1965).

Both are part of the so called *adaptive or acquired immunity*, a highly effective complex network between host and pathogen, involving cell-cell contacts as well as factors such as cytokines, that lead to a highly specific immune response to particular pathogens. This kind of immunity is achieved through exposure and develops out of either recovery from disease or medical intervention, a phenomenon commonly known as immunological memory. By contrast, the *innate immunity* could be considered as the first line of defense against infection. It is roughly divided into external and internal defense, the first one consisting of natural mechanical barriers to infection such as skin, mucous membranes, cilia, mucous, cough reflex and acid lining which all prevent the penetration of pathogens into tissues. The internal innate immunity offers many forms of protection after pathogens enter the body. It consists of phagocytic cells, such as the monocytes, macrophages and polymorphonuclear neutrophils, all using primitive non-specific recognition systems which allow them to bind to a variety of microbial products, to internalize and to digest them.

2.2 The Humoral Immunity

Humoral immunity is provided by the plasma cell also called AFC (antibody forming cell) as effector cell of the B-cell lineage. B-cells carry membrane-bound immunoglobulins (Igs) on their surface, the B-cell antigen receptors that have the same binding specificity as the antibody finally produced by the mature AFC. After contact with an appropriate antigen, they produce and secrete large amounts of circulating antibodies/immunoglobulins that recognize and neutralize free antigen in serum and tissue fluids. These immunocomplexes activate the complement system and facilitate phagocytosis by opsonization.

There are five major antibody-classes (isotypes): IgA, IgD, IgE, IgG and IgM. They are all glycoproteins, but each class has its own physicochemical properties. Their structure is different, IgG typically occurring as a monomer (see figure 1), IgA occurring as a monomer (extravascular sites) and a dimer (secretions across epithelia) and IgM as a pentamer. This results in a distinct molecular weight, intravascular distribution and biological activity. Some of them are further divided into different subgroups, such as IgG₁, IgG₂, IgG₃, IgG₄ as well as IgA₁ and IgA₂. The IgG is the main antibody fraction in vertebrate serum, accounting for 70-75% of the total immunoglobulin pool. Furthermore, it is the most stable, persisting for over 15 days in serum (Woolley and Landon 1995) and the only one that is able to cross the placental barrier (all subgroups of IgG, especially IgG₁ and IgG₃), thus conferring a high degree of passive immunity to the

newborn. Each terminally differentiated plasma cell is derived from one specific B cell and produces antibodies of just one of those classes or subclasses.

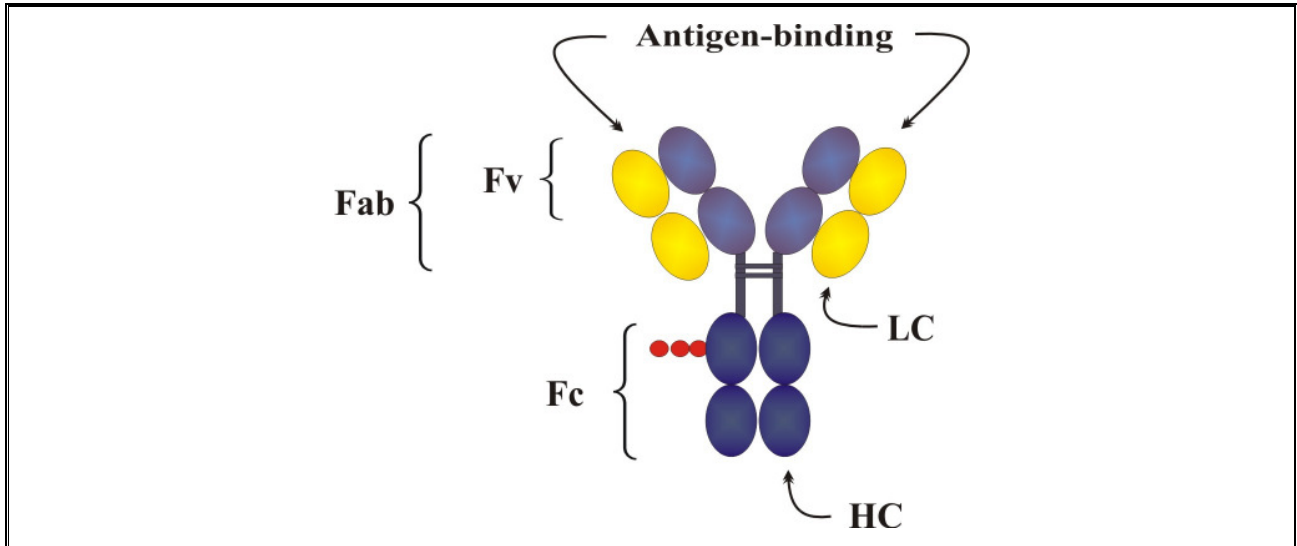


Figure 1: The Molecular Structure of Immunoglobulins. - The figure shows a single monomer immunoglobulin (Ig) molecule. All immunoglobulins are bifunctional molecules: each Ig-monomer functionally consists of two antigen-binding fragments (Fabs), which are linked via a flexible region (the hinge) to a constant (Fc) region. While the Fab-region is in charge of binding to the antigen, the Fc-region mediates effector functions such as binding of the Ig to host tissue or cells of the immune system, to phagocytic cells and to the first component (C1q) of the classical complement system. The Fc-region is glycosylated, to a very variable degree, depending on the antibody-subclass. Chemically, they are composed of two identical light chains (LC, yellow) and two identical heavy chains (HC, blue). The heavy chains are covalently linked together in the hinge region and the light chains are covalently linked to one heavy chain each. The variable domains of both the heavy and light chains constitute the antigen-binding portion of the molecule, termed Fv. Compare figure 3 p. 21; (Figure modified from Brekke and Sandlie 2003; Hadge, Fiebig et al. 1980b; Kendall 1981)

2.3 The Cellular Immunity

All cells of the immune system arise from the same pluripotent stem cells through two main lines of differentiation: the lymphoid lineage produces lymphocytes, and the myeloid lineage produces phagocytes and other cells.

The major lymphoid organs and tissues are divided into primary (central) and secondary (peripheral) lymphoid organs. Thymus and bone marrow are the mammals' primary lymphoid organs where the lymphoid cells acquire their repertoire of specific antigen receptors that enable them to meet the innumerable antigenic challenges throughout life. The thymus is the site where T-lymphocytes differentiate from lymphoid stem cells and proliferate and mature into functional cells, whereas the B-lymphocytes develop and mature in the bone marrow. Briefly, lymphocytes develop

in the primary organs, but migrate to and function in secondary organs and tissues such as the spleen, lymph nodes and mucosa-associated lymphoid tissue (MALT).

Unlike humoral immunity which is mediated by B-cells, cellular immunity is ensured by T-cells. These T-cells carry various molecules on their surface: the antigen is recognized by the T-cell receptor (TCR) consisting of two membrane-bound and covalently linked polypeptide chains α and β . In the peripheral blood some T-cells with slightly different chains (γ and δ) were detected, but the function of these cells is not yet fully understood. The T-cell activation is supported by CD3, a molecule closely associated with the TCR. Other coreceptors are CD4 and CD8. The two main effector cells are CD4⁺ T-helper cells and CD8⁺ T-cytotoxic cells. CD4⁺ T-cells differentiate upon activation into either T_H1- or T_H2-cells which differ in the cytokines they produce and therefore in their function. This differentiation is itself mediated by cytokines (IFN- γ , IL-12, IL-4) produced in response to pathogens by cells of the innate immune system. With selective production of T_H1-cells a cell-mediated immunity with macrophage-activation and the production of opsonizing IgG-Abs (IgG₁ and IgG₃ in human) are favored, with T_H2-cell production humoral immunity is supported by B-cell activation and IgM, IgA and IgE production (see figure 3). All T-cell effector functions involve the interaction of an effector T-cell with a target cell displaying a specific antigen. In contrast to humoral immunity, the cellular immunity cannot be executed on free antigens. CD4⁺ T-helper cells (T_H) interact with specialized glycoproteins (major histocompatibility complex (MHC)-II) on the surface of antigen-presenting cells (APC). They recognize exogeneous antigen fragments that have previously been internalized and processed, and then exposed together with MHC-II on the surface of APC. The CD8⁺ cytotoxic T-cells (T_C) interact with other glycoproteins (MHC-I), present on all nucleated cells, and kill the target cell by cytolysis in case it displays non-self endogen peptide fragments (i.e. following viral infection).

