CHAPTER V: Discussion

The role of the thymic hormone thymulin for the immune system was studied intensively in the ‘70s and ‘80s. In order to prove the strong impact of thymulin on the neuroendocrine system and its crucial role for the neuroendocrine hormone balance, *in vivo* studies of specific immunoneutralization (quenching) of this hormone were carried out in mice during early life. In a series of preliminary studies, the antibodies to be used were assessed, and the experimental design was set up. Below, the experimental results are discussed.

1 The Antibodies

1.1 Antibody Generation and Purification

The use of avian antibodies and the IgY-technology is becoming more and more widespread in biomedical research and human as well as veterinary medicine. The expectation of obtaining a pool of highly specific and sensitive antibodies led to the introduction of those avian antibodies into the present quenching experiments. In addition to differences between the mammalian and the avian immune systems themselves, a significant phylogenetic distance between these two animal classes contributes to the different antibody specificities and qualities. Both aspects have been intensively described and discussed in chapter I (see table 1). Several authors have reported that chickens often produce antibodies against phylogenetically highly conserved mammalian proteins or peptides more efficiently than rabbits do (Larsson, Karlsson-Parra et al. 1991). Furthermore, the antibody production of a hen roughly corresponds to that of a large mammal, such as a sheep or a goat. Thus, an extraordinary amount of antibody can be produced from only one hen, opening possibilities for new fields of research where large amounts of antibodies are required, such as immunotherapy and immunoprophylaxis for viral and bacterial infections in clinical medicine, or for laboratory research such as immunoneutralization studies in particular.

Antibodies were generated in chickens and rabbits using the synthetic metallopeptide FTS-Zn^{2+} coupled to KLH. Being a hapten with only nine aminoacids, the small molecule FTS alone would not induce the immunoresponse necessary for the generation of antibodies. Keyhole limpet hemocyanine (KLH), which is isolated from the giant sea mollusc *Megathura crenulata,* is one of the most popular carrier proteins used to create an immunogen for injection. KLH induces a strong antibody response because of its large mass (>10^6 M.W.) and because it is a non-mammalian

protein. Additionally, the immunization was supported by the use of so called adjuvants. Freund's complete/incomplete adjuvants were used in our case, but specol and PCSL (Pam_3Cys-Ser-$(Lys)_4$) have also been recommended (Schade, Calzado et al. 2005). These adjuvants facilitate polyclonal antibody-generation by antigen-independent, non-specific B-cell-stimulation.

Different antibody-extraction methods are required according to the origin of the antibody. Furthermore, various available processes may be used alone or in combination according to criteria like yielded amount, purity and biological activity of the antibody, or difficulty and cost involved. But every purification step leads to a certain loss of total amount of antibody (Staak 2001). Most of those methods make use of a phenomenon called 'salting out'. By increasing the ionic strength (salt concentration) of an aqueous solution the proteins begin to precipitate. However, they are easily resolubilized because this precipitation is the result of a decreased solubility and not of denaturation.

The principle of affinity chromatography consists of immobilizing a reaction-partner (ligand) on a matrix to which a sample solution containing the specific reaction partner is added. Specific non-covalent bonds are formed while unbound, non-specific substances are washed away. The bound partner is finally detached and isolated through certain manipulations such as addition of acid media or elevated salt concentrations (0.5M-1.0M). In our experiments we did not succeed in detaching the antibodies from the coupled ligand FTS, perhaps due to highly elevated avidity.

1.2 Characterization and Comparison of the Ab Generated – Assessment of Immunoreactivity

1.2.1 Dot Blot

All mammalian antibodies (see Table 2) gave a positive result on Dot Blot. In testing those antibodies for immunoreactivity against biological active thymulin (zinc-bound FTS) and the zinc-free FTS, no differences were detected. This finding is in line with former experiments that resulted in the same immunoreactivity of mammalian anti-thymulin-specific antibodies for thymulin and FTS (Metreau, Pleau et al. 1987). Dot Blot turned out not to be sufficiently sensitive to allow comparison between the different mammalian antibodies.

All avian antibodies (see Table 2) gave a positive result when tested on Dot Blot. In analogy to mammalian antibodies, no differences within the group of tested avian antibodies could be detected. In comparison to the mammalian antibodies, all avian antibodies showed less intensive reactions on Dot Blot when tested under similar assay-conditions. This could be due to a lower antibody titer of the avian antibodies, or even due to a weaker sensitivity or specificity (see below).

