ANTI-TSH ANTI-IDIOTYPIC ANTIBODIES: CHARACTERISTICS AND APPLICATIONS

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by

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ABSTRACT

Antibody combining sites represent mirror images of their antigen epitopes. Anti-idiotypic antibodies specific for the combining site for the first antibody could exhibit similar tridimentional structure to that of the original epitope. With this background, internal image anti-idiotypic antibodies have been tested by numerous groups of investigators in several biological systems. Some of these were without any specific biological activity but others such as anti-insulin, anti-acetylcholine and anti-beta adrenergic receptor agonists have been found to have agonistic activity at the level of their specific receptors.

Graves' Disease is an autoimmune disorder involving the thyroid gland. Its etiological agent is thought to be an antibody with agonistic properties at the level of the thyroid stimulating hormone (TSH) receptor. The receptor itself has been thought to be the antigen responsible for the production of this antibody. However, considering the above mentioned premises, it is possible to speculate that the thyroid stimulating antibody of Graves' Disease could, at least in some cases, be an anti-TSH anti-idiotypic antibody.

This work was designed to investigate the possibility of producing anti-TSH anti-idiotypic antibodies and to test their biological activities. Anti-TSH antibodies were raised by injecting rats with highly purified TSH. This first antibody was later purified and used in the production of anti-idiotypic antibodies this time in rabbits. The activity of the anti- TSH anti-idiotypic antibodies was tested in experiments in vitro. It was demonstrated that the antibodies produced were true agonists at the TSH receptor level. They were capable of interfering with the binding of TSH to its receptor, activating adenylate cyclase, promoting adenylate cyclase mediated cellular processes and recognizing the TSH receptor protein in thyroid plasma membrane protein blots.

A second group of experiments explored the activity of anti-idiotypic antibodies directed to the individual subunits of the TSH molecule. The information

obtained appears to favor different requirements for receptor binding and post binding events. Antibodies with activity specific for each of the subunits of TSH were individually capable of binding to thyroid plasma membranes. On the contrary, there was absolute requirement for the participation of both antibodies for the activation of adenylate cyclase.

The third part of this work explores the influence that TSH may have on the synthesis and turnover of its receptor. These experiments were possible thanks to a detection system that benefits from the capacity of anti-TSH anti-idiotypic antibodies to recognize the TSH-receptor on thyroid plasma membrane protein blots. The results suggested definite participation of TSH in the regulation of receptor synthesis and turnover. TSH accelerates the synthesis and prolongs the half life of its own receptor.

In summary this work has demonstrated that it is possible to raise anti-TSH anti-idiotypic antibodies. The antibodies produced had agonistic effects at the level of the TSH receptor. It was also shown that these antibodies can be useful tools in the detection of receptor proteins and in the investigation of the hormone-receptor interaction.

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And last, but not least, my heart felt gratitude to my parents for their love and understanding. Despite geographical distances their support has been extremely important for me.

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LIST OF ABBREVIATIONS

α-anti-id ..... Anti-idiotypic antibody raised against monoclonal antibodies specific for the alpha subunit of TSH
ATP ..... Adenosin tri phosphate
AC ..... Adenylate cyclase
β-anti-id ..... Anti-idiotypic antibody raised against monoclonal antibodies specific for the beta subunit of TSH
BB ..... Binding buffer
BSA ..... Bovine serum albumin
bTSH ..... Bovine thyroid stimulating hormone
cAMP ..... Cyclic adenosine monophosphate
CFA ..... Complete Freund's adjuvant
CG ..... Chorionic gonadotrophin
cGMP ..... Cyclic guanosine monophosphate
cpm ..... Counts per minute
Da ..... Daltons
FSH ..... Follicle stimulating hormone
GC73 ..... Monoclonal antibody specific for the beta subunit of TSH
Gpp[NH]p ..... Guanosine-5'-{$\beta'\gamma$-imido} triphosphate
GTP ..... Guanosine triphosphate
HBSS ..... Hanks balanced salt solution
HT ..... Hashimoto's thyroiditis
hTSH ..... Human thyroid stimulating hormone
Id-IgG ..... Idiotypic immunoglobulin
Ig ..... Immunoglobulin
Igs ..... Immunoglobulins
IgG ..... Immunoglobulin of the G class
IgM ..... Immunoglobulin of the M class
Ir genes ..... Immune response genes
LATS ..... Long acting thyroid stimulator
LH ..... Lutheinizing hormone
MEM ..... Minimal essential medium
MEM/FCS ... Minimal essential medium with fetal calf serum
MHC ..... Major histocompatibility complex
MIF ..... Migration inhibition factor
N-rat-Ig ..... Normal rat immunoglobulin
NR-Ig ..... Normal rabbit immunoglobulin
PAS ..... Periodic acid Schiff
PBS ..... Phosphate buffered saline
RER ..... Rough endoplasmic reticulum
RNA ..... Ribonucleic acid
SD ..... Standard deviation
SDS ..... Sodium dodecyl sulphate
SDS-PAGE .. Sodium dodecyl sulphate polyacrylamide electrophoresis gel

STEM ..... Buffer containing sucrose, tris HCl, $MgCl_2$ and EGTA
TBS..... Buffer containing tris HCl and NaCl
TCA..... Trichloro acetic acid
TEM ..... Buffer containing tris HCl, $MgCl_2$ and EGTA
TSAb ..... Thyroid stimulating antibody
TSH ..... Thyroid stimulating hormone (thyrotropin)
TSH-anti-id.. Anti-idiotypic antibody raised against anti-TSH
TS28 ..... A monoclonal antibody specific for the alpha subunit of TSH
TSH-R..... Thyroid stimulating hormone receptor
w/v ..... Weight/volume

Chapter 1
INTRODUCTION

1.1. ANATOMY AND PHYSIOLOGY OF THE THYROID GLAND

1.1.1. Gross Anatomy

The thyroid gland, in man and many other mammals (Halmi,1978; Ekholm,1979; Ingbar and Woeber,1981) consists of two pyramidal shaped lobes connected by a narrow isthmus. It is localized in the anterior and lateral aspects of the trachea extending from the middle of the thyroid cartilage to the fifth and sixth cartilage rings. In the normal human it weighs on average twenty grams.

The thyroid is a well vascularized organ. Its blood supply derives from the superior and inferior thyroid arteries, which enter the respective poles, branch profusely and form a complicated capillary network. The lymphatic drainage is also rich. The thyroid is supplied by nerves of both the sympathetic and parasympathetic systems. The thyroid gland is surrounded by two capsules of connective tissue, the outer and inner capsules. The outer capsule encloses the gland and is continuous with the pretracheal fascia. The inner capsule covers the gland and emits prolongations within the tissue. The space between these two capsules contains the vessels, the recurrent laryngeal nerves and the parathyroid glands.

1.1.2. Light Microscopy

Under light microscopy (Halmi,1978; Ekholm,1979; Ingbar and Woeber,1981) it is possible to see that the thyroid gland is divided into lobules surrounded by fibrous septa derived from the inner capsule. The lobules are composed of a variable number of follicles. The follicles are circular, spheriele masses in which a single layer of epithelial cells enclose a viscous proteinaceous material called the colloid. The average diameter of the follicles is 200 μm, but there are considerable variations. Each follicle is surrounded by a basement membrane, a rich capillary network, and sympathetic and parasympathetic nerve fibers.

Two types of epithelial cells are found in the thyroid gland of mammals, the follicular and the parafollicular cells. The parafollicular cells are involved in the production of the hormone calcitonin. The follicular cells are the most abundant and are involved in thyroid hormone production. The thyroid follicular cells vary in height depending on the animal species and the degree of thyrotropin (TSH) stimulation. In man they generally vary from low cuboidal to tall columnar. In the absence of TSH stimulation the cells become low cuboidal and the colloid content increases. Under marked TSH stimulation follicular cells hypertrophy and colloid volume decreases.