2.4 The Physiologic Immune Response

2.4.1 Protection Against Infection

As pathogens enter the body, its immune system must select appropriate effector mechanisms to combat the infection. Depending on the 'enemy' (bacteria, viruses, fungi and parasites but also tumors and transplanted organs), very different and perfectly adapted defense mechanisms are activated. T-helper cells play an important role in this selection process (see figure 2). The three most easily recognizable patterns are:

2.4.1.1 Antibody Production by B-Cells Directed by T_H2-Cells

The B-cell antigen receptor has the structure of membrane-bound monomer IgM and IgD in some cases. After antigen-binding, this single B-cell is stimulated and proliferates. This phenomenon is called *clonal expansion*. B-cell-stimulation is supported in most cases by interaction with T-helper-cells. Both can recognize the same antigen, but they detect different parts of it: whereas B-cells recognize native antigen, T_H-cells respond to processed antigen bound to MHC on APC such as dendritic cells or B-cells. B-cells first provide T_H-cell activation and then get stimulated themselves by those T_H-cells by mediation of cytokines such as IL-2, IL-4, IL-6, TNF α and IFN γ . There is a small number of antigens capable of activating B-cells without the help of T-cells. They are called *T-independent antigens*, most commonly large polymeric molecules with repeating antigenic determinants (e.g. bacterial proteins such as flagellin or endotoxin).

After differentiation, the resulting cells become memory cells and plasma cells, the latter being the immunoglobulin-secreting effector cells of the B-cell lineage. The secretion of pentamer IgM occurs as a *primary response*. Only after a second encounter with the same antigen the class switches to mainly IgG and an increased antibody affinity occurs. This *class switch* is mediated by IL-4 in memory cells, and is only observed with T-dependent antigens.

2.4.1.2 Cytotoxicity Mediated by CD8⁺-T-Cells

T-cells undergo proliferation (*clonal expansion*) and differentiation into memory and specific effector cells as well. CD8⁺-cells remain functionally inactive when they leave the thymus. Prior to full activation they require two signals: first, the contact with their specific antigen via TCR-MHC-I-interaction, together with costimulatory B7-CD28-interaction, and second, specific cytokines (IL-2 and IFN- γ) produced by T-helper cells (paracrine *T-cell-T-cell-cooperation*) and the CD8⁺ T-cell itself (autocrine stimulation), both leading to proliferation and differentiation. Later, the activated T-cell does not need any costimulatory signal to recognize pathogens and to perform its function: after binding to the target cell, the T_C-cell has a variety of mechanisms available to destroy the identified cell. These include cell-cell signaling via surface molecules that lead to receptor-mediated killing through Fas or TNF as well as indirect signaling via cytokines or granule exocytosis discharging perforins and granzymes into the cleft between T_C-cell and target cell.

2.4.1.3 Macrophage Activation Directed by T_H1-Cells

Macrophages are involved in all stages of the immune response. They are part of the innate immunity, combating infection before T- and B-cell-enhanced immunity can act, and they are important helpers in the acquired immunity, providing antigen presentation, lymphocyte activation and effector functions with anti-tumoricidal and anti-microbial activity.

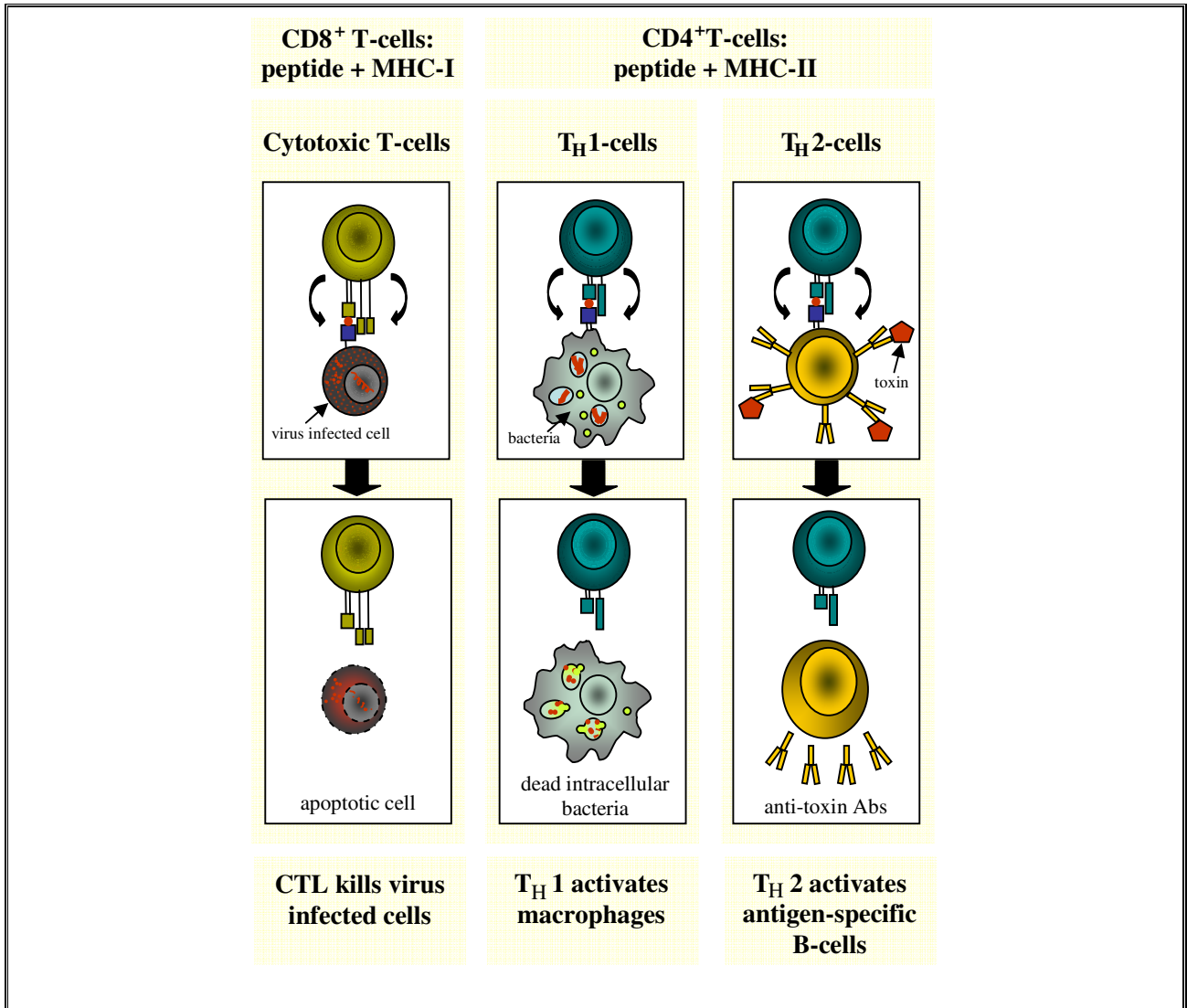


Figure 2: Three classes of effector T-cells are specialized to deal with three classes of pathogen. – CD8⁺ cytotoxic cells kill target cells that display non-self peptide fragments (of cytosolic pathogens such as viruses, or of tumor cells or transplanted allogeneic tissues) bound to MHC-I at their surface. CD4⁺ T-cells are divided into T_H1- and T_H2-cells, both recognizing peptide fragments bound to surface-MHC-II after processing within intracellular vesicles (modified from Janeway 2005).

2.4.2 Mechanisms of Transplant Rejection

In clinical practice, especially allogeneic tissue transplantation is becoming more and more important, strong genetic disparity between donor and recipient being responsible for transplant rejection. Transplantation immunobiology encompasses virtually all aspects of immune function: Transplantation can stimulate all of the various active mechanisms of humoral and cellular immunity, both specific and non-specific. This stimulation has to be seen as a physiologic reaction of the host's T-cells against a non-physiologic introduction of foreign peptide antigens associated with the foreign MHC molecules on the grafted cells, or even associated with the host's APCs that present antigens that had shed from the transplant. These APCs activate T_H-cells that produce a mixture of different lymphokines stimulating cell-mediated and antibody-mediated immune pathways as well as non-specific inflammatory reactions (see figure 2).

T-cells are pivotal in transplant rejection, and much of our knowledge of T-cell physiology and function, of self tolerance and autoimmunity, and of the role of the thymus in T-cell education, is derived from studies of transplantation. From a historical perspective it should be mentioned that it was the study of mouse skin-graft rejection that led to the discovery of the MHC-molecules (Snell 1948).

The *host versus graft reaction* can only be avoided by careful typing (autografting, isografting) and the use of immunosuppressive drugs. Another complication often resulting in the death of the recipient is the *graft versus host reaction* where immunocompetent cells contained in the grafted tissue attack the host-tissue. Removal of mature T-cells from the graft can decrease this risk.