1.2.2 ELISA

Since the concurrent introduction of enzymes as markers for the labeling of antigens and antibodies by Avrameas and Uriel in France as well as Nakane and Pierce in the United States (Avrameas and Uriel 1966; Nakane 1966), immunoenzymatic techniques have been considerably developed and diversified. In general, immunoenzymatic procedures based on the use of enzyme-antibody or enzyme-antigen conjugates and on the use of chromogenic substrates are sufficiently sensitive for the routine detection, titration and quantification of most constituents of biological interest. However, in order to detect constituents present in very low amounts, different techniques capable of strongly amplifying the enzymatic signal had to be established. Several attempts were undertaken in the past for the detection of thymulin whose concentration in serum is about three orders of magnitude (10^3) lower than that of other hormones (see below). In 1987, Métreau et al. made use of an amplification system including Ellman's reagent (Ach + DTNB). A monoclonal mouse anti-rabbit antibody was bound to ELISA plates. Rabbit antibodies were preincubated with FTS and G4-acetylcholinesterase-labeled FTS and afterwards added to the plate (see figure 27/A). Considerably lower detection limits of 5pg/ml were achieved (see below, figure 28). Later attempts in the early 90s using other techniques did not allow better amplification. Thus, Goya et al. proposed two systems, both including peroxidase and ABTS as chromogenic substrate (Goya et al. 1994, Goya et al. 1995, unpublished data). In figure 27 those systems are outlined. In 27/B a donkey antibody linking two rabbit antibodies was applied (one recognizing FTS that is bound to the plate, and the other one recognizing free peroxidase), whereas in 27/C a peroxidase-labeled secondary antibody (goat) was used. Both systems led to an amplification and detection limit that were around three orders of magnitude (10^3) inferior to that attained by Métreau et al. (see figure 28).