The nucleus located in the basal part of the follicular cell, is large, spheroidal in shape, contains one or more nucleoli and has a low chromatin content.

The cytoplasm of the thyroid follicular cell is basophilic, contains numerous ingested luminal colloid droplets, which stain positively with the periodic-acid Schiff (PAS) reagent. The colloid is also PAS positive due to the high glycoprotein content of its main component, thyroglobulin.

1.1.3. Ultrastructure

Electron microscopical studies in conjunction with several other techniques such as immuno-staining and autoradiography, have made it possible not only to describe static structures present in the thyroid cell but also to establish the structural-functional relationship of the different cell components (Fawcett, Long and Jones,1969).

The thyroid cell has well defined apical and basal aspects, this fact has been termed "polarization" and has important functional implications. Numerous microvilli extend from the apical aspect of the cell into the colloid. The apical plasma membrane is very dynamic. Under TSH stimulation it becomes expanded by fusion with the material surrounding the newly synthesized thyroglobulin, and later forms pseudopodia that are involved in the endocytosis of colloid, which is the first step in the process of hormone release.

Microtubules are numerous in the apical part of the cell, but are also found elsewhere, and run in all directions taking straight or slightly curved courses.They have diameters of about 250 Angstroms and are thought to be involved in endocytosis (Gabrion, 1981). Colloid droplets are spheroidal structures formed through endocytosis. They vary in size, are limited by a membrane similar to the apical plasma membrane and contain colloid. These droplets travel from the apical cell region to the middle of the cell and occasionally are seen around the basal aspect. Lysosomes are diffusely distributed throughout the epithelial cell and often are closely associated with colloid droplets. These two structures have been observed to fuse. Fusion allows for the peptidases contained in the lysosomes to degrade thyroglobulin and achieve the release of free, i.e. non-peptide linked, hormones like tetraiodothyronine and tri-iodothyronine and other products such as diiodotyrosine and monoiodotyrosine.

The basal cell membrane has been implicated in hormone release into the capillaries. Although there is no direct evidence that an exocytic process is

involved, microtubules and microfilaments are thought to contribute to this process (Wolff and Williams, 1973). Recent experimental data has shown that the basal cell membrane probably contains the sodium/potassium dependent ATPase system known as the iodide pump and the TSH receptor-adenylate cyclase system (Chambard, Verrier, Gabrion and Mauchamp,1983).

In the active follicular cell the most prominent cytoplasmic structure is the rough endoplasmic reticulum (RER); its distribution is preferentially basal and perinuclear. The cisternae of the RER are wide and contain a moderately dense material, most likely thyroglobulin components. The thyroglobulin peptide chains produced in the ribosomes are transferred into the cisternae of the RER where the signal peptide is cleaved. The almost fully processed thyroglobulin is tranferred to the Golgi apparatus by a process of fusion. The Golgi complexes are found apically to the nucleus, they contain a varied number of Golgi saccules and small vesicles. Most of the thyroglobulin carbohydrate side chains seem to be added within the Golgi apparatus. Thyroglobulin, when fully processed, is transported to the apical aspect of the cell in vesicles. These exocytotic vesicles fuse with the apical membrane as previously described and empty their content into the lumen where it is stored. Mitochondria, the other important cellular organelles, are scattered throughout the cytoplasm. The nucleus of the thyroid cell, under electron microscopy, does not exhibit any unusual characteristics.

1.1.4. Thyroid Gland Physiology

Production of thyroid hormones is the main function of the thyroid epithelial cell. (Taurog,1978a; Taurog, 1978b; Ingbar and Woeber,1981.) Their synthesis can be summarized as follows: the trapping of iodide; the synthesis and iodination of thyroglobulin and lastly the secretion of thyroid hormones.

The thyroid gland is very efficient in the use of iodide, basically due to its limited availability in the natural environment, particularly in areas located away from the sea. The concentration of iodide by the thyroid gland involves an active

transport mechanism. The exact biochemical events involved are not yet fully understood but there seems to be a participation of the sodium-potassium-dependant ATPase pump. Sodium, with the involvement of some membrane phospholipid carriers, is able to increase the influx and decrease the efflux of iodide. Concentration of iodide requires cellular integrity, oxidative metabolism, phosphorylations and occurs against both concentration and electrical gradients. Transport is inhibited by certain ions such as perchlorate, pertechnetate and thiocyanate. It is also influenced by an internal autoregulatory system based on the availability of iodide. TSH and cyclic 3'5' adenosine monophophate (cAMP) also play an important role; these aspects will be discussed later.

Iodide (I^-) enters the thyroid gland and is converted to an oxidised form by a thyroid peroxidase. Tyrosines in fully mature thyroglobulin are iodinated by this product. The process of iodination seems to take place at the cell-colloid interface very close to the apical membrane (Taurog, 1974; Ui, 1974). The iodination of thyroglobulin is inhibited acutely and transiently by large amounts of iodide. A number of mechanisms have been proposed. These include inhibition of thyroid peroxidase catalysed iodination or alterations in the peroxidase generation systems. Iodotyrosine coupling mechanisms are dependent on the integrity of the native structure of the thyroglobulin, efficient iodination, and the coupling catalytic effect of thyroid peroxidase. Two mechanisms for coupling have been proposed: intramolecular coupling and intermolecular coupling. In vitro, both seem possible; in vivo, intramolecular coupling seems to be favored (Taurog, 1978b).

Hormone release is initiated by endocytosis of iodinated thyroglobulin from the follicular lumen. Endocytotic vesicles migrate from the apical towards the basal aspects of the cell. Numerous lysosomes migrate towards these vesicles and the fusion of these structures permits the access of proteolytic enzymes to the thyroglobulin. Subsequent digestion of thyroglobulin leads to hormone release. The main products secreted by the thyroid gland are tetraiodothyronine (T4),

3,5,3'triiodothyronine (T3) and small quantities of 3,3',5'triiodothyronine (reverse T3). Iodotyrosines, such as mono and di-iodotyrosine, have also been detected in serum but they are mainly retained inside the thyroid follicular cell where they are deiodinated. Thyroglobulin is mainly intrathyroidal but it has been detected extrathyroidally in the lymphatic drainage, and in small amounts in the serum, even in normal individuals.

TSH is involved in the control of thyroid hormone secretion; it stimulates endocytosis, thyroglobulin digestion, and hormone release.

Sympathetic stimulation and sympathomimetic amines are capable of increasing hormone secretion under certain experimental circumstances (Waldstein, 1966; Melander, 1970; Ganong, 1974). Their role under physiological conditions is uncertain and still remains under investigation (Williams, Lefkowitz, Watanabe, Hathaway and Besch, 1977; Bilezikian and Loeb, 1983)

1.1.5. Control of Thyroid Gland Activity

Several factors are involved in the control of thyroid gland activity (Dumont and Vassart,1978; Ingbar and Woeber,1981). Among these are TSH, thyroid hormones, iodide supply, cholinergic agents and catecholamines.

By far the most important of these regulators is the glycoprotein hormone TSH, secreted by the pituitary gland. TSH (Pierce and Parsons, 1981) is composed of two different glycopeptide subunits designated as α and β. These subunits are held together by strong non-covalent forces. Within the same animal species, the amino acid sequence of the α subunit of TSH is almost identical to that of the α subunits of other glycoprotein hormones [luteinizing hormone (LH), follicle-stimulating hormone (FSH) and chorionic gonadotropin (CG)] though with minor differences in their oligosaccharide components. The composition of the β subunits of these hormones also show certain homology, but the differences observed are more evident than those described for the α subunits.

The dissociated TSH subunits are inactive. Hormonal activity is expressed only after re-association between the α and β subunits. It is of interest to mention that recombinant hormones produced using α and β chains of different hormones, as well as of different species are active. The biological activity of the recombinant is dictated by the particular β subunit present, suggesting that this subunit determines the hormone specificity . As to how this specificity is mediated no clear explanation has been presented.