2.5 Disorders of the Immune System

The more complex a system and its control mechanisms become, the higher the risk of eventual breakdowns. The immune system can malfunction in two main modes, namely by overreacting or underreacting; inappropriate activation of an effector function can either lead to failure to combat an enemy or to chronic immunopathology, i.e. chronic infection, hypersensitivity-reactions or autoimmunity.

2.5.1 Immunodeficiency

Immunodeficiency disease results from the decrease, absence or failure of normal function of one or more elements of the immune system. All elements of the immune system can be affected.

Examples are non-specific immunodeficiency diseases involving abnormalities of components of the innate immune system such as complement or phagocytes, and specific immunodeficiency diseases involving dysfunction of B- or T-cells. In the main, *primary immunodeficiency* diseases are determined genetically (IgA deficiency, CVID, SCID, DiGeorge anomaly), whereas *secondary immunodeficiency* diseases are acquired during life (caused by drugs or HIV). Immunodeficiency diseases cause increased susceptibility to infection in patients. Broadly speaking, patients with defects in immunoglobulins, complement proteins or phagocytes are especially threatened by pyogenic infections with encapsulated bacteria such as *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Staphylococcus aureus*. On the other hand, defective cell-mediated immunity leads to overwhelming, even lethal, infections with opportunistic microorganisms such as yeast and common viruses such as chickenpox.

2.5.2 Hypersensitivity and Autoimmune Phenomena

When an adaptive immune response occurs in an exaggerated form, the term hypersensitivity is applied. Gell and Coombs (Gell and Coombs 1963) described four types of hypersensitivity reaction, the first three being antibody-mediated, and the fourth type mediated mainly by T-cells and macrophages. The Type I is known as immediate hypersensitivity and is characterized by an allergic reaction. The term *allergy*, meaning ‘changed reactivity’ of the host when meeting an agent, was initially applied by Clemens von Pirquet (1874-1929) in 1906 (von Pirquet 1906).

When errors occur in the negative selection process when immune-cells mature and learn to recognize the difference between self and non-self, then autoantibodies and autoreactive T-cells can persist and cause autoimmune diseases. One of the earliest examples in which disease was associated with the production of autoantibodies was Hashimoto’s thyroiditis. Nowadays, there are an increasing number of diseases found to be of autoimmune nature. Organ-specific autoimmune diseases like Hashimoto’s thyroiditis, Addison’s disease and insulin-dependent diabetes mellitus produce symptoms in particular organs. Non-organ-specific autoimmune diseases, which include rheumatological disorders, systemic lupus erythematosus (SLE) and dermatomyositis, characteristically involve skin, kidneys, joints and muscles, and their antibodies are directed against widespread antigens.

2.6 The Avian Immune System

All species in the animal kingdom possess an immune system to protect them from foreign substances. There are not many substantial differences between species, however since birds and mammals developed two distinct evolutionary lines more than 300 million years ago, some slight variations can only be expected to be found.

Analogously to mammals, the thymus of the chicken functions as the maturation center where stem cells differentiate into T-lymphocytes. Their bone marrow is only the source of the T- and B-cell lineage stem cells. B-cell maturation takes place in the bursa of Fabricius, an organ situated at the dorsal side of the cloaca, and their proliferation and memory B-cell storage takes place in the chicken's spleen. It is of historical interest that it was in chickens that Cooper et al. demonstrated in 1965 the existence of those two immunological compartments: one thymus-derived, the second bursa-derived (Cooper, Peterson et al. 1965).

The highly diverse antibody repertoire of around 10^8 different immunoglobulins (Janeway 2005) is achieved by a different strategy in chickens. Whereas in mammals recombination of V-J-genes and somatic mutations after antigen stimulation are responsible for this diversification, in chickens mainly somatic hyperconversion leads to the necessary antibody-pool (Reynaud, Dahan et al. 1989).

Interestingly, other classes of immunoglobulin can be found in chickens (see figure 3). Apart from serum-IgA and -IgM that are nearly similar in mammals and chickens, the major low molecular weight Ig class present in avian serum and in the egg yolk is the so called IgY (Warr, Magor et al. 1995). This immunoglobulin is unique to birds and the only one that is found in substantial amounts in the egg yolk, hence its name. The function of this immunoglobulin class is comparable to that of mammalian IgG. On the other hand, the structure of the IgY was identified as comparable to IgE or IgM-monomers (Hadge 1985). Warr et al. postulated the avian antibody IgY to be a phylogenetic progenitor of mammalian IgE, IgA and IgG (Warr, Magor et al. 1995).

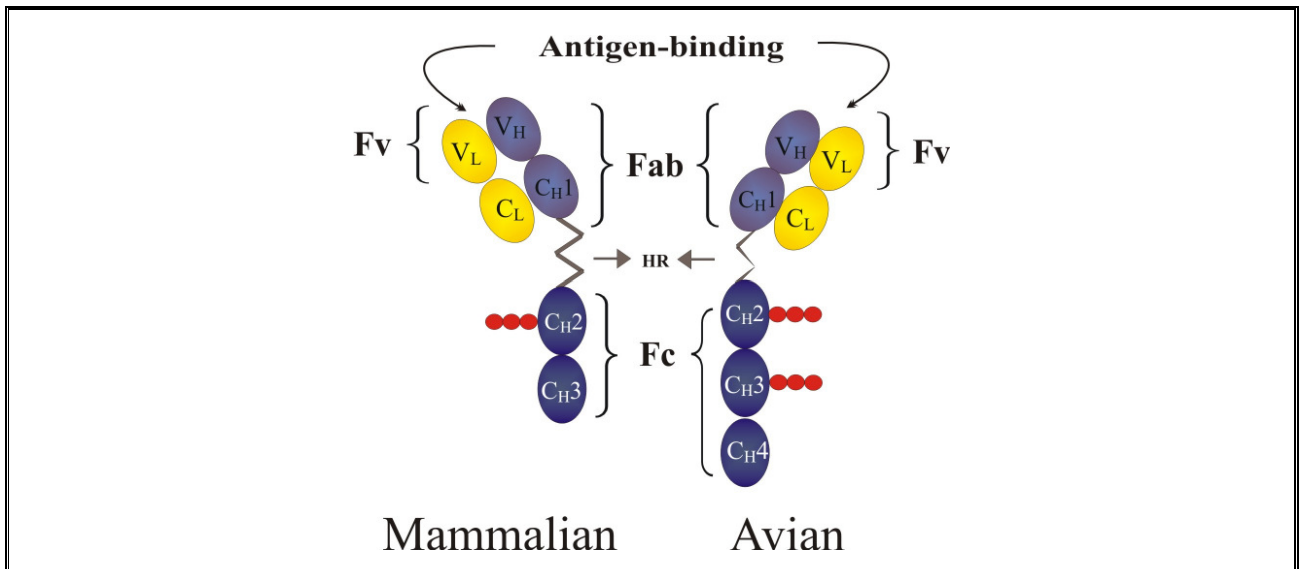


Figure 3: Structural Features of Avian IgY in Contrast to Mammalian IgG. – All antibody-monomers are composed of two identical light chains (LC, *yellow*) being common to all classes of antibodies (LC- κ/λ) and two identical heavy chains (HC, *blue*) being structurally different for each class or subclass (HC- α , HC- δ , HC- ϵ , HC- γ , HC- μ for mammalian Ig-isotypes and HC- ν for IgY). Every light chain contains one constant domain (C_L) and one variable domain (V_L), the latter being called so due to its high degree of sequence variability. Every heavy chain contains one variable domain (V_H) as well as three constant domains C_{H1}, C_{H2} and C_{H3} in the case of IgA, IgD and IgG, and four (with an additional C_{H4}) in the case of avian IgY (but also IgM, IgE). The hinge region (HR) is situated between the C_{H1}- and C_{H2}-domain. It gives considerable flexibility to the two Fab fragments. Sequence comparisons between IgG and IgY have shown that the C_{H2}- and C_{H3}-domains of IgG are closely related to the C_{H3}- and C_{H4}-domains, respectively, of IgY, and that the additional C_{H2}-domain of IgY was integrated into the hinge-region in IgG. (Figure modified according to Schade, Henklein et al. 1996; Shimizu, Nagashima et al. 1992; Warr, Magor et al. 1995)

The IgY transfer from serum to egg yolk is exclusively receptor-mediated and an intact Fc-part and hinge region were both shown to be essential for this transfer (Mohammed, Morrison et al. 1998; Morrison, Mohammed et al. 2002). The Ab's concentration in the yolk is directly proportional to the Ab's concentration in the serum, but the amount of transferred IgY seems to be independent of the egg size (Bollen and Hau 1997; Morrison, Mohammed et al. 2002). Woolley and Landon detected 1.23 times more Ab in the yolk than in the serum (Woolley and Landon 1995), but here contradictory information is given by different authors. Many reported no differences between them. However, significant variations among individuals, genetic lines or breeds were consensually found, as well as a septadian biorhythm (Schade 1991). This Ab-transfer to the yolk ensures a certain passive immunity to the newborn, in analogy to the mammalian transplacental IgG₁/IgG₃-transfer (which is also receptor-mediated: FcRn binds to the Fc portion of IgG).