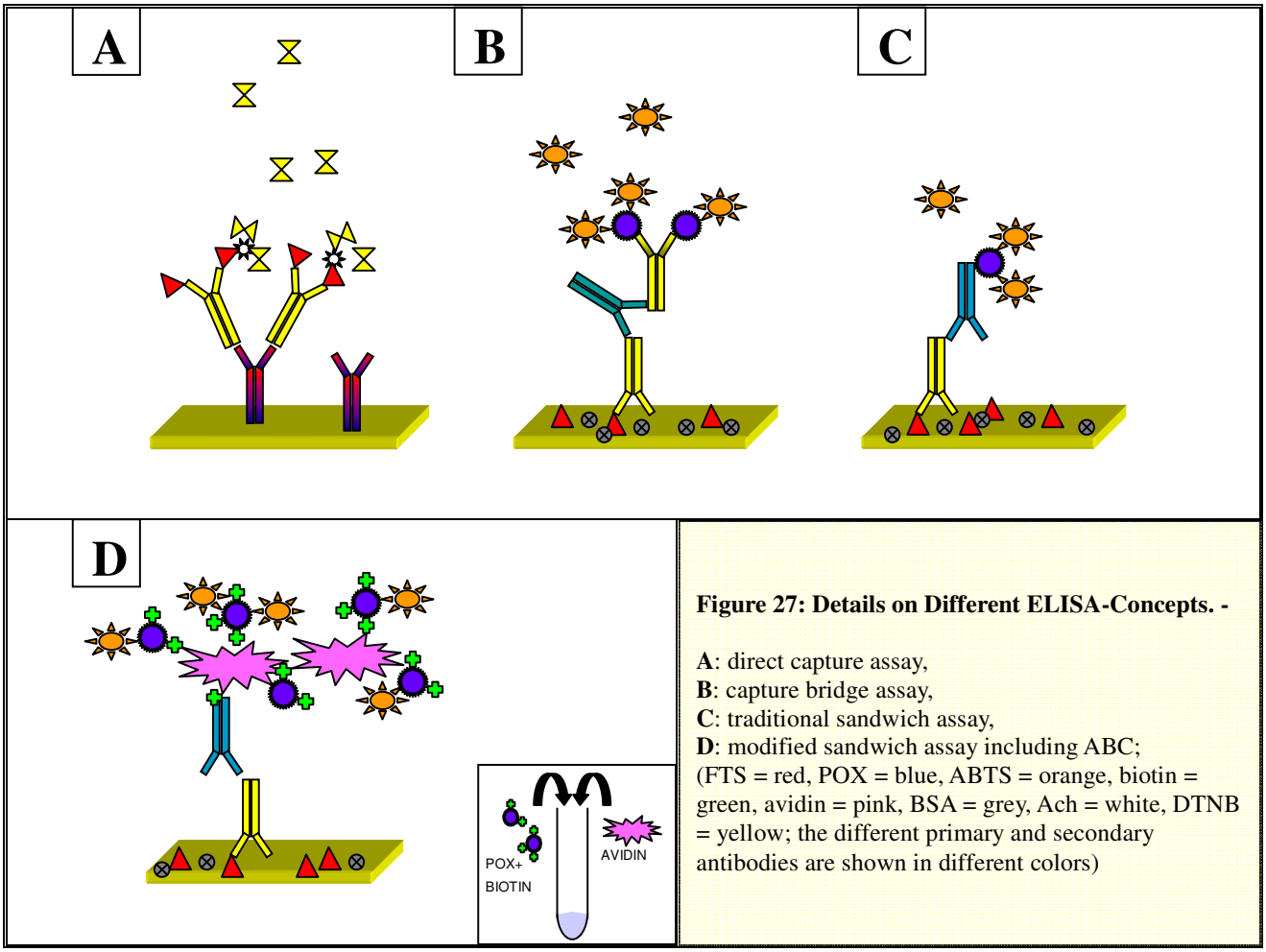

Figure 27: Details on Different ELISA-Concepts. -

A: direct capture assay,
B: capture bridge assay,
C: traditional sandwich assay,
D: modified sandwich assay including ABC;
(FTS = red, POX = blue, ABTS = orange, biotin = green, avidin = pink, BSA = grey, Ach = white, DTNB = yellow; the different primary and secondary antibodies are shown in different colors)

In 2003 we took up this subject and modified the ELISA for thymulin based on the scheme displayed in figure 27/C. By introducing a procedure based on avidin-biotin interaction, a far better amplification was obtained (see chapter IV, figure 14). Avidin is a glycoprotein of 67 kDa and is found in egg white, whereas biotin, also known as vitamin H, is present in almost all cells, even though in small amounts. The procedure takes advantage of the fact that avidin has an extremely high affinity for biotin with an association constant of 10^{-15} M (Wilchek 1984). Furthermore it is based on the principle that avidin has four active binding-sites. Because of steric hindrance not all of these four combining sites are involved in the interaction with the biotinylated antibody, so that the remaining free active sites can act as acceptors for the subsequently added biotinylated enzymes (POX). The avidin-biotin system was first described for use in immunoenzymatic assays in 1979 (Guesdon, Ternynck et al. 1979). We used a modified and somehow simplified protocol according

to Hsu et al., with preformation of soluble avidin-biotinylated POX-complexes, a technique also known as ABC-procedure (Hsu, Raine et al. 1981) (see figure 27/D).

After setting up the protocol described in chapter III, a series of experiments was performed with the mammalian antibodies listed in table 2. All antibodies showed a slightly weaker sensitivity to FTS-Zn^{2+} than to FTS alone. This could be due to crystallization phenomena hampering antibody-binding to coated thymulin, since Zn^{2+} was found to crystallize on Dot Blot membranes (see figure 13). In earlier studies, the same immunoreactivity of anti-thymulin monoclonal antibodies was found for FTS and FTS-Zn^{2+} (Dardenne, Pleau et al. 1982b). As expected, ammonium sulfate purified mammalian antibodies led to increased signals in this ELISA (see figure 16). The antibodies R-Ab_12 and R-Ab_23 are detected as the best performing antibodies (note corresponding titers in figure 16), with an ED50 calculated from figure 17 of 5040 pg/ml and 3560 pg/ml, respectively. The titer of an antibody is defined as the highest dilution of the sample that can be detected at a predetermined cut-off. Titers can provide a considerable amount of information about the sample. Furthermore it can be concluded that the antibody generated in Paris in 1986 seems better preserved when stored in glycerol (1:1) than when frozen at -20°C. In spite of highly approved signals after the introduction of a new application system (the kit ABC), the sensitivity reached with this ELISA remained too weak for the detection of thymulin in both rodents and humans (see figure 28).

Efforts to set up an ELISA for avian antibodies were given up when strong non-specific interference was encountered. In analogy to mammalian antibodies, a protocol including the ABC-procedure has also been used for avian antibodies. In addition, when the ABC-procedure was employed with a biotinylated anti-rabbit-antibody, the use of an intermediate rabbit anti-chicken antibody was necessary. It has previously been documented that the ABC procedure may result in a relatively high non-specific binding. This background is most often due to non-specific interactions induced by the positively charged avidin molecule (isoionic point 10) (Avrameas 1992). Streptavidin, a neutral protein found in Streptomyces avidinii and expressing the same highly amplifying characteristics as avidin but proving to minimize background signals, could be tried in future assays (Wilchek and Bayer 1990).