Thyrotropin's actions on thyroid follicular cells can be divided into rapid and slow effects. Rapid effects are related to the activation of secretion of thyroid hormones with acceleration of exocytosis, pseudopod formation and endocytosis. There is also increased binding of organic iodide, increased iodothyronine formation and activation of cellular metabolic processes such as the pentose phosphate shunt and mitochondrial respiration. Slow TSH effects are related to increased ribonucleic acid (RNA) accumulation due to activation of transcription, increased protein synthesis and cell division.

TSH, both exogenous and endogenous, is known to increase the ratio of intrathyroidal/serum iodide, demonstrating increased thyroidal intracellular iodide accumulation. This increase was observed, however, to be preceded by a distinct drop of the intrathyroidal/serum iodide ratio suggesting that the TSH effects on iodide uptake are biphasic (Halmi et al, 1960). Later studies carried out in vitro using cultured bovine thyroid cells also showed increased intracellular iodide accumulation after TSH stimulation (Knopp, Stolc and Tong, 1970). These authors suggested that these actions were mediated through cAMP and probably required the participation of some enzymatic products capable of enhancing the activity of certain membrane bound iodide carriers.

TSH actions are initiated by its binding to a specific receptor localized in the plasma membrane of thyroid cells. This binding appears to produce conformational changes within the receptor and the surrounding membrane lipid environment permitting interaction of the hormone-receptor complex with the

regulatory protein with subsequent activation of membrane-bound adenylate cyclase. This activation requires the presence of divalent cations, mainly calcium, and guanine nucleotides such as guanosine tri-phosphate (GTP) to activate the regulatory protein (Saltiel, Powell-Jones, Thomas and Nayfeh,1981). Adenylate cyclase acts on its substrate adenosine tri-phosphate (ATP) to produce cAMP, the second messenger for many TSH actions. Cyclic AMP activates cAMP-dependent protein kinases which phosphorylate a series of other enzymes or proteins which are thus either activated or inactivated. cAMP's action is terminated as a result of its degradation by intracellular phosphodiesterase.

Even though cAMP is an important mediator of thyrotropin's actions, it is unlikely that all hormonal effects depend on its generation. So far, the stimulation of phosphatidyl inositol turnover and glucose oxidation have been found to be cAMP independent processes. The importance of cAMP in growth promoting actions under physiological conditions is still unclear. Some studies in vitro have shown that cAMP is capable of promoting increased transcription and protein synthesis (Wilson, Raghupathy, Tonove and Tong, 1968; Adiga, Murthy and Mc Kenzie,1971). Other recent studies using cultured thyroid cells suggest that the TSH effect on cell growth is independent of the activation of adenylate cyclase (Valente, Vitti, Kohn, Brandi, Rotella, Toccafondi, Tramontano, Aloj and Ambesi-Impiombato,1983).

1.1.6. Characteristics of thyroid cells in culture

Cultured thyroid epithelial cells are useful tools in the study of the structure and the physiology of the thyroid gland. Isolated cells in monolayer cultures retain many functional and morphological characteristics of the differentiated thyroid cell within the intact organ. Addition of TSH to primary confluent cultures leads to the formation of follicle-like structures. These follicles concentrate iodide and synthesize thyroglobulin and thyroid hormones. As in normal follicles the apical pole of the cells face the follicular cavity in which thyroglobulin accumulates (Kerkof, Long and Chiakoff,1964; Fayet, Pacheco and Tixier,1970; Kondo, Horiuchi and Inoue,1980).

When cultured in the absence of TSH, cells organize into a uniform monolayer and appear to lose their capacity to concentrate iodide and to produce thyroid hormones. Thyroglobulin synthesis continues but at a very low level. TSH responsiveness is greatly depressed but the capacity to produce TSH-responsive adenylate cyclase is maintained. In these cells the basal aspect faces the culture plate and the apical aspect faces the culture medium. This inverted orientation may at least partially explain the loss of certain physiological responsiveness to TSH (Mauchamp, Margotat, Chambard, Charlier, Remy and Michel-Bechet, 1970; Chambard, Gabrion and Mauchamp, 1981; Chambard et al., 1983). Ultrastructurally, isolated thyroid cells maintain the general characteristics previously described for the thyroid gland.

1.2. THE THYROTROPIN RECEPTOR

1.2.1. Characteristics of hormone receptors in general

Receptors are specialized cellular molecules capable of recognizing specific ligands and binding them with high affinity and specificity (Baxter and Funder, 1979; Lefkowitz and Michel, 1983). After binding, hormone-specific signals are transmitted to trigger the appropriate physiological responses. According to their cellular localization, receptors can be: cytosolic receptors which following their binding to specific hormones migrate to the nucleus to interact with chromatin, nuclear receptors which are present in chromatin even in the absence of specific hormone and plasma membrane-bound receptors.

Activated hormone-receptor complexes interacting with chromatin regulate the levels of specific messenger RNAs. As a consequence they change the rate of synthesis of proteins coded for by these hormone-regulated messengers. Membrane-bound receptors bind hormone and the hormone-receptor complexes transmit signals through changes in membrane mobility to activate "second messengers". cAMP is the best studied second messenger, others like cyclic guanosine monophosphate (cGMP) and calcium have been invoked but their

involvement has been less extensively studied. Second messengers, principally cAMP, are known to activate protein kinases that initiate protein and enzyme phosphorylations, activating or inactivating them, to elicit further physiological responses.

Many membrane bound hormone-receptor complexes have been observed to internalize as endosomes after aggregation. The role of internalization is still not clear. It is thought to be associated in some instances with hormone degradation, receptor degradation, may be followed by receptor recycling, or may be necessary for the activation of certain biological responses (Hopkins, 1983).

Receptors are continuously being synthesized and degraded. Synthesis of cell surface receptors as well as other receptors, is thought to resemble the process involved in the synthesis of "exported" proteins. It starts in ribosomes associated with the RER, processing continues in the cisternae of the RER and later in the Golgi complexes. Association of receptor subunits and association of the receptor with the membrane proteins are still being investigated.

Radiolabeled hormones or drugs have contributed greatly to the study of receptor-ligand interaction. Data obtained from these studies have traditionally been analyzed with the use of Scatchard plots (Scatchard,1949). Better interpretation of data is now being obtained with innovative computer-assisted non-linear least square curve fitting programs that apply the law of mass action in a more direct and accurate fashion (Munson and Rodbard, 1980). Ligand-binding studies have yielded information related to receptor number and affinity under varied circumstances. Through this information it is now known that the number of receptors found in a cell is not constant, the numbers are dynamically regulated through ligand binding. It is also known that these variations in numbers can dictate the sensitivity of the cell to stimulation. Changes in affinity can also occur, even though they are less frequent, the phenomenon of "negative cooperativity" is an example of such changes.

The next step in the study of receptors is the determination of their molecular and structural characteristics. In several systems this has been achieved by purification or by their identification with irreversibly-bound ligands. Through experimental procedures like affinity chromatography (Lowe, 1979; Drummond, McQuade, Grunwald, Thomas and Nayfeh, 1982), photo-affinity labeling (Hazum, 1983), and anti-receptor antibody-receptor interactions (Van Obberghen, Kasuga, Le Cam, Hedo, Itin and Harrison, 1981), the structural characteristics of several receptors are now known. This knowledge has lead to the construction of hypothetical models of these receptors. These models have proven very useful in the interpretation of several physiological events.

1.2.2. Strutture of the Thyrotropin Receptor

Thyrotropin binding activity is almost exclusively localized to thyroid follicular epithelial cells, but extrathyroidal binding sites have also been described in testes (Davies, Rees-Smith and Hall, 1978), adipose tissue (Teng, Rees-Smith, Anderson and Hall, 1975) and lymphocytes (Pekonen and Weintraub, 1978). Binding at non-thyroidal sites seems to exhibit different characteristics from thyroidal binding according to the authors quoted above. Recently certain structural similarities as well as differences between the adipose and thyroidal receptors have been described (Buckland and Rees Smith, 1984). The physiological role of these extrathyroidal sites is still unknown.