3 The Thymus Gland

3.1 The Thymus – Morphological and Physiological Aspects

In the second century, Galen of Pergamum gave the name ‘thymus’ to this pinkish-gray organ in the chest because, it is said, it reminded him of a bunch of thyme (Diamond 1979; Nishino, Ashiku et al. 2006).

The thymus is derived from the endoderm of the ventral part of the third pharyngeal pouch on each side. First, two elongated diverticula are formed on each side, soon becoming solid cellular masses and growing caudally until they meet ventral to the aortic pouch, being united by connective tissue only. Vascularized mesenchyme that includes the lymphoid stem cells invades the cellular mass of the endodermal thymus during the third gestational month approximately. Immediately after birth the thymus is largest relative to body weight. During the first 2 years of life its absolute weight increases, remaining from then on fairly constant at about 20g until the 6th decade when reduction occurs. In spite of this fairly constant weight, increasing infiltration by adipose tissue can be noted from birth on.

Even though this fatty infiltration, leading to the so called physiologic involution of the thymus, is usually completed by the fourth decade, the capacity of thymic hormone-secreting cells, thymocyte production and differentiation persist at a basic level throughout life, so that T-cells from this source continue, at a very low grade, to populate the peripheral lymphoid tissues, blood and

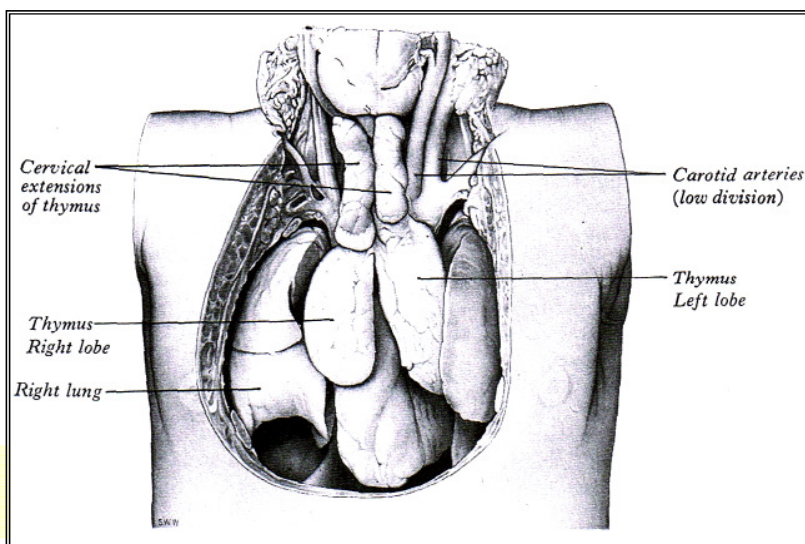


Figure 4: Dissection to display the neonatal thymus in human. -

(Bannister 1995)

lymph (Steinmann and Muller-Hermelink 1984).

The thymus is situated in the superior and anterior inferior mediastinum, reaching down to the 4th costal cartilages and sometimes even up to the inferior poles of the thyroid gland. It is an encapsulated organ that consists of two lobes lying close together and being joined by

connective tissue. Each of the two lobes is partially divided by the ingrowth of shallow septa so that superficially it appears lobulated. Each thymic lobule consists of cortical and medullary areas. The cortex is further divided into the subcapsular region, outer and deep cortex. It is densely packed with cells, mainly thymocytes. The medulla that is separated from the cortex by the so called cortico-medullary junction is composed of connective tissue with only few lymphoid cells. Here, the typical Hassall's corpuscles are found as well as APC. Both, cortex and medulla are permeated by a loose network of interconnected thymic epithelial cells (TEC) (see figure 5).

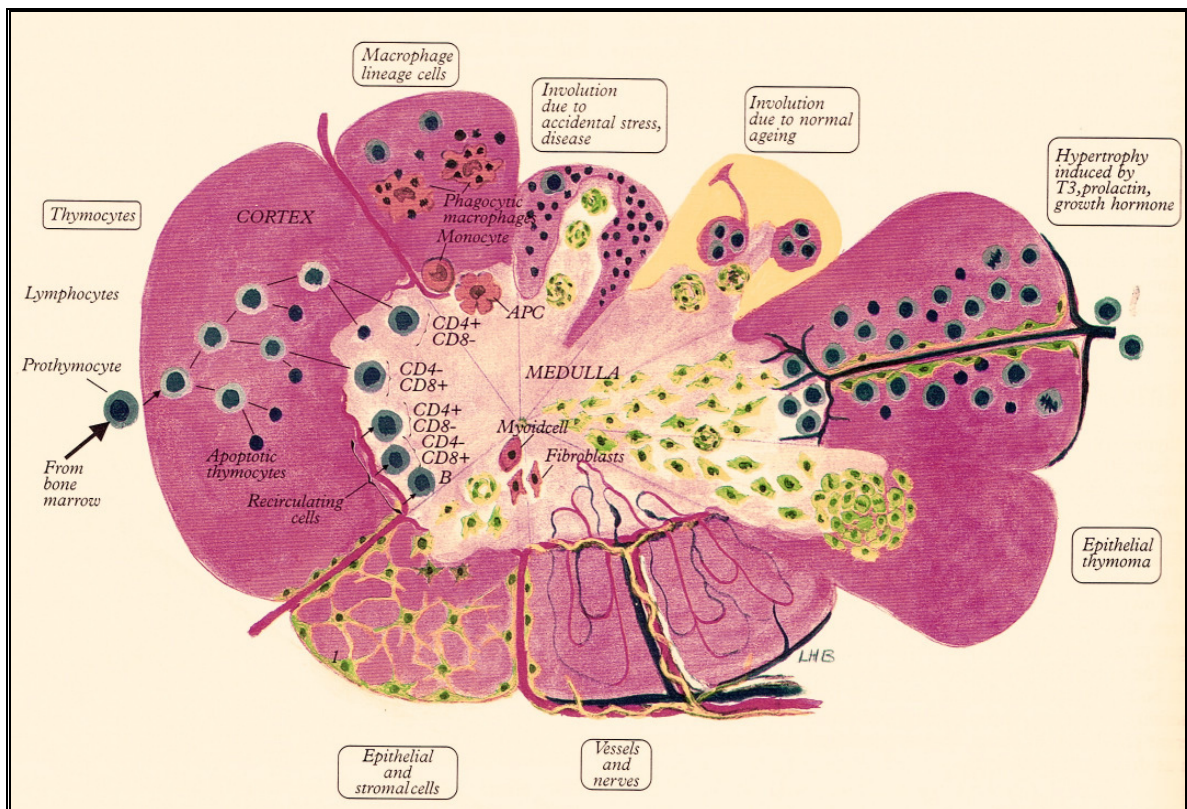


Figure 5: Thymus in Health and Disease - Scheme illustrating the microscopic organization (cortex/medulla and their cells, vessels and nerves) of the thymus at various stages of life and under different conditions. Physiologic and pathologic involution are indicated as well as hypertrophy induced by hormones and thymoma (Bannister 1995).

3.2 The Thymus as an Immune Organ

The thymus provides a unique microenvironment in which the T-cell precursors undergo development, differentiation and clonal expansion. During this process, the exquisite specificity of T-cell responses is acquired, as is their immune tolerance to the body's own components: the stem cells arriving from the bone marrow first colonize the subcapsular region where they proliferate, actively generating the thymocyte population. Maturation of those thymocytes takes place while

journeying inwards via the outer and deep cortex, through the cortico-medullary junction towards the medulla, presenting a constantly changing set of differentiation markers of functional significance on their surface. This is when they progressively generate their T-cell receptor diversity by somatic recombination, and where positive and negative selection take place. Positive selection, also called *thymic education*, ensures that only those TCRs with a moderate affinity for self MHC are allowed to develop further. Negative selection, also known as *central tolerance*, ensures that only those thymocytes that fail to recognize self antigen are allowed to proceed in their development, the rest undergoing apoptosis (*clonal deletion*).