It should be pointed out, however, that many authors have previously reported that the use of avian antibodies generated lower background signals when compared to rabbit antibodies (Cipolla, Cordeviola et al. 2001). The higher the signal an immunoassay generates, the higher the risk of elevated background signals. Every single constituent in the ELISA-system could cross-react or

show nonspecific binding with other constituents. Even though biotinylation of antibodies itself does not affect their biological activity, it must be kept in mind that biotinylation of even a few NH_2-groups in certain monoclonal antibodies was able to sharply decrease their antigen-binding capacity (Avrameas 1992).

Apart from these difficulties with background blocking conditions, in all our assays performed with avian antibodies the working dilution was always about 100 times lower in comparison with an equivalent assay using mammalian antibodies. This is consistent with observations made in RIA for the quantification of CCK-8, where a different working dilution of the antibodies needed to be applied in order to reach comparable sensitivities for both antibodies (Schade, Henklein et al. 1996). However, a number of authors reported that, in principle, there were no significant differences between the avidities and affinities of IgY and rabbit IgG (Bollen and Hau 1997; Woolley and Landon 1995).

All immunological methods involve non-specific interactions to a greater or lesser degree. Considering all facts together, the background noise induced by non-specific interactions has to be seen as a limiting factor in the development of sensitive immunoassays. Figure 14 shows that even NRS applied to the wells as a control was also associated with elevated signals when the ABC-procedure was introduced. On the other hand, any reduction of an apparent background noise, beyond a given point, also results in a decrease of the specific signal. A delicate balance exists between specificity and background noise.

1.3 Characterization and Comparison of the Ab Generated – Assessment of Biological Activity In Vitro

1.3.1 In Vitro Quenching: ELISA & SCRA

R-Ab_12 showed a higher titer than R-Ab_23 when immunoreactivity was assessed by ELISA. In line with this finding, a better biological activity was found for this antibody in comparison with R-Ab_23 in immunoneutralization studies performed with ELISA and SCRA. Results for R-Ab_31 and R-Ab_32 in figure 18 give an estimate of the loss of biological activity through purification (see also chapter V, 1).

1.3.2 Immunohistochemistry

Many years before enzyme immunoassay techniques were introduced by Avrameas et al. (Avrameas and Uriel 1966), A. Coons had established an indirect or ‘sandwich’-procedure for

immunofluorescence which made use of fluorescein-labeled antibodies to identify and localize cellular antigens (Coons 1956; Coons 1941). A specific native unlabeled antibody was applied followed by the addition of a fluorescein-labeled antibody against this first antibody. Since the first antibody possesses many epitopes, it will bind more than one molecule of labeled antibody and thus result in an increase of the enzymatic signal associated with the antigen. In immunohistochemistry, background noise problems have to be dealt with and discussed in a similar way to ELISA. It is not surprising that the risk of nonspecific interactions increases when the antigen is not a single peptide bound to the bottom of a plate, but a whole cell exposed to the antibodies.

1.4 Characterization and Comparison of the Ab Generated - Assessment of Biological Activity In Vivo

Although the titer of R-Ab_12 was shown to be the best, assessment of biological activity *in vitro* by means of SCRA revealed only slight differences between all mammalian antibodies tested. It has to be pointed out that, even though intrinsically quantitative, the SCRA is more precise when the dilution factor for samples and standard is smaller and the number of dilutions tested increases. Since a huge amount of antibody is required for *in vivo* quenching experiments, the availability of the antibody limited our choice. R-Ab_12 was excluded because samples were lacking.

Finally, the R-Ab_21 was chosen for all of the following *in vivo* experiments. R-Ab_23 had been stored in glycerol and needed to be dialyzed against PBS prior to in vivo application. A single application of the antibody led to an immunoneutralization of 80% and >90% in adult and infant mice respectively, which remained unchanged for the whole period of observation (10 days in pups, 27 days in adult mice). Based on these results the injection protocol was designed: injection of 0.01 ml FTS-hyperimmune serum/g animal weight, repeated every seven days until the end of the experiment.

Summing up, avian anti-thymulin specific antibodies have to be seen as less powerful in comparison with mammalian antibodies. The antibody generation and the titer-development as a result of immunization are not easily predictable. At least four variables influence the immune response: the antigen (dose, MW), the type of adjuvant used, the application method, as well as the animal itself (animal species, age, breed) and the conditions under which it is kept. While keeping conditions, the adjuvant and the application method were standardized in our studies, the antigen and the immune response of the particular animal were actuating variables that could not be changed in the case of thymulin or predicted in the case of the animal's immune response. Immunogenicity

has to be understood as a very complex phenomenon, the same peptide being able to lead to very different antibody-titers in different animals of the same species (Patterson, Youngner et al. 1962). This can be due to the immune system of the individual animal itself. But in chickens two different pathways appear to exist in the secondary immune response. A response pattern similar to that of mammals has been observed in some chickens, with strongly increasing titers with every booster immunization; in others, an increase in the antibody titer following the first immunization was observed, without any effect of further boosters on the titer development (Patterson, Youngner et al. 1962).