Radio-iodinated ligand binding studies have helped in the identification of TSH binding sites in the surface of intact thyrocytes in culture (Lissitzky, Fayet, Vernier, Hennen and Jaquet,1973), in thyroid cell membrane preparations (Mehdi and Nussey,1975) and in detergent solubilized cell membranes (Manley, Bourke and Hawker,1974). Scatchard analysis of TSH binding at near physiological pH and salt concentrations was found to give a curvilinear plot suggesting two classes of binding sites, high affinity sites and low affinity sites (Tate, Holmes, Kohn and Winard, 1975; Peterson, Dawes, Rees Smith and Hall, 1977; Iida, Konishi, Kasagi, Ikekubo, Kuma and Torizuka, 1981). The physiological involvement of each of

these sites is difficult to assess but it seems that the high affinity site represents the biologically relevant TSH receptor and the low affinity site is related to the high affinity site either as a precursor or by aggregation (McQuade, Thomas and Nayfeh, 1983; Islam and Farid, 1985). Occupancy of the receptor by ligand appears to be involved in the regulation of receptor numbers. Studies of thyroid cell responsiveness to repeated TSH stimulation in vitro have demonstrated that this response is biphasic, at low doses there appears to be a constant stimulation whereas at high concentrations there seems to be a desensitization with a lesser number of receptors available for binding (Takasu, Charrier, Mauchamp and Lissitzky, 1978; Davies and Catt, 1978; Witte and Mc Kenzie, 1981). The different degrees of cell responsiveness to TSH stimulation are reflected in changes in cAMP generation.

It has been maintained that TSH-receptor interaction was transient and readily reversible. Studies with thyroid plasma membrane preparations have demonstrated that this is not so; the interaction of TSH with its receptor becomes more stable with time (Brennan, Peterson, Peterson, Rees Smith and Hall, 1980). These findings suggest a more permanent association between the hormone and a sub-population of the specific binding sites.

Studies involving cytological techniques with rhodamine conjugated TSH have demonstrated that TSH receptors are diffusely distributed in the surface of thyroid cells. Binding of hormone to its receptor is followed by aggregation and endocytosis in a manner typical of many other peptide hormone receptors (Avivi, Tramontano, Ambesi-Impiombato and Schlessinger, 1982). The role of internalization is still to be fully investigated, although it has been suggested that it is associated with the termination of the AC activation (Goldfine, Jones, Hradek, Wong and Mooney, 1978).

Several attempts by different groups of investigators to establish the biochemical structure of the TSH receptor have yielded a number of conflicting results; none of which has given unequivocal data as to its molecular mass or its organization.

The molecular size has been reported to be from 15,000 Daltons (Da) to 500,000 Da (Manley et al., 1974; Tate et al., 1975; Dawes, Peterson, Rees Smith and Hall, 1978; Czarnocka, Nauman, Adler and Kietczynski, 1979; Iida et al., 1981; Koizumi, Zakarija and Mc Kenzie, 1982; Iida, Konishi, Kasagi, Endo, Misaki, Kuma and Torizuka, 1983. Buckland and Rees Smith, 1984; Pekonen and Weintraub, 1979).

1.2.3. Structure of the porcine thyroidal TSH receptor

The Thyroid Research Laboratory at Memorial University of Newfoundland has been involved in the study of the structure of the porcine thyroidal TSH receptor (Islam and Farid, 1985). Some experimental procedures are described in a detailed fashion in this subsection because of the close relationship between these results and the work to be described in this thesis.

The purification procedure, briefly described, is as follows: porcine thyroid plasma membrane preparations were solubilized with lithium diiodosalicylate, applied to a DEAE-sephacel column and eluted with 1 M NaCl. After desalting the eluate was next applied to a second DEAE-sephacel column and further eluted with a 0.0- 1.0 M NaCl gradient. Several protein peaks were obtained. Peak VIII, which eluated between 0.35 and 0.56 M NaCl, contained the ^{125}I-bTSH binding activity. Peak VIII was further purified by applying to a TSH-Affigel column and eluating with 3 M NaCl. After desalting the product was made isotonic in 2 M sucrose. The preparation was resolved by 7.5-15 % linear gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis in the absence of reductant and stained with silver nitrate. The predominant product obtained was an Mr$\sim$ 197,000 polypeptide which corresponds to the holoreceptor. The recovery of this polypeptide was greatly dependent on the buffer osmolality. Several other minor bands were also observed at Mr$\sim$ 130,000; 105,000; 70,000 and 66,000; these were found to correspond to different receptor breakdown products (Figure 1.1:a). The subunit composition of the receptor was studied by resolving the eluted product by electrophoresis in a 10% sodium dodecyl sulphate polyacrylamide gel in the

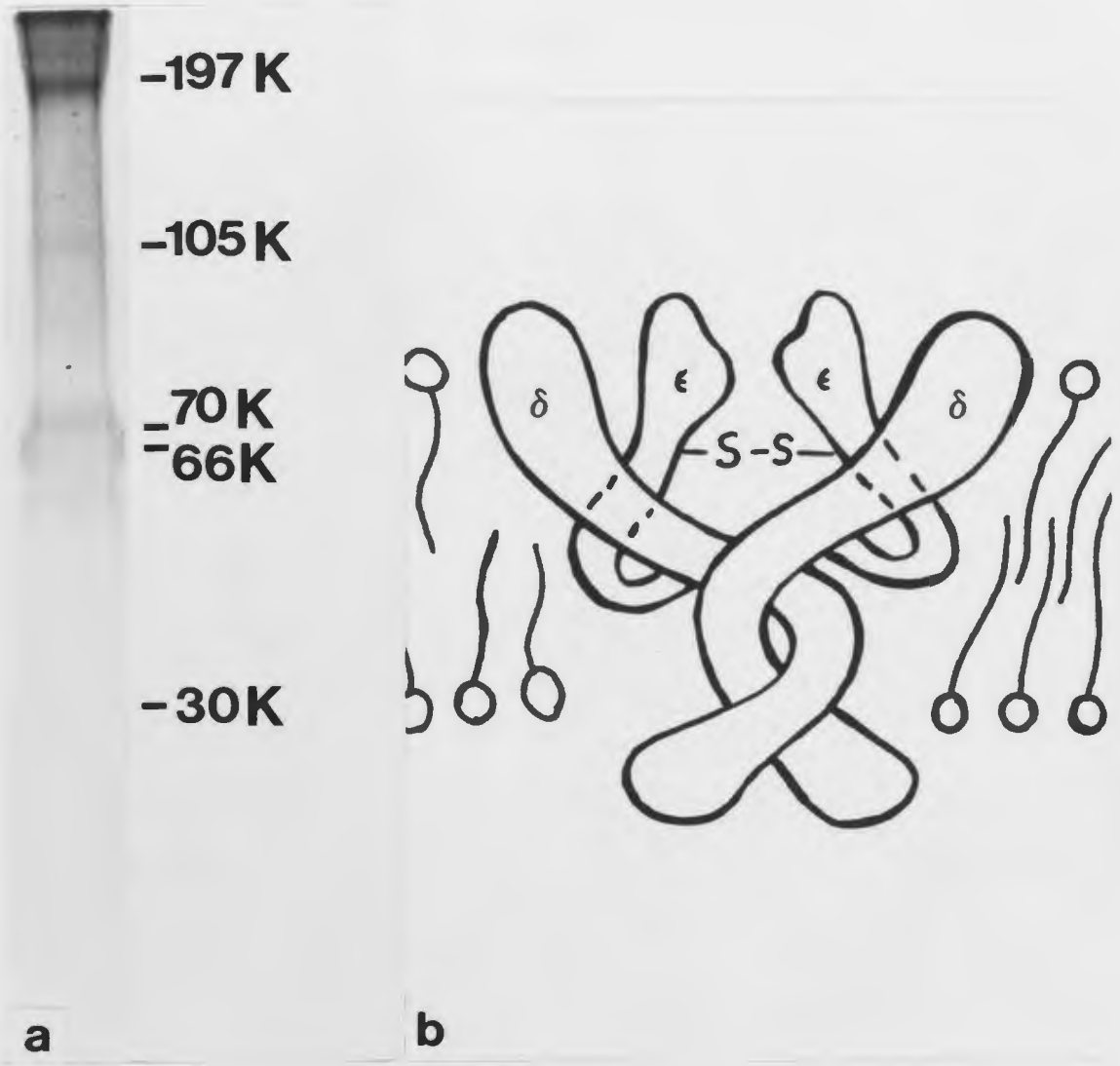


Figure 1-1: The TSH Receptor (from: Islam & Farid, 1985)

a) Electrophoretic pattern of the TSH-affinity column purified thyrotropin receptor.