Those key events in intrathymic T-cell differentiation are driven by the influence of the thymic microenvironment, a three-dimensional network comprising TEC, macrophages and dendritic cells as well as extracellular matrix (ECM)-elements (Boyd, Tucek et al. 1993). Intimate interactions between the TECs and thymocytes could be demonstrated to be of special importance: TECs secrete a variety of polypeptides, including thymic hormones and cytokines, directly affecting thymocytes by binding to specific receptors. It is important to mention that receptors for thymulin were only found on two leukemia cell lines, but not on normal thymocytes (Gastinel, Pleau et al. 1982; Pleau, Fuentes et al. 1980). Furthermore, there are many types of cell-cell contacts also playing an important role, such as between classical adhesion molecules, between ECM ligands and their respective receptors, or the interaction of MHC-I or MHC-II molecules with T-cell receptors in the context of CD8 or CD4, respectively. Whereas early stages of maturation depend mainly on cell-cell contacts, later stages are under thymic hormone control. It was concluded that an important intrinsic biological circuitry exists, based on the fact that those TEC-thymocyte interactions were found to be bi-directional (Ritter and Boyd 1993).

3.3 The Endocrine Thymus

The Thymus has long been recognized as an endocrine organ. Albrecht von Haller (1708-1777) described it as “gland without duct, pouring special substances into the blood” (Sebastian 1999). Many soluble factors were found to be present in the thymic microenvironment, next to more or less thymus-specific hormones; typical neuroendocrine hormones and cytokines have been detected.

The thymic epithelial cells are the site of production of the classical thymic hormones thymulin, thymosins (isolated from thymosin fraction 5, especially α_1 and β_4), thymopoietin and thymic humoral factor (all of them have been isolated and sequenced, none of them showing

structural homology). Generally speaking, the thymic hormones are all modulators of T-cell differentiation acting at different stages: they showed immune-stimulating effects in a variety of clinical trials (Bach 1983; Hadden 1993), they are able to induce terminal deoxynucleotidyl transferase (TdT) activity in immature thymocytes, they stimulate the expression of membrane markers such as Thy-1, and they modulate T-helper and T-cytotoxic functions.

Thymulin-production was shown early on to be under a negative feedback autoregulation (Cohen, Berrih et al. 1986; Savino, Dardenne et al. 1983b) as is the case for other hormones produced by endocrine glands. Additionally, Savino et al. demonstrated in 1983 that thymulin-containing cells (TECs) would increase in number and content of the hormone after long-term-elimination of circulating thymulin with anti-thymulin monoclonal antibodies (Savino, Dardenne et al. 1983a).

Intriguingly, a number of hormones primarily produced by different endocrine glands as well as their receptors are produced intrathymically, albeit in trace amounts, thus giving rise to a complete intrathymic biological circuitry with autocrine and paracrine pathways: adenohipophyseal hormones (GH, ACTH, FSH/LH, TSH) and neurohipophyseal hormones such as oxytocin (OT) and arginine vasopressin (AVP) were found to be produced by TEC, and some of them (GH, LH) as well as PRL and LHRH were even found to be produced by thymocytes, at least in trace amounts. On both cell types the corresponding receptors have been demonstrated for most of those peptide hormones (Blalock 1992). Interestingly, a certain cell-type-specificity with respect to the intrathymic site of production of the different hormones could be demonstrated: OT and AVP seem to be exclusively produced by TEC, whereas PRL expression is apparently restricted to thymic lymphocytes.

3.4 The Thymus-Neuroendocrine Network

3.4.1 The Physiological Interplay Between the Immune and Neuroendocrine Systems

In human physiology three main integrative systems can be identified: the nervous, the endocrine and the immune system. Each of them was long thought to possess its own specific messengers and corresponding receptors: neurotransmitters, peptide hormones and cytokines. In the early nineties overwhelming evidence emerged supporting the idea that those ligands and receptors were shared by the three systems and that they were used as a common chemical language for communication within and between the immune and neuroendocrine systems. In complex

multicellular organisms like mammals, communication among distant cells and organs provided distinct survival advantages, so that evolutionary pressure across the last billion years layered complexity upon complexity in those systems. Primitive systems were gradually modified in favor of more adequate ones, new receptor molecules evolved so that old hormones took on new functions, or new ligands were devised for old receptors. Interestingly, somehow surprising similarities had earlier been noted between those two systems, for example in terms of total number of cells or their memory function (Blalock 1984; Jerne 1985). From then on it was concluded that the immune and neuroendocrine systems exert profound and biologically relevant effects on one another in vivo, and that such crosstalk was undoubtedly important for homeostasis.

The molecular basis for the immune-neuroendocrine crosstalk in terms of ‘common language’ between the immunological and neuroendocrine circuits has been proposed by several authors in the second half of the last century (Besedovsky and Sorkin 1977; Blalock 1994a; Blalock 1994b; Comsa, Leonhardt et al. 1982; Deschaux and Rouabhia 1987; Fabris 1983; Fabris, Pierpaoli et al. 1971). *Neuroendocrino-immunology (NEI)* was born as a fast-growing field of research. Its ideas were supported by findings in different pathologies in which both systems showed imbalances and in which the same or similar treatment was successful. Clinical and Laboratory parameters were shown to be paralleled in patients with systemic lupus erythematosus (SLE) and others with hyperprolactinemia, and amelioration of biological parameters as well as longevity could be demonstrated treating both patients with bromocriptine, an inhibitor of PRL secretion (McMurray, Keisler et al. 1991). Folch et al. (1991) and many others proved in the 90s that the immune system was easily altered by different stressors affecting the central nervous system (Folch, Ojeda et al. 1991).

The relevance of the thymus in this network first became evident observing congenitally athymic subjects or models of neonatal thymectomy, and its immune and neuroendocrine consequences (Goya, Console et al. 2001; Goya, Sosa et al. 1995; Michael, Taguchi et al. 1980).

3.4.2 Neuroendocrine Control of Thymus Physiology – A Pleiotropic Action upon Thymic Epithelial Cells (TEC), Thymocytes and Thymic Stromal Cells

Many adenopituitary hormones exert a stimulatory effect on thymulin serum levels. Thus, thyroid hormones upregulate thymulin secretion by TEC, as do growth hormone and prolactin (Dardenne, Savino et al. 1989; Timsit, Savino et al. 1992; Villa-Verde, de Mello-Coelho et al. 1993). Mocchegiani et al. hypothesized that thymulin secretion is dependent on *de novo* synthesis

since the observed T_3 -effect was prevented by *in vitro* treatment with cycloheximide (Mocchegiani, Amadio et al. 1990). Sometimes, the stimulating effect is mediated by other messengers, such as IGF-I in the case of GH where anti-IGF-I and anti-IGF-I-receptor antibodies were able to block those modulatory effects of GH (Timsit, Savino et al. 1992). Pituitary hormones appear to act directly upon TECs since their effect can be observed in cultures of pure TEC lines. Furthermore, specific hormone receptors have been found on TEC for all of the discussed hormones (Ban, Gagnerault et al. 1991; Dardenne, Kelly et al. 1991). In order to underline those experimental data, patients with hyperthyroidism or adenopituitary hyperactivity states such as prolactinoma or acromegaly were studied: increased serum levels of T_3 , PRL or GH were found to be paralleled by increased serum thymulin levels (Timsit, Safieh et al. 1990; Travaglini, Mocchegiani et al. 1990). Conversely, cases of hypothyroidism and GH-deficiency were accompanied by low thymulin levels (Mocchegiani and Fabris 1990; Mocchegiani, Santarelli et al. 1994; Timsit, Savino et al. 1992). The effects of adrenal and gonadal steroids on thymulin secretion appear to be rather more complex. Addition of glucocorticoid hormones, estradiol, progesterone or testosterone enhanced thymulin release into the TEC-culture supernatants (Savino, Bartoccioni et al. 1988). Following adrenalectomy or gonadectomy in male and female mice, a transient fall of thymulin serum levels was observed in both sexes, accompanied by an increased intrathymic thymulin-content and the appearance of a thymus-dependent thymulin inhibitor (Dardenne, Savino et al. 1986). Folch et al. showed evidence in 1986 for a hypothalamic dependency of serum FTS concentrations. Hypothalamic extracts from young mice injected into old mice were able to induce the reappearance of detectable thymulin levels (Folch, Eller et al. 1986). Other thymic hormones were partially demonstrated to be influenced by similar mechanisms and in similar ways, but extensive data are lacking.

In addition to this influence upon thymic endocrine function, other aspects of TEC physiology can be modulated by the same hormones, thus characterizing their pleiotropic action: several experiments have shown that cytoskeletal protein-, ECM (extracellular matrix) ligand- and receptor-expression are enhanced by direct effects of the pituitary gland upon TEC, and that TEC growth is therefore positively affected (Dardenne, Savino et al. 1989; Kendall, Loxley et al. 1994; Savino, Ban et al. 1990). Among the most striking *in-vivo* findings the thymic atrophy following hypophysectomy experiments has to be mentioned (Gala 1991).