Even though many reports have been published about avian antibodies being more sensitive and specific than mammalian antibodies, the quality of every immune response depends unequivocally on the type of antigen.

As a consequence of these facts, the best immunization protocol for a particular antigen should be found out by experimental testing, or at least recommendations on the immunization and sample-collection should be followed (for chicken see Schade, Calzado et al. 2005). Furthermore, a maximum number of animals have to be immunized in order to be able to choose the best antibody after careful assessment and characterization of all antibodies.

2 The Thymic Hormone Thymulin

2.1 What is Thymulin? What is its Function?

Thymulin could be suspected to be nothing else but the circulating cleavage-product of another protein, acting on sensitive cells in a non-specific manner. The difficulty to prove the existence of thymulin-specific receptors could be considered an argument in support of this hypothesis. However, amino acid (aa) sequence-homology studies using the algorithm FASTA (FASTA@EMBL-Heildelberg.de) that allows simultaneous exploration in Swiss-Prot and NBRF/PIR, thereby giving access to all known amino acid sequences, revealed no significant aa - sequence homologies with any other mammalian protein. Only eight proteins, most of them from simple metazoan or unicellular animals, were found to have significant sequence homology with the nonapeptide FTS (see table 4). It should be pointed out that following this research, thymulin has to be seen as a molecule with its own identity, and not just a fragment of another protein.

Table 4: Amino Acid Sequence Homology Studies Applying the Algorithm FASTA. The last column shows the percentage of sequence homology of different proteins with the nonapeptide FTS. (*Caenorhabitis* = a nematode, *Centruroides* = a scorpion species, *Crustacean* = a large group of arthropods, *Trypanosoma* = a parasite provoking the African sleeping sickness and Chagas Disease in South America.)

GENERO	***PROTEIN***	***% OF IDENTITY***
Caenorhabitis	mab-5 6/94P10038 (353aa)	77.77
Centruroides	Neurotoxin 1 2/95P01492 (65aa)	75.00
Centruroides	Neurotoxin 3 6/94P01494 (65aa)	75.00
Crustacean	Toxin 1 11/95P45667 (66aa)	75.00
Centruroides	Neurotoxin 2 3/92P01493 (66aa)	75.00
Centruroides	Precursor of toxins 11/95P45663 (87aa)	75.00
Centruroides	Precursor of toxins 11/95P45664 (87aa)	75.00
Trypanosoma	Cytochrome C 5/92P18822 (93aa)	63.57

2.2 Thymulin Circulates at Very Low Concentrations – Detection Methods for Thymulin

As shown by Consolini et al. in 2000 (see figure 7), thymulin titers in normal subjects are especially low throughout the course of life. Compared to concentrations of other hormones, serum thymulin levels are about 10^3 times lower. This fact seriously complicates most investigations about this hormone. Despite a number of attempts, no reliable and sufficiently sensitive immunoassay could be established for routine measurement of thymulin (see figure 28). The time-consuming sheep cell rosette inhibition assay (SCRA) described by Dardenne and Bach (1975) is the only time-proven method available to evaluate the biologically active form of this hormone (thymulin or Zn^{2+}-facteur thymique serique, Zn^{2+}-FTS) since immunoassays cannot discriminate between thymulin and the inactive form of the hormone not containing Zn^{2+} (FTS).

The SCRA is based on the ability of thymulin to confer azathioprine-sensitivity to spleen rosette-forming cells from adult thymectomized mice. Initially it was reported to have a detection limit of 0.144pg (0.17fmol). However, the sensitivity of the SCRA used during the present work was

consistently 1 fg/ml (Goya 2004)[1]. Unfortunately, routine use of this bioassay is limited by the fact that it is time consuming and of low accuracy under normal laboratory conditions.

Several radioimmunoassay (RIA)-designs have been reported for measuring serum or plasma thymulin levels (Ikeyama, Kato et al. 1984; Ohga, Incefy et al. 1983; Pléau 1977; Safieh, Kendall et al. 1990). In 1987, Métreau et al. first approached the problem by means of an enzyme-immunoassay (EIA).