b) Hypothetical model of the TSH receptor. A heterotetradimeric glycoprotein composed of 2 subunits, delta (δ) and epsilon (ϵ).

presence and in the absence of β-mercaptoethanol. Under non-reducing conditions the Mr~ 70,000 and the Mr~ 66,000 were identified. These peptides maintained a 1:2 stoichiometry. Under reducing conditions the Mr~ 66,000 persisted, while the Mr~ 70,000 disappeared and a new band at the level of Mr~ 35,000 was observed.

It was therefore concluded that the holoreceptor is a glycoprotein heterotetradimer with a Mr~ 200,000. It is composed of two different glycoprotein subunits, the first with a Mr~ 66,000 and the second with a Mr~ 35,000. Two 35,000 subunits are linked by one or more disulfide bonds to give a 70,000 form. The 66,000 and 70,000 subunits are held together by strong non-covalent forces. The integrity of the disulphide bond was found to be essential for the integrity of the holoreceptor and for hormone binding confirming previous studies which demonstrated that ^{125}I-bTSH binding is diminished in the presence of S-S bond reducing agents (Ozawa, Chopra, Solomon and Smith, 1979; Ginsberg, Rees Smith and Hall, 1982). Moreover the occupancy of the receptor by TSH protects it against the disruptive effect of the reducing agents (Davies and Platzer, 1981).

Using all the available information acquired and considering the stoichiometric ratios deduced from the relative degree of radiostaining of the two receptor components, a model for the receptor was proposed (Figure 1.1:b). Surface labeling of viable thyrocytes with radio-iodine showed that both receptor chains are exposed on the surface of the thyroid follicular cells (Islam, Briones-Urbina, Bako and Farid, 1983 a).

Several lines of evidence have supported the conclusion that the TSH holoreceptor is a glycoprotein with a Mr~ 200,000. Native and radiolabeled TSH bind specifically to the Mr~200,000 polypeptide on nitrocellulose paper thyroid plasma membrane protein blots (Islam and Farid, 1985). When this polypeptide was cross-linked covalently with radio-iodinated TSH, the interaction was inhibited quantitatively by native TSH but not by other hormones (Bako, Islam

and Farid, 1985). In a solid phase radiometric assay, the purified receptor preparation exhibits only one high affinity binding site with a $K_D = 1.2 \times 10^{-12}$ M (Islam and Farid, 1985).

1.3. AUTOIMMUNE THYROID DISORDERS

1.3.1. Autoimmunity and disease

The primary role of the immune system is to recognize "non-self" within the context of "self". Under certain circumstances the immune system becomes "auto-aggressive" attacking self determinants, a process that has been called autoimmunity.

One important question that has to be addressed is whether autoimmunity itself is necessarily an abnormal or pathological process. It has been suggested that the capability of reacting against self is part of the normal immune response. According to this hypothesis, it is suggested that recognizing non-self is closely related to the capacity of recognizing self. The production of low titres of auto-antibodies can be viewed as a normal physiological process. Exaggerated responses, often associated with tissue injury, result in autoimmune disorders. The normal immune response is maintained by a delicate balance of immuno-regulatory mechanisms. This principle has been supported by the demonstration that the potential for autoimmunity is present in all normal individuals. Many healthy subjects have antibodies to several normal body components (DNA and thyroglobulin among others) but that pathology will only develop in a limited number of cases where an alteration of the normal balance occurs (Talal, 1977).

Immune-responsiveness is determined, among other factors, by immune response genes and efficient immune regulatory mechanisms. The immunocompetent cells involved in the immune response include antigen presenting cells (macrophages, Langerhans and endothelial cells), T-cells (thymus dependent lymphocytes) and B-cells (bursa derived lymphocytes in birds and bone marrow derived in mammals). Subsets of T-cells either helper or suppressor are capable of regulating B-cell function (Julius, 1982). Helper T-cells trigger the differentiation of B-cells into antibody producing cell (Saxon, Stevens and Ashman, 1977). Suppressor T-cells on the other hand "down-regulate" an ongoing immune response or ensure that such

response does not occur at all (Golub, 1981). Excessive help or deficient suppression lead to the expression of abnormal antibody and cell-mediated immunity. Idiotypic- anti-idiotypic networks have been demonstrated to be very important in immuno-regulatory mechanisms. Their characteristics and participation will be described in a later section of this introduction.

Immune responsiveness depends greatly on the expression of the products of two clusters of genes: the Major Histocompatibility Complex (MHC) and the Immunoglobulin (Ig) system (Blanden, 1980). Genes of the MHC encode for molecules that control interactions between macrophages and T-cells and between helper and suppressor T-cells and B-cells. This communication requires that the interacting cells have identical MHC structures. The MHC-coded gene products are highly polymorphic transmembrane glycoproteins which are identical in the same individual, although distinctive determinants may be expressed not only by different T-cell subsets but also in different tissues.

Molecules encoded by Ig genes control specific interactions between the different clones of lymphocytes. Apparently several immune response (Ir) genes map close to Ig heavy chain gene clusters and at least in the mouse, one or more of these Ir genes determine the idiotypic repertoire of the antibody response (Bach, Greene, Benacerraf, and Nisonoff, 1979; Hirai, and Nisonoff, 1980).

Theories explaining the origin of autoimmunity are multiple and their concepts have changed throughout the years (Talal, 1980; Fauci, 1981; Rose, 1981). These include the "forbidden clone" theory and the "clonal abortion" theory (Burnet, 1969); cross-reactive antigens due to tissue damage caused by bacterial or viral infections and/or toxins (Allison, 1977) and disordered immune regulation (Talal, 1980).

Development of autoimmunity requires the influence of several factors, both host related and environmental. The familial aggregation found in certain autoimmune disorders has suggested a genetic predisposition for these diseases.

The inheritance of these disorders does not comply with Mendelian patterns, reflecting their polygenic nature. Certainly, in autoimmune thyroid disease there is evidence for an interactive predisposition linked to MHC alleles and IgG heavy chain markers. Decreased or increased efficiency in foreign antigen recognition in association with some MHC alleles is probably the basis of the variations in immune responsiveness that can potentially predispose to pathological autoimmunity.

Failure of immune regulation can occur after functional alteration of any of the three groups of cell populations involved in this response. Most of the attention has been concentrated on the role of the T-cell as potential cause of the regulatory derangement. Experimentally, suppressor T-cell deficiency has been documented in some autoimmune disorders. It is still argued whether in several human diseases and their animal model counterparts this defect is secondary to the auto-immune response or its causative agent. Because B-cell response to self antigens is highly dependent on T-cell regulation, immuno-regulatory T-cell dysfunction may lead to altered control of B-cell function with increased antibody production. Additional defects in the ability of B-cells to respond to suppressor signals have also been postulated.