Regarding thyroid hormones, not only the adhesion of thymic lymphocytes to cultured TECs could be enhanced by T_3 , but also their spontaneous release by cultured thymic nurse cells (TNCs), a lymphoepithelial complex located in the thymic cortex (Villa-Verde, de Mello-Coelho et al. 1993). Some hormones appeared to be able to stimulate thymocyte proliferation, T_3 by upregulating cellular Ca^{2+} concentration in thymic lymphocytes (Segal, Hardiman et al. 1989) and PRL by enhancing IL-2 production and IL-2-receptor expression (Mukherjee, Mastro et al. 1990). Specific receptors for nearly all pituitary hormones were found on thymocytes, including for neurohypophyseal hormones OT and AVP. Last but not least, steroid and peptide hormones influence the secretion of various other cytokines from thymic stromal cells, as shown by *in vitro* experiments (Tseng, Kessler et al. 1997).

3.4.3 Thymic Humoral Factors Acting on the Neuroendocrine System

Even though a number of thymic hormones seem to interact with the neuroendocrine system, an emerging core of information is identifying the thymic hormone thymulin as a key link between the thymus and the anterior pituitary gland. The first evidence for strong thymic influence on the neuroendocrine system was given by experiments with neonatal thymectomy: developmental atrophy of female sexual organs as well as a decrease in the number of secretory granules in acidophilic cells of the adenopituitary were detected (Besedovsky and Sorkin 1974; Ruitenbergh and Berkvens 1977). Similar findings were made in athymic nude mice, exhibiting significantly low levels of various pituitary hormones, including PRL, GH, LH and FSH (Daneva, Spinedi et al. 1995; Goya, Console et al. 2001). Thus, the thymus seems to affect the whole neuroendocrine axis, including the hypothalamus, pituitary and peripheral endocrine glands. Goya et al. showed in 1994 that thymulin possesses an *in vitro* stimulatory effect on perfused rat pituitaries, enhancing the release of GH, PRL and, to a lesser extent, TSH. Zaidi et al. demonstrated in 1988 a similar effect on LH-release (Goya, Sosa et al. 1994; Zaidi, Kendall et al. 1988). A somewhat contradictory finding was made by Hadley et al. (1997) who found ACTH- and LH-levels increased, but PRL-release inhibited after thymulin-application (Hadley, Rantle et al. 1997). The *in vivo* injection of thymulin in mice had led to enhanced circulating progesterone levels, but the authors suggested that this effect occurred via the action of LH on Leydig cells. It should be pointed out that the existence of direct effects of thymic hormones on target endocrine glands of the hypothalamus-pituitary axis has not yet been sufficiently studied. Some other thymic hormones were equally shown to stimulate neuroendocrine hormone secretion, thymosin- α 1 was apparently able to down-regulate TSH, ACTH

and PRL secretion in vivo (Milenkovic and McCann 1992). As the corresponding releasing hormones were also decreased by the same hormone, this inhibitory effect of thymosin- α 1 seems to occur through hypothalamic pathways.

Interestingly, none of the receptors for thymulin or any other thymus specific hormone have ever been detected on the cells of the neuroendocrine axis.

4 The Thymic Hormone Thymulin

4.1 Thymulin – the Facts

The thymic hormone thymulin is a metallononapeptide with the following amino acid sequence: pyroGlu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-AsnOH (Bach, Dardenne et al. 1976; Pleau, Dardenne et al. 1977). The biologically inactive peptide component (molecular weight: 857D) was initially characterized in pig serum (Bach and Dardenne 1973) and therefore called 'facteur thymique sérique (FTS)'. It was later found to be coupled in an equimolecular ratio to the ion zinc which confers biological activity to the molecule (Dardenne, Pleau et al. 1982a; Gastinel, Dardenne et al. 1984) (see figure 6).

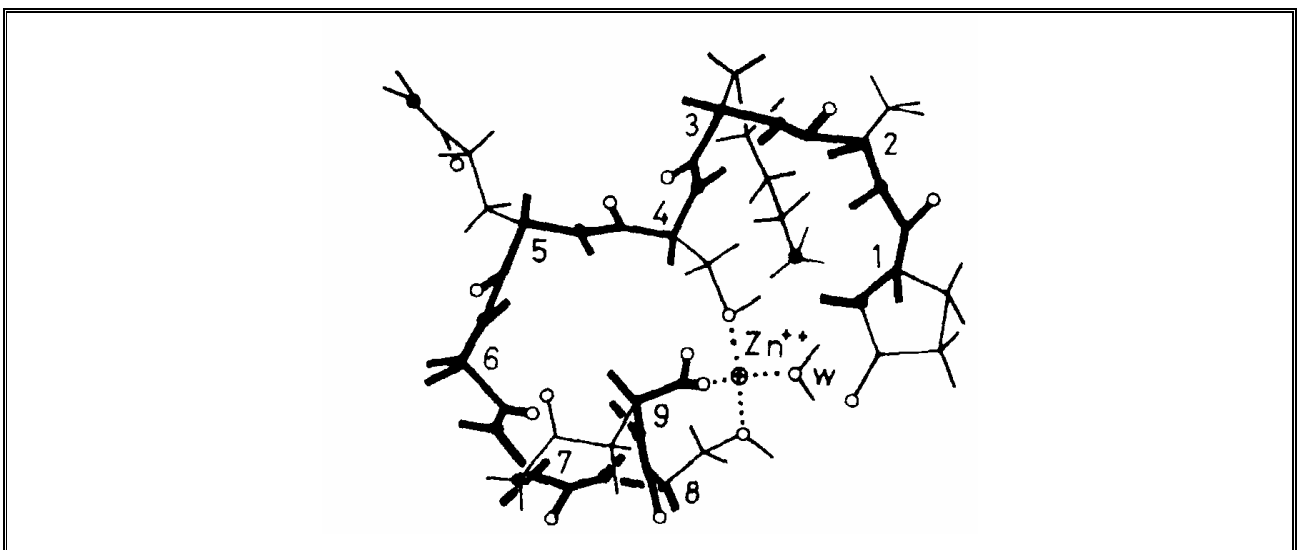


Figure 6: NMR Spatial Structure of Thymulin Showing its tetrahedral coordination with Zn²⁺ in an Equimolecular Ratio. - The numbers represent the amino acids composing the nonapeptide FTS, beginning with the N-terminal group, and zinc is represented by Zn²⁺ being covalently bound to Ser⁴-OH, Ser⁸-OH and Asn⁹-COOH, the fourth ligand being estimated to be a molecule of water (w). (Modified from Cung, Marraud et al. 1988); NMR - nuclear magnetic resonance

There is no apparent species specificity. Folkers and Wan demonstrated in 1978 that thymulin is initially translated as a bigger precursor molecule that gives rise to thymulin *and* thymopietin after splitting. However, this finding has never been confirmed. Thymulin is exclusively produced by the thymic epithelial cells (TEC), accumulated in intraplasmatic vacuoles and secreted by exocytosis (Savino and Dardenne 1986; Savino, Dardenne et al. 1982). A carrier was proposed to exist which binds thymulin in serum, its estimated molecular weight is 40-60kD. Prealbumin was mentioned as a presumable candidate, but its relationship to the ill-defined carrier proteins remained a matter of speculation (Dardenne, Pleau et al. 1980). Thymulin undergoes a circadian rhythm, with peak concentrations being found to occur at night (Molinero, Soutto et al. 2000). Studies on older subjects revealed its rapid decline with age (Consolini, Legitimo et al. 2000) consistent with the physiological involution of the thymus (see figure 7), and the simultaneous increase of a serum-inhibitor. The molecule responsible for inhibition is not known, but it has been shown to be active at very low concentrations (Bach and Dardenne 1973). The gene for thymulin has never been cloned.