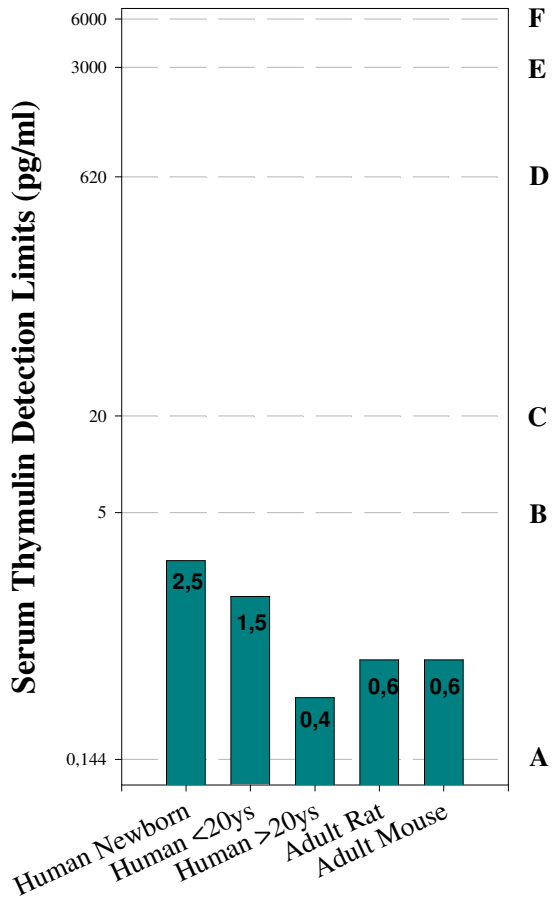

Figure 28: Demonstration of Minimal Assay Sensitivity Necessary for the Detection of Serum Thymulin Levels in Selected Species. –

Average serum thymulin-concentrations are given for humans of different ages, adult rats and adult mice, as determined by Safieh et al. 1990 (Safieh, Kendall et al. 1990). The dash-lines indicate the detection limits of five different ImmunoAssays, and a single bioassay available for thymulin. (**A** – SCRA, Dardenne et al., 1975; **B** – ELISA by Métreau et al., 1987, **C** - RIA by Pléau et al. 1977 and ELISA by Ohga et al. 1983; **D** – ELISA using kit ABC by Vesenbeckh et al. 2003 (unpublished data), **E** – ELISA by Goya et al. 1994 (unpublished data); **F** – ELISA by Goya et al. 1995, unpublished data.)

- Data plotted by SigmaPlot 4.0 -

[1] This data was published during the course of this thesis work: *Immunoneutralization of serum thymulin during early life reduces circulating gonadotropin and prolactin levels at puberty in mice (Abstract); Goya RG, Vesenbeckh SM, Sosa YE, Pléau JM, Cónsole GM, Schade R, Dardenne M; 12th International Congress of Endocrinology, Lisbon, Portugal, (30/08/2004 - 04/09/2004).*

Although interestingly low detection limits were achieved in some cases when artificial thymulin solutions were tested, all attempts to apply these assays to the measurement of thymulin in plasma or serum samples revealed high levels of interference. The mechanisms responsible for this interference have not, to our knowledge, been documented. More recently, a commercial ELISA-kit for thymulin was introduced into the market by Immundiagnostik AG (Immundiagnostik 2001). However, through informal contacts with the company we learned that the problem of severe interference with serum and plasma has also emerged in the case of this kit.

3 Quenching Experiments

3.1 The Concept

Mice and rats are both common animal models in laboratory research. The immune system of mice, however, is significantly more immature at the time of birth than the immune system of rats, and the same is true for the neuroendocrine system of mice. This is why the mouse model is especially suitable when questions about the immune system are approached. Countless experiments have already been carried out on athymic nude mice. With the purpose of mimicking those congenitally athymic mice, newborn mice were often thymectomized immediately after birth which leads to the same immunodeficiency and other symptoms (except hairlessness) seen in nude mice. It is well established that a maturational perinatal time-window exists at which the thymus is essential for a proper immune and neuroendocrine maturation. In mice, this window extends until postnatal day 3-4. Thymectomy in older animals could not induce the typical alterations found in nude mice, since here the thymus had enough time to release immunocompetent lymphocytes into the blood and peripheral organs, and its TECs had already produced and released relevant amounts of thymulin acting on hypothalamus, pituitary and other distant neuroendocrine glands. It has to be underlined that in the present experiment, a complete serum thymulin quenching that is defined as complete immunoneutralization of all circulating thymulin could not be achieved, so that a remaining 10% of the initial thymulin-concentration remained in the serum of the experimental animals.