The fact that some organs are more prone than others to the attack by auto-antibodies has lead to the belief that there are some special characteristics that make these tissues more susceptible to this type of destruction. Little information is available to explain this auto-antibody specificity for certain organs. Recently an aberrant expression of HLA-DR antigens on the surface of thyroid cells of patients with Graves' Disease was found (Hanafusa, Pujol-Borrell, Chiovato, Russell, Doniach and Bottazzo, 1983). This finding suggested a new potential explanation as to how certain organs trigger the production of autoantibodies by presenting their own antigens within the context of these aberrantly expressed MHC antigens. These auto-antigens can influence the immune-response by enhancing immuno-regulatory cell communications through specific products like

interleukins. Non-specific lymphocyte factors, such as interferon, may facilitate aberrant expressions of DR antigens (Bottazzo, Pujol-Borrell and Hanafusa, 1983).

It is possible,however, that the induction of DR antigen expression is secondary to the organ lesions due to the autoimmune process. It is important to mention that even though it is generally thought that autoantibodies react only against specific target tissues, patients suffering from certain organ-specific autoimmune diseases frequently have autoantibodies directed against organs other than those primarily affected. Naturally occurring multiple organ autoimmunity is well documented (Allison, 1977). Experimentally, polyendocrine disease with pancreatic anti-islet βcell, anti-anterior pituitary, anti-thymus and anti-gastric mucosa antibodies has been produced in reovirus infected mice (Onodera, Toniolo, Ray, Jensen, Knazec and Notkins, 1981). In the search for an explanation for the origin of multiple organ autoimmunity, monoclonal antibodies were raised using spleen cells of mice with multiple endocrine autoimmune disorders. These antibodies cross-reacted with antigens in multiple endocrine tissues. Further studies, using monoclonal antibodies prepared from lymphocytes from patients with polyendocrine diseases, were also found to react with multiple normal endocrine and non-endocrine human tissues. It is conceivable then, that molecules specific to one organ can share common antigenic determinants with molecules specific to other organs. The antibodies raised against the first organ can cross-react with all other similar molecules. These findings can, at least partially, explain the appearence of multiple organ autoimmunity (Haspel, Onodera, Prabhakar, Mc Clintok, Essani, Ray, Yagihashi and Notkins, 1983). Despite the evidence described above suggesting that cross-reactivity is one of the major issues in the development of multiple organ autoimmunity, several questions remain unanswered. One of them relates to the discordance in the time of onset of the different autoimmune disorders in the same individual. Knight and Knight (1984) have commented on the importance that host-related and genetically influenced somatic mutations may have in producing an array of different antibody generating clones of cells. They suggest that these clones are likely to have arisen

by separate somatic mutations from either a common or a separate precursor or precursors. Their antibody products could, then, share some idiotypic determinants that are directed to closely related antigens. This theory could at least explain the time lapse in between the appearance of the autoimmune problems affecting different organs and also the cross-reactivity observed during experimental work.

The development of autoimmunity also requires the presence of environmental triggering factors. The role of bacterial and viral infections, as well as some other environmental elements may prove of great importance in many disorders. Some other host related factors deserve mention. Under experimental conditions, it has been found that sex hormones influence the immune response and antibody production in certain strains of mice susceptible to autoimmunity. This influence of sex hormones has been related more to the absence of testosterone than to the presence of estrogen (Theofilopoulus and Dixon, 1981; Steinberg, Huston, Taurog, Cowdery and Raveche, 1981; Roubinian, Talal, Greenspan, Goodman and Siiter, 1978). The higher susceptibility to autoimmune disease present in females, as compared to males, seems to agree with these findings. Aging, with its associated decrement in feedback regulation of immune responses, may play an important role in the development of autoimmunity.

In conclusion, autoimmune responses alone do not necessarily lead to autoimmune disease. Pathological changes occur only when a variety of factors coincide in the same individual.

1.3.2. Hashimoto's Thyroiditis

Hashimoto's, or chronic lymphocytic thyroiditis, is one of the most common endocrine organ-specific autoimmune disorders (Volpe, 1978; Werner, 1978; Kidd, Okita, Row and Volpe, 1980; Kahn and Flier, 1980; Ingbar and Woeber, 1981). Clinically it most commonly presents with goiter. About 70% of the patients are euthyroid, 20% hypothyroid and 10% hyperthyroid at the time of clinical

diagnosis.There is familial aggregation of cases. No clear association with HLA-A or B locus has been described. However, a significant association of HLA-DR5 with goitrous thyroiditis (Farid, Sampson, Moens and Barnard, 1981) and HLA-B8 and DR3 with atrophic thyroiditis (Moens, Barnard, Bear and Farid, 1979) was found in studies performed in Caucasian inhabitants of Newfoundland.

Histologically the affected glands demonstrate the presence of small follicles depleted of thyroglobulin. The thyroid epithelial cells are abnormal in size and shape. Normal tissue is extensively replaced by infiltrating lymphocytes and fibrous tissue.

Circulating antibodies to different components of the thyroid tissue have been found in these cases. Approximately 90% of patients with Hashimoto's thyroiditis are positive for anti-thyroglobulin antibodies when examined by the tanned red cell agglutination test (Hall, 1962). Anti-microsomal antibodies have been detected by a variety of tests including complement fixation, immunofluorescence, immunoperoxidase and hemagglutination. When tested by the latter method up to 90% of patients were found to be positive for these antibodies (Irvine, 1975).Antibodies to the second colloid antigen (Irvine, 1975) and to nuclear components (Hall, 1962) have also been described. Positive titers are variable, but in the adult population, titers higher that 1:100 are of diagnostic significance. Antibodies to thyroid cell surface antigens have also been detected in the sera of patients with Hashimoto's thyroiditis using immunofluorescent techniques and human thyroid cells in culture (Fagraeus and Jonsson, 1970; Khoury, Hammond, Bottazzo and Doniach, 1981). Using an assay based on the ability of immunoglobulins of certain patients to compete with radio-iodinated TSH for binding to its receptor or to human thyroid plasma membranes, it was found that up to 10% of patients with Hashimoto's thyroiditis have antibodies capable of inhibiting TSH binding (Endo, Kasagi, Konishi, Ikekobu, Okuno, Tokeda, Mori and Torisuka, 1978). These findings suggested the possibility that some of the antibodies present in patients with Hashimoto's thyroiditis are directed against the TSH receptor.

The mechanisms by which antibodies to different thyroid cell components develop is still unknown. Theories that suggested that thyroid antigens normally have no access to immunocompetent cells have been invalidated by the demonstration of circulating thyroglobulin in the serum of normal subjects. Alterations in thyroid components due to viral infection have been proposed as etiological factors in Hashimoto's thyroiditis since under electron microscopy particles that resemble viruses have been observed (Kahn and Dale, 1973). On the other hand, certain disorders known to be caused by infective organisms, like subacute thyroiditis, do not predispose to autoimmune thyroiditis. However, the additional participation of certain types of viruses in a predisposed host cannot be excluded as being responsable for changes leading to chronic lymphocytic thyroiditis.

Cell-mediated immunity has also been implicated in the pathogenesis of Hashimoto's thyroiditis. Enhanced mitogenic responses of circulating lymphocytes to thyroid antigens and/or thyroglobulin have been observed (Ehrenfield, Klein and Benezra, 1971) as well as increased numbers of circulating lymphocytes capable of binding thyroglobulin and thyroid microsomes (Allison, 1976). The total number of T and B cells in the peripheral blood of these patients was not found to be different from normal by some authors (Kidd, et al. 1980), while others found a decreased number of T-cells (Fournier, Chen, Eger and Charriere, 1983). Direct cytotoxic effects of T-cells have also been experimentally observed (Calder, Mc Leman and Irvine, 1973). T-cells of patients with Hashimoto's thyroiditis have been found capable of producing migration inhibition factor (MIF) in the presence of crude and detergent solubilized thyroid antigens (Okita, Kidd, Row and Volpe, 1980), suggesting antigen specific T-cell sensitization in autoimmune thyroid disease.