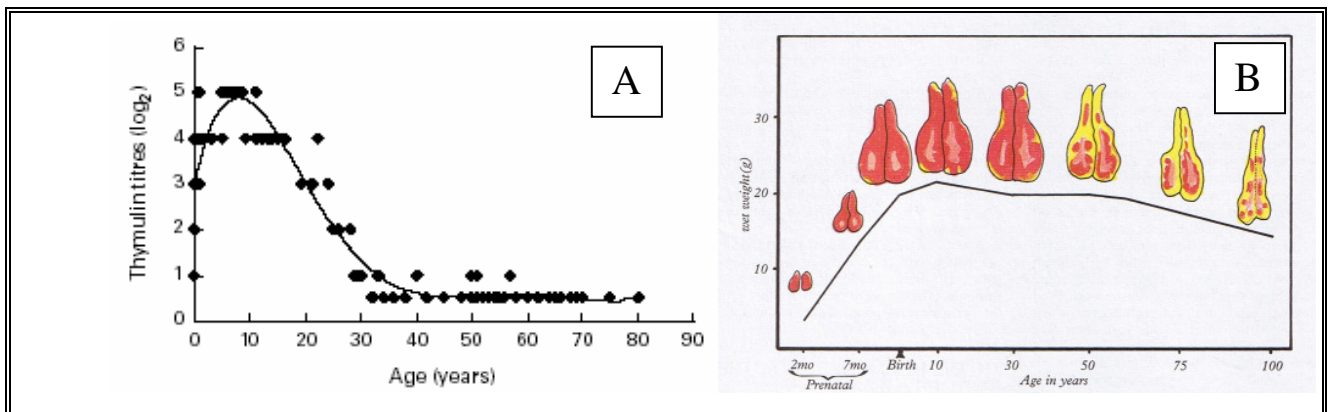


Figure 7: Decreasing Serum Thymulin-Levels are Paralleled with Involution of Thymic Gland. - Diagram illustrating the trend of thymic function through the course of the life. **A)** The thymulin titres of 93 subjects are plotted for each increment in age. Data are fitted to polynomial function using non-linear regression analysis (solid line; $r^2 = 0.8857$). **B)** The thymus organ with its age-related changes in weight and composition is shown at different ages (*pink* = lymphoid medulla, *red* = cortex, *yellow* = fatty infiltration). (Modified from Consolini, Legitimo et al. 2000; Kendall 1981)

4.2 The Outstanding Role of Thymulin as a Thymic Hormone

Thymulin is undoubtedly the best characterized of all thymic hormones. Over the years, a growing number of studies point to thymulin as the most important of all thymic factors in the thymus-brain-pituitary network. Jean-Francois Bach, Mireille Dardenne and Jean Marie Pléau

among others, pioneered the structural, biochemical and physiological characterization of this hormone in the seventies and eighties, the latter in relation to its immunological actions. Since the early 90s, Goya and Dardenne have focused their efforts on the thymus-pituitary axis with special emphasis on the impact of aging. More recently, Safieh-Garabedian et al. (2003) have demonstrated an analgesic and anti-inflammatory activity of thymulin in the CNS (Safieh-Garabedian, Ochoa-Chaar et al. 2003). The fact that thymulin was first detected in pig serum and later in every species tested, whereas the other thymic hormones were originally isolated from bovine thymus glands only, strengthens the idea that thymulin is a phylogenetically widespread molecule. Using a rosette bioassay, thymulin was measured in several human body fluids (Dardenne 1975), and a number of initiatives were started to evaluate thymulin in health and disease. Its possible therapeutic use in thymus-related immunodeficiencies became subject of several clinical trials (see 4.3).

4.3 Clinical Aspects

4.3.1 Thymulin-Alterations in Associated Diseases

4.3.1.1 Diseases of the Immune System

The thymulin status has been assessed in a great variety of patients with primary or secondary immunodeficiency diseases as well as with autoimmune diseases. Circulating thymulin levels were shown to fall sharply both in animals and humans affected by immunodepressing pathologies such as AIDS (Incefy, Pahwa et al. 1986), DiGeorge Syndrome (Incefy, Dardenne et al. 1977; Iwata, Incefy et al. 1981) and mouse congenital athymia as well as by chronic or acute stress. The effect of stress on thymulin serum levels is thought to be mediated via classical stress hormones like cortisol (corticosterone in rodents), which are well established immunodepressants. The levels of thymulin were found to be lower than in normal age-matched subjects for a high proportion of patients with common variable immunodeficiency (CVID), severe combined immunodeficiency (SCID), IgA deficiency, ataxia telangectasia and Wiskott-Aldrich syndrome (Iwata, Incefy et al. 1981 and others). Autoimmune mice (NZB and B/W) exhibited premature decline in thymulin production (Bach, Dardenne et al. 1973) and a sharp reduction in the number of thymulin-containing cells in the thymus (Savino, Dardenne et al. 1983a). In patients with systemic lupus erythematosus (SLE), thymulin levels were shown to be decreased (Bach, Dardenne et al. 1975; Iwata, Incefy et al. 1981), whereas in other autoimmune diseases such as myasthenia gravis, increased values were

found at least in some older subjects, in others thymulin levels remained normal (Savino, Manganella et al. 1985).

4.3.1.2 Neuroendocrine Imbalances

Thymulin levels have been determined in a number of pathologies involving neuroendocrine alterations. In cases of hyperproduction of hormones like GH, PRL and thyroxin, increased levels of thymulin were detected, and vice versa (see 3.4.2).

4.3.1.3 Other Diseases

Thymulin levels in some non-endocrine diseases have been evaluated over the years. In many cases they were found to be decreased: zinc deficiency (Iwata, Incefy et al. 1979), chronic graft-versus-host-disease (Atkinson, Incefy et al. 1982), Cockayne's syndrome (Bensman, Dardenne et al. 1982), in children with nephrotic syndrome (Bensman, Dardenne et al. 1984), Down's syndrome (Franceschi, Licastro et al. 1981). In patients with osteopetrosis or protein energy malnutrition normal or decreased levels have been detected (Chandra 1979; Iwata, Incefy et al. 1981), whereas some patients with tumors (mycosis fungoides + Sezary syndrome and thymoma) have displayed increased levels (Chollet, Plagne et al. 1981; Safai, Dardenne et al. 1979; Savino, Manganella et al. 1985).

4.3.2 Therapeutic Potential of Thymulin

The restoration of immunity with thymic extracts was assessed experimentally as early as 1896 when Abelous and Billard administered crude thymic extracts to thymectomized frogs and restored muscle tone (Abelous JE 1896). About 70 years later, with the first description of a syndrome including congenital dysplasia of the thymus by DiGeorge in 1965, fetal thymus transplantation was able to restore cellular immunity in several of those patients (August, Rosen et al. 1968).

Only from the seventies on were experiments with well defined thymic factors carried out: a number of T cell alterations consequential to congenital athymia or neonatal thymectomy in mice, were found to be reversed by *in vitro* or *in vivo* treatment with thymulin (Bach 1983; Dardenne, Savino et al. 1984). In autoimmune mice a favorable effect of thymulin was observed, but paradoxically the disease could either be improved or aggravated according to the recipient mouse (NZB or B/W), its age and sex, the dose administered and the autoimmune manifestations considered.

In humans, thymulin has been used to treat a limited number of patients. In the course of an open clinical trial, no signs of toxicity were observed, and normalization of deficient T-cell numbers or functions was attained in some cases. In children presenting IgA deficiency, detectable IgA appeared in their serum after two to six weeks of thymulin-treatment, and infections occurred with less frequency or severity. After vaccination, specific antibodies appeared for the first time or increased to titers higher than ever before.

Two randomized, double blind, placebo-controlled trials were conducted in patients with rheumatoid arthritis (RA). Thymulin provided significant clinical improvement as evaluated by the global assessment of all patients and by four objective parameters (Amor, Dougados et al. 1987). Unfortunately, in these trials, as in many cases of clinical application of thymic hormones, the correlation between immunological reconstitution and clinical improvement was not clear.

As mentioned above, recent evidence suggests that thymulin may act as an anti-inflammatory agent in the brain as well as a modulator of some peripheral nervous sensory functions such as those related to pain sensitivity. Safieh-Garabedian et al. found in 1996 that experimental hyperalgesia could be reduced by injections of thymulin in a dose-related manner (Safieh-Garabedian, Jalakhian et al. 1996). Subsequent experiments were designed to examine the effects of ICV injections of thymulin on cerebral inflammation induced by ICV injection of endotoxin (ET). Pretreatment with thymulin reduced, in a dose-dependant manner, the ET-induced hyperalgesia, and exerted differential effects on the up-regulated levels of cytokines in different areas of the brain, suggesting a neuroprotective role for thymulin in the CNS (Safieh-Garabedian, Ochoa-Chaar et al. 2003).

5 Methods for Thymulin Quantification: Immunoglobulins as a Biotechnological Tool

5.1 Significance of Immunoglobulins in Science

Long before the nature of the immune system was understood Paul Ehrlich hypothesized that the use of antibodies (antitoxins) in medicine would contribute to the recovery of sick patients. Soon after this, the first pharmacotherapy for diphtheria was developed. It was made out of the serum of immunized animals (cited according to von Behring and Kitasato 1991). Even though the existence as well as the effectiveness of those antibodies had been proved, only G.M. Edelman and R.R. Porter successfully analyzed their chemical structure in 1972 (Edelman, Cunningham et al. 1969; Porter 1973). They both earned the Nobel Prize for their groundbreaking discoveries.