3.2 The Consequences of a 90%-Quenching of Circulating Serum Thymulin

3.2.1 The Impact on Pituitary

These data are published elsewhere and therefore not shown here. Please refer to the original publication:

Camihort G, Luna G, Vesenbeckh S, Ferese C, Dardenne M, Goya R, Console G. Morphometric assessment of the impact of serum thymulin immunoneutralization on pituitary cell populations in peripubertal mice. Cells Tissues Organs. 2006;184(1):23-30. PMID:17190977

Goya RG, Reggiani PC, Vesenbeckh SM, Pleau JM, Sosa YE, Console GM, Schade R, Henklein P, Dardenne M. Thymulin gene therapy prevents the reduction in circulation gonadotropins induced by thymulin deficiency in mice. Am J Physiol Endocrinol Metab. 2007 Mar 27; [Epub ahead of print] PMID:17389714

3.2.2 Hypogonadism Subsequent to a 90%-Quenching of Thymulin

Hormone measurements had revealed that a 90%-thymulin-quenching caused a functional hypopituitarism. Presuming the axis between pituitary and peripheral organs, this state is invariably followed by hypogonadotropic hypogonadism. Unfortunately, neither testosterone nor estrogens were measured due to insufficient serum sample volume.

Mammalian spermatogenesis and oogenesis are finely tuned and complex processes involving intimate interactions among cells in the two compartments of the testis and the ovary respectively. The two glycoprotein hormones FSH and LH, which are produced by the pituitary gland in response to the stimulus from the hypothalamic GnRH, are involved in the process. These gonadotropins have a key role in the differentiation and maturation of mammalian sexual organs and functions. Fu-Ping Zhang et al. (2001) found out that the intrauterine sex differentiation was independent of LH action in both sexes, but that LH had a crucial role postnatally for attaining sexual maturity. In his study, LH-receptor knockout mice were found to be born phenotypically normal with testes, ovaries and genital structures indistinguishable from their wild-type littermates, but with severely affected postnatal sexual development (Zhang, Poutanen et al. 2001). In male and female FSH-receptor knockout mice an early qualitative and quantitative decline in spermatogenesis and ovarian development respectively, was found (Danilovich, Maysinger et al. 2004; Sairam and Krishnamurthy 2001). Interestingly, gonadotropin receptors occur in rodent ovaries only for several days after birth (Sokka, Hamalainen et al. 1996).

3.2.3 Testicular And Ovarian Histology After a 90%-Quenching of Thymulin with Subsequent Hypogonadotropic Hypogonadism, Leading to Arrested Postnatal Sexual Development?

In initial experiments it was found that neonatal thymectomy promotes developmental atrophy of female sexual organs (Besedovsky and Sorkin 1974). It was initially argued that such an effect could reflect an autoimmune process rather than the direct action of thymic hormones on the neuroendocrine system. This assumption was based on the fact that perinatal thymectomy in BALB/c mice was able to induce autoimmune disease (Bonomo, Kehn et al. 1995). However, thymectomy in this case was carried out on the third postnatal day, when the thymus had already released a significant amount of thymocytes to peripheral lymphoid organs. This detail taken

together with the fact that the production of sex steroids was enhanced *in vivo* and *in vitro* by the thymic hormone thymulin makes an autoimmune genesis less probable.

Effects of both neonatal thymectomy and congenital athymia on sexual development in mice have been shown by many authors: nude female mice showed significantly reduced levels of circulating and pituitary gonadotropins, a fact that seems to be causally related to a number of reproductive derangements described in these mutants (Rebar, Morandini et al. 1981). Thus, in homozygous (nu/nu) females the times of vaginal opening and first ovulation were delayed (Besedovsky and Sorkin 1974), fertility was reduced (Rebar, Morandini et al. 1981), and follicular atresia was increased such that premature ovarian failure resulted (Lintern-Moore and Pantelouris 1975b). Similar abnormalities resulted from neonatal thymectomy of normal female mice (Michael, Taguchi et al. 1980; Nishizuka and Sakakura 1971).

Since our animals did not attain puberty during these experiments, no evaluation of first ovulation, vaginal opening or premature ovarian failure could be undertaken.