Evidence for the involvement of both cell-mediated and humoral immunity in the destruction of the thyroid gland in chronic lymphocytic thyroiditis has been presented. An antibody-dependent-cell mediated cytotoxicity may , however,

prove to be the most important mechanism for tissue injury in this autoimmune thyroid disorder (Dessaint and Wemeau, 1982). In this system killer cells, bearing the Fc receptor, will bind the Fc portion of the immunoglobulin molecule forming part of an antigen-antibody complex localized on the surface of thyroid cells and will promote the lysis of the latter.

Since demonstrable abnormalities in both cell-mediated and humoral immunity are observed in Hashimoto's thyroiditis and because humoral immunity is highly dependent on T-cell function, possible alterations in immuno-regulatory T-cell subsets in these patients were investigated. Several authors found mitogen-triggered suppressor T-cell function to be normal in patients with Hashimoto's thyroiditis (Aoki, Pinnamaneni and De Groot, 1979; Beall and Kruger, 1979; Mc Lachlan, Wee, Mc Gregor, Rees Smith and Hall, 1980). On the contrary, when studying antigen-specific T-cell function inhibition of migration of T-cells in response to crude thyroid antigens was described (Okita, Row and Volpe, 1981)

Determinations of T-cell numbers in Hashimoto's thyroiditis have also yielded conflicting results. When detected by subset-specific monoclonal antibodies, a decreased ratio of circulating suppressor to helper T-cells was found by some authors (Thielemans, Vanhaelst, De Waele, Jonckheer and Van Camp, 1981; Sridama, Pacini and De Groot, 1982) but not by others (Jansson, Totterman, Sallstrom and Dahlberg, 1982). T-cells bearing Ig receptors were enumerated and found normal in autoimmune thyroiditis (Canonica, Bagnasco, Moretta, Cocco, Ferrini and Giordano, 1981). As observed, the results reported in the literature regarding T-cell function and number are quite conflicting. Definitive answers to these questions require further investigation.

Several other variants of thyroiditis deserve mention. Primary hypothyroidism associated with an atrophic thyroid is the most common. Despite the absence of goiter, the histological and immunological characteristics are very similar to Hashimoto's thyroiditis. The fibrous variant of Hashimoto's thyroiditis can only be differentiated from the lymphocytic variant through biopsy and because it is

more frequent in the older age group. Transient postpartum hypothyroidism occurs three to five months postpartum in women who had previously noted to have a goiter. This syndrome often follows a transient hyperthyroid phase related to the release of stored thyroglobulin. The reasons for the transient nature of this disorder and the temporal relationship to pregnancy are still intriguing questions.

1.3.3. Graves' Disease

Graves' disease (Werner, 1978b. Kidd, et al. 1980; Kahn and Flier, 1980; Ingbar and Woeber, 1981; Farid, 1981; Strakosch, Wenzel, Row and Volpe, 1982; Farid, Briones-Urbina and Bear 1983) is an autoimmune, multisystemic disorder with a prevalence of approximately 1% of the general population. It has three main components; hyperthyroidism, infiltrative ophthalmopathy and infiltrative dermopathy. These components can coexist or may appear individually.

The hyperthyroidism of Graves' disease is associated with diffuse enlargement of the thyroid gland. The clinical symptoms and signs include heat intolerance, increased nervousness, weight loss despite increased food intake, polyuria, polydypsia, increased heart rate and brisk reflexes. These symptoms are thought to be directly dependent on increased levels of the thyroid hormones, thyroxine and tri-iodothyronine. It has also been suggested that some of these symptoms may be due to the amplification of tissue responses to the actions of catecholamines but no direct proof is readily available (Landsberg, 1977; Melander, Westgren, Ericson and Sundler, 1977). The major manifestations of Graves' ophthalmopathy are proptosis, periorbital edema and chemosis. Opthalmopathy can occur with or without hyperthyroidism. The dermopathy, with pretibial myxedema occurs in 3 to 5% of patients with Graves' disease. Almost all these patients have concomitant opthalmopathy.

A family history of Graves' disease is fairly common among these patients. Despite this familial association and studies that have included large numbers of patients, no clear inheritance patterns for the disease have been found. The risk of

the siblings and parents of propositus patients for developing Graves' disease has been reported to be of about 2-8 % (Friedman and Fialkow, 1978). However, studies carried out at The Memorial University of Newfoundland have analyzed the incidence of the disease among first-degree relatives of 624 patients with Graves' disease and have found that the occurence of this disorder is not higher than 3% (Farid, N.R., personal communication). Twin studies have demonstrated that about 50% of monozygotic pairs are concordant for hyperthyroidism, as compared to less than 5% of dizygotic pairs. These results demonstrate the importance of the genetic involvement but also imply the participation of non-genetic elements in the development of the disease (Farid, 1981).

The association of Graves' disease with HLA antigens has been extensively studied (Farid, 1981; Farid and Bear, 1981). Several groups of investigators have found increased frequency of HLA-B8 in caucasian individuals with Graves' disease. An even closer relationship has been found between this disease and the presence of HLA-DR3 (Farid, Stone, Johnson, 1980; Farid, 1981). In other ethnic groups, however, Graves' disease has been found to be associated with other HLA antigens (Farid and Bear, 1981; Stenszky, Balasz, Kozma, Leovey and Farid, 1981).

The hyperthyroidism of Graves' disease is caused by the overstimulation of the thyroid gland by a circulating immunoglobulin directed against the TSH receptor (TS Ab) (McKenzie and Zakarija, 1978. Kidd, et al., 1980). The first evidence of a circulating thyroid stimulator was established by Adams and Purves in 1956 (Adams and Purves, 1956). The assay depends on the ability of patient's sera to cause release of radioactive iodine from ^{131}I prelabeled guinea pig thyroid glands in vivo. A similar assay was later developed using mice (McKenzie, 1958). Since TSH caused a peak response at 2 hours and Graves' disease serum at 9-12 hours in the mouse the activity was called long-acting thyroid stimulator (LATS). LATS was later characterized as an immunoglobulin of the G class (Kriss, Plashokov and Chien, 1964; Meek, Jones, Lewis and Vanderlaan, 1964). Because

up to 50% of patients with active Graves' disease had a negative response in the McKenzie assay, the importance of this immunoglobulin as the etiological agent of the disease was in doubt for some time. The immune basis of the disease was again favored when IgG from up to 90% of patients with Graves' disease was found to "protect" a LATS-positive serum from being absorbed by human thyroid extracts. It was concluded that these sera contained immunoglobulins which, even though unable to bind or stimulate mouse thyroid, could bind to human thyroid and competitively inhibit the binding of other thyroid stimulating immunoglobulins (Adams and Kennedy, 1971).

An assay based on the ability of serum or immunoglobulins of patients with Graves'disease to compete with ^{125}I-bTSH for binding sites on the receptor of human thyroid membranes was developed. Sixty to one hundred percent of Graves' disease patients sera showed evidence of anti-TSH receptor antibodies as manifested by inhibition of TSH binding (Rees Smith and Hall, 1974; Endo et al., 1978). Some normal sera, however, showed a major inhibitory effect on the TSH-receptor interaction (Sato, Zakarija and McKenzie, 1977). Non-specific binding was suggested, but it is also possible that some normal individuals have low levels of anti-TSH-receptor antibodies but exhibit no pathology. Some actions of the thyroid stimulating antibodies have been measured in different systems. It was found that they are capable of stimulating cAMP production (Onaya, Kotani, Yamada and Ochi, 1973), radioactive iodine uptake (Endo et al., 1978) and colloid droplet formation (Onaya et al., 1973), all known effects of TSH. Persistently present TSAb after treatment has been said to be useful in predicting relapse of the disease (Zakarija, McKenzie and Banovac, 1980; Karlson and Dahlberg, 1981).