The therapeutic usage of antibodies in human diseases underwent a rapid development. Fractionated and purified sera (polyclonal antibodies) have been used since 1940 for various infections and diseases. The multi-specificity of the polyclonal sera can be of great interest, multiple antibodies can recognize more epitopes thus enhancing the efficiency of the used serum. Unfortunately, polyclonal sera undergo great fluctuations in terms of availability and antibody concentration, due to the individual characteristics of the immunized animal as well as to the need of isolation and purification from animal fluids. In the past 30 years of antibody research, the technology has improved immensely. The mouse hybridoma technology (Kohler and Milstein 1975) gave rise to a new generation of antibodies. The development of monoclonal antibodies is more expensive and time consuming than the immunization of suitable animals for production of antisera.

Today, an ever growing variety and quantity of antibodies is used in therapy or clinical trials for diseases like cancer, transplant rejection, rheumatoid arthritis and Crohn's disease. Laboratory routine work and diagnostics are no longer conceivable without the use of antibodies as biological tools. Without the use of antibodies in research and the refinement of immune techniques, many of the discoveries that have led us to our present knowledge of nature and man would simply not have been possible.

5.2 The Generation and Purification of Polyclonal Antibodies

Immunogenicity is defined as the ability to induce an immune response such as antibody formation in a competent host. Most molecules inducing an immune response are proteins or peptides, but even polysaccharides and nucleic acid can be immunogenic in some cases. Small molecules (<1000-3000 Da) seldom elicit an immune response; they are called 'haptens'. However, coupled to a carrier macromolecule like serum albumin or keyhole limpet haemocyanin (KLH), most haptens become immunogenic and, among the polyclonal antibodies generated against the complex, a small subgroup will recognize the hapten's epitope(s).

The ability of a molecule to react specifically with the functional binding site (Fab) of a complementary antibody is known as its antigenic reactivity or *antigenicity*. Rather than the entire surface of a large molecule being required for the initiation of an immune response, the system detects only a small group of atoms, called antigenic determinants or epitopes, located on a number of sites on a molecular surface. In proteins, an epitope is composed of five to eight amino acids. Thus, a large protein usually possesses many epitopes and may induce the formation of a variety of specific antibodies from different B-cell clones. They are called polyclonal antibodies. Small

peptides often expose only one epitope. It has to be stressed that any change in molecule-conformation could lead to the exposure of a different group of atoms and therefore to the formation of another epitope.

Whereas antigenicity can be understood taking into consideration mostly principles of chemistry, immunogenicity is a very complex phenomenon. The same peptide can lead to very different antibody-titers in different animals of the same species (Patterson, Youngner et al. 1962). The particularities of the host immune system cannot be controlled by adjusting the structure of the peptides in a predetermined manner (Van Regenmortel 2001).

Different methods exist to concentrate polyclonal serum-immunoglobulins. After bleeding of the animal, the serum has to be obtained by centrifugation. The serum contains proteins, the proportion of immunoglobulins is estimated to be around 15%, the amount of specific antibody being even less (2-10%, depending on the animal species).

The ionic strength (salt concentration) of an aqueous solution influences the solubility of proteins. At pH values above or below their isoelectric point proteins have multiple positively and negatively charged groups and are referred to as polyampholytes. The electrostatic interactions between the charged groups and ions of opposite charge shield the protein from other polyampholytes. As such, increasing the ionic strength of an aqueous solution increases the solubility of proteins. However, as the ionic strength continues to increase, proteins will begin to precipitate because most of the molecules of solvation (i.e. water) are involved in solvating the ions and there is not enough bulk solvent to dissolve the proteins. This phenomenon, referred to as 'salting out', is commonly used to purify or concentrate proteins, here IgG-antibodies. The proteins are easily resolubilized because precipitation is the result of decreased solubility and not denaturation.

Different antibody extraction methods (i.e. using PEG, ammonium sulphate or caprylic acid as active agent) are required according to the origin and amount of the antibody. Finally, different chromatographic methods (affinity chromatography) against a single protein allow a very specific purification of the antibody. Various available processes may be used alone or in combination according to criteria like yielded amount, purity and biological activity of the antibody, or effort and cost involved. Every purification step leads to a certain loss of antibody mass and activity. Thus, an isolation-process entails a choice between yield and purity (Staak 2001).

5.3 Chicken Egg-Yolk Antibodies

As mentioned in section 2.6, the avian immune system shows some remarkable differences in comparison to the immune system of mammals. Those particularities have been exploited by science. In the last ten years more and more evidence has been generated to justify the use of avian antibodies in vastly different areas of investigation. The so called IgY technology, a term initially introduced by Dr. Claus Staak in 1995 (Schade and Hlinak 1996), was increasingly accepted worldwide as a biochemical tool. Whereas industrialized countries became more and more interested in it for its strong contribution to animal welfare, emerging Latin American and Asian countries especially were attracted by economic interests: the antibodies are sampled in a non-invasive, painless way, reducing the animal's suffering formally by 50%, with the yielded amount of antibody using chickens for immunization being approximately seven times higher than using rabbits, and therefore more cost effective (Schade and Hlinak 1996). Last but not least, more and more scientific reasons justify the use of this alternative method. Due to the wide phylogenetic difference between avian and mammalian species, many advantages arise (see table 1): whereas mammalian antibodies frequently show cross-reactivity between themselves, even though they come from different species, there is no immunological cross-reactivity between avian IgY and mammalian IgG (Hadge and Ambrosius 1984). A new assay for the determination of human serum C-Reactive Protein (CRP) using avian instead of mammalian antibodies could successfully avoid interferences of IgG with rheumatoid factors, thus lowering false positive reactions significantly (Larsson, Karlsson-Parra et al. 1991; Rieger, Burger et al. 1996). Background staining could be minimized for the same reasons, avoiding the use of IgG as primary and secondary antibody. It should be noted that in clinical laboratories most analyses are performed on serum samples. Here, the prevalence of human anti-mammalian antibodies can reach up to 80% in the general population (Kricka 1999) and those samples contain complement components that are usually activated by IgG, both facts leading to erroneous results that can be avoided by implementation of IgY.

It has been shown that 3-5 times more chicken antibody than swine antibody was bound to rabbit IgG which amplified the signal in immunological assays (Hadge and Ambrosius 1984; Olovsson and Larsson 1993). It could be proved that avian antibodies were able to recognize a wider range of epitopes than mammalian antibodies (Song, Yu et al. 1985) and that in some cases only the introduction of those avian antibodies allowed further approaches and solutions to certain questions (Schade, Henklein et al. 1996). Thus, due to phylogenetic reasons, the binding capacity of IgY can

be interestingly different from that of IgG. Those findings are especially relevant for highly conserved mammalian proteins (Larsson and Sjoquist 1990). The advantages of the IgY allow diagnostic tools to be improved, especially in wise combination with mammalian antibodies, enriching the traditional techniques.

Table 1: Characteristics of mammalian antibody compared with avian antibody. – Note the difference in antibody output, which is the basis for new applications in human and veterinary medicine, such as in the treatment of intestinal diseases, caries and intoxications (for example, as a result of snake bites or food contamination). (Data summarized according to a review published in *ATLA*, Schade et al. 1991 (Schade 1991); *Data from Hadge et al. 1980 (Hadge, Fiebig et al. 1980a; Kendall 1981))

	IgG (rabbit)	IgY (chicken)	
Antibody sampling	invasive	non-invasive	
Antibody amount	200mg/40ml blood	50-100mg/egg 5-7 eggs/week	
Antibody amount/month	200mg	ca. 1000-2800mg	
Amount of specific antibody	ca. 5 %	2-10 %	
Interference with mammalian IgG	Yes	No	
Interference with rheumatoid factors	Yes	No	
Activation of mammalian complement	Yes	No	
Protein A/G-binding	Yes	No	
Carbohydrate Composition (g%)	Hexosamines*	1.3 (human IgG)	3.2
	Sialic acid*	0.1 (human IgG)	0.2
	Total*	2.4 (human IgG)	5.2

The constantly growing number of publications on IgY technology, as well as commercially available avian antibodies prove the success of this method, formerly considered as alternative but progressively becoming a primary choice. In the 7th Edition of Linscott's Directory of Immunological and Biological Reagents (1994/1995) the proportion of 'chicken antibodies' was only 0.007%, whereas a few years later, in the 10th Edition (1998/1999), the percentage rose up to 0.11. The Chemicon International Communications Update (9, N°6) recorded 12 chicken antibodies out of 100 antibodies offered for the year 1999 (personal communication R.Schade). Recent developments even make the application of monoclonal avian antibodies now feasible (Matsuda, Mitsuda et al. 1999; Nakamura, Shuyama et al. 2004; Nishinaka, Ravindranath et al. 1996).