4 Conclusions

4.1 The Immune and Endocrine Systems as Our Sixth Sense?

We are blind to viruses, bacteria and antigens with regard to our classic sensory systems. Our immune system perceives, however, through antigenic recognition, an internal image of the macromolecular and cellular constituents of the body and our neuroendocrine system monitors and controls the physical and chemical characteristics of the internal milieu (Goya 1991). Both are able to react to particular distortions of the image by converting the recognition of non-cognitive stimuli into biochemical information. Thus, a physiological response to such threats is ensured by the release and transmission of peptide neurotransmitters, hormones and cytokines, each of them being the classic representative of the neurological, endocrine and immune systems respectively. All of these three systems, finely linked together, ensure a perfectly adapted physiologic response to environmental alterations, and thereby homeostasis. Moreover, these systems could be usefully regarded as a sensory organ.

4.2 The Role of the Thymus Gland Throughout Life

Modern textbooks of disease and anatomy stress that the gland undergoes fatty involution with age in man but omit reference to the statements here and there in the literature that the gland is active and produces lymphocytes throughout life. To suggest that the bone marrow, which also

builds up fat throughout life, is atrophic and not important to adult man would deny all modern hematological concepts. Yet few people today take a parallel view of the thymus except perhaps those investigating aging and thymic hormones. In both of these areas of research it is obvious that the thymus must be active throughout life for continued good health. The vital importance of the thymus for the infant as well as in adult life should be considered seriously. A new philosophy on the treatment of immune diseases in both the young (SCID and AIDS patients) and in the aged (autoimmune conditions and cancers) would result, improving the treatment of patients recovering from illness and from many drug treatments.

Since the thymus gland extends its influence to several non-immunologic components of the body, the early onset of its involution might even act as a triggering event which would initiate the gradual decline in homeostatic potential that characterizes the aging process. Thus, aging was found to reduce most of the stimulatory actions of thymulin upon anterior pituitary cells (Brown, Sosa et al. 1998; Brown, Sosa et al. 1999). Certainly an age-dependent desensitization of the neuroendocrine system and other integrative centers of the body to thymic signals could act as one of the pacemakers of homeostatic decline (Goya, Brown et al. 1999). Age-related immunodeficiency was found to go along with cognitive deterioration. Thymectomy in mice not only led to reduced immune responses, but also resulted in deteriorated learning performances. Cytokines such as IL-1, IL-6 and TNF, and corticosterone were shown to affect the induction of hippocampal long-term potentiation, a synaptic model of memory (Nishiyama 2001). In addition to the well known deficit in cognitive function of patients affected by Alzheimer disease, those patients presented abnormal immune reactions that were held responsible for brain lesions occurring in the course of this disease. For this reason a direct causal connection between those abnormal immune reactions and the disease itself was suggested.

4.3 The Thymus-Neuroendocrine Interactions Dominated by Thymulin Influences

In this thesis work, the hypothesis that thymulin is crucial during early life for the development of a proper neuroendocrine balance was shown to be right. It can furthermore be concluded that the thymic hormone thymulin is strongly involved in the neuroendocrine alterations observed in athymic nude mice.

Injection of our rabbit anti-thymulin serum (raised against synthetic FTS-Zn^{2+} covalently linked to Keyhole Limpet Hemocyanin) did not completely eliminate thymulin from circulation but caused a drastic and long-lasting fall in serum thymulin levels in mice. Still, this

immunoneutralization of serum thymulin from birth to near-puberty in mice seems to have a slightly negative impact on growth rate which is consistent with what has been reported for nude and neonatally thymectomized mice. The influence of thymulin on body growth might be effected through the neuroendocrine system. The depressing effect that thymulin quenching has on serum PRL and gonadotropins is in line with observations in nude and neonatally thymectomized mice (Goya 1996; Michael, Taguchi et al. 1980). Furthermore, since thymulin has been shown to stimulate LH, FSH and PRL release from perfused rat pituitaries, the present *in vivo* findings were not unexpected. They are particularly significant however, because they demonstrate a physiological (i.e. *in vivo*) role for thymulin as a neuroendocrine modulator (Brown, Sosa et al. 1998; Brown, Sosa et al. 1999; Brown, Sosa et al. 2000).

The effect of thymulin-immunoneutralization on gonadotropic and lactotropic cell populations is not in line with previous studies in nude mice where pituitary morphology was found to be unaffected by athymia despite significant functional derangements in neuroendocrine function (Goya 1996; Goya, Console et al. 2001).

Taken together, the data point to a general facilitatory activity of thymulin on pituitary hormone secretion. In this regard, the nature of thymulin action on the pituitary gland seems different from that of cell-specific hypothalamic hypophysiotropic factors.

The observation that other findings typically associated with athymia (e.g. the aspect of the 'wasted animal') remained unaffected by immunoneutralization of thymulin is probably due to the fact that all other thymic hormones and cellular components were able to maintain part of the gland's immune functions in the latter animal model.