Despite the initial findings that demonstrated normal B-cell proportions in patients with Graves' disease (Mulaisho, Abdou and Utiger, 1975; Calder, Irvine, Davidson and Wu, 1976; Lundell, Wasserman, Granberg and Blomgren, 1976), several investigators have recently described increased numbers of B-cells when detected by either EAE-rosette formation or specific monoclonal antibodies (Mori,

Amino, Iwatani, Kabutomori, Asari, Motoi, Miyai and Kumahara, 1980; Fournier, et al., 1983).

Apart from the humoral abnormalities described above, the involvement of cell-mediated immunity has also been suggested for the hyperthyroidism of Graves' disease. T-cell production of lymphokines in response to crude thyroid antigens (Lamki, Row and Volpe, 1973) and the mitogenic effect of thyroid membrane preparations on lymphocytes from patients with Graves' Disease support this belief (Makinen, Wagar, Apter, von Willebrand and Pekonen, 1978). As in Hashimoto's thyroiditis, alterations in the balance between suppressor and helper T-lymphocytes has been proposed as responsible for autoantibody production in Graves' disease. Again, results have been conflicting. Several reports point to defects in suppressor T-lymphocyte function in Graves' disease. Mitogen-triggered suppressor T-cell function was found to be impaired in these patients (Aoki, et al., 1979; Balazs, Leovey and Bordan, 1979). A good correlation between the clinical stage of the disease and the degree of T-cell dysfunction was described. Furthermore the defects were normalized after adequate treatment with anti-thyroid drugs. Inhibition of migration of T-cells from patients with Graves' disease in response to thyroid antigens also suggest antigen-specific defects in suppressor T-cell function (Okita, et al., 1981).

Regarding suppressor T-cell numbers, some authors have reported a decreased proportion of circulating suppressor T-cells relative to helper T-cells when tested with subset-specific monoclonal antibodies (Thielmans, et al. 1981; Sridama, et al., 1982). Contrary to these findings some authors have found suppressor T-cells to be normal both in number and function (MacLean, Miller, Brown and Reichlin, 1981). So far, however, most of the evidence accumulated supports the existence of suppressor T-cell dysfunction in Graves' disease. This dysfunction seems to be antigen-specific to the extent that lymphocytes from patients with autoimmune disorders other than thyroiditis have been able to correct the T-cell defects (Topliss, How, Strakosch, Lewis, Row and Volpe, 1982). Recently, subpopulations

of lymphocytes derived from patients with Graves' disease were enumerated using specific monoclonal antibodies. The authors found that a subset of inducer T-cells is diminished in these patients. Based on this, they hypothesized that the missing cells might be part of a subpopulation necessary in the activation of suppressor cells (Fournier, et al. 1983). Further studies will be necessary to confirm or refute this hypothesis. Defects in T-cell function and ratio in Graves' disease appear to be genetically linked, and possibly related to organic and emotional stress (Monjan and Collector, 1977).

Ophthalmopathy and dermopathy associated with hyperthyroidism appear to be closely related but independent immunological disorders. These 2 problems have also been considered to be autoimmune in origin and involvement of both cellular and humoral immunity have been suggested (Jacobson and Gorman, 1984).

It is very important to address the close relationships existing among the different autoimmune thyroid disorders. In all of them, alterations at the level of both humoral and cellular immunity have been demonstrated. However, a major problem that remains to be solved is the origin of the antigenicity of affected tissues. New findings seem to bring some light with regards to this problem. Recently, thyroid follicular epithelial cells from patients with autoimmune thyroid disorders were found to aberrantly express HLA-DR antigens (Hanafusa, et al., 1983). According to this hypothesis, increased DR antigen expression in thyroid epithelial cells will facilitate the presentation of local autoantigens to T-cells; certain T-cell subsets proliferate and regulate B-cell maturation into antibody-producing cells. These findings, however, have not excluded the possibility that the induction of aberrant DR antigen expression is secondary to the appearance of the autoimmune process.

1.4. IMMUNE-REGULATION VIA IDIOTYPIC-ANTI-IDIOTYPIC INTERACTIONS

Communication among the different components of the immune system is very important for the maintenance of an adequate balance among immunoregulatory pathways. This "cross-talk" is achieved through complex interactions that involve immuno-regulatory T-cells, their soluble mediators,effector T-cells, B-cells as well as immunoglobulin molecules produced by plasma cells (terminally differentiated B-cells) (Rowley, 1980; Miller and Schwartz, 1982).

T-cell/B-cell interaction and antigen recognition are dependent on specific cell membrane surface receptors found in these two types of cells. Both types of cells recognize the same antigens, however, the antigen domains that are identified appear to be different (Cone, 1981).

Most T-cells simultaneously recognize antigen and a self molecule coded for by genes of the Major Histocompatibility Complex. MHC restricted T-cell receptors have been recently identified with the help of highly specific antibodies (Haskins, Kappler, Marrack, 1984; Samuelson and Schwartz, 1983; Kaye, Porcelli, Tite, Jones and Janeway, 1983). Cellular products precipitated by these antibodies were analyzed. Disulfide linked proteins with molecular weights between 80,000 and 90,000 Da were identified and thought to constitute the receptor for antigen on MHC-restricted T-cells. Peptide mapping of these proteins has shown that this receptor consists of both constant and variable peptide regions (Acuto, Meuer, Hodgon, Schlossman and Reinherz, 1983).

The molecular genetics of the T-cell receptor for antigen has been approached by using substractive or differential hybridization techniques. In 1984 two independant groups of investigators published the first results describing T-cell specific cDNA clones that encode for proteins that have an extensive sequence homology with the light chain of the immunoglobulin molecule over the entire legth of the variable, joining and constant regions (Yanagi, Yoshikai, Leggett,

Clark, Aleksander and Mak, 1984; Hedrick, Cohen, Nielsen and Davis, 1984). Since that time a number of research groups have published data concerning the T-cell receptor. Most of these results have been recently reviewed by Kronenborg, Siu, Hood and Shastri (1986). In summary, the genes encoding for the α and β chains of the T-cell receptor and the γ gene have been cloned, their structure, organization as well as their patterns of rearrangement and diversification are now understood. Many similarities have been found between the α and β heterodimeric T-cell receptor and the immunoglobulin molecule, however, marked differences regarding the mechanisms of activation and antigen recognition are known to be present (Kronenberg, et al., 1986). The recently acquired knowledge of the structure of the T-cell receptor for antigen has still not provided with the answer as to how this receptor recognizes a foreign antigen in association with a MHC antigen. Reseach in this field continues, for now, this area remains controversial.

B-cells are stimulated by the helper subset of T-cells through the mediation of specific growth factors to differentiate into plasma cells or antibody producing cells. The basis for antibody diversity, which involves three clusters of genes (one heavy chain and two light chain clusters) derives from the re-arrangement of selectable somatic elements into functional immunoglobulin molecules of a given specificity (Leder, 1982).

Immunoglobulins (Igs) are Y shaped molecules formed by two identical light chains and two identical heavy chains, held together by disulfide bridges. All antibody molecules have regions that exhibit a constant amino acid sequence (constant regions of the heavy and light chains) and others that have heterogeneous amino acid sequences that correspond to the antigen combining sites (variable regions of the heavy and light chains). Amino acid sequences forming the constant regions of the heavy and light chains of the Ig molecules are each encoded by multiple genetic segments. The variable domain of the light chains is encoded by segments of two distinct genes, the V and the J genes. The

variable domains of the heavy chain are created by the combination of three types of germline genes, the V, D and J genes. These genes undergo multiple rearrangements resulting in the expression of the antibody diversity required for highly specific antigen recognition (Binz, Wigzell and Bazin, 1976; Kraig, Konenberg, Kapp, Pierce, Abruzzini, Sorensen, Samelson, Schwartz and Hood, 1983; Krammer and Eichmann, 1977). V_H and V_L chains form the antigen binding portion of the immunoglobulin molecules; as well as the receptors for antigen on the surface of B lymphocytes.

To explain the mechanisms through which the different groups of immunocompetent cells and their products interact, Jerne proposed the "Network Theory"(Jerne, 1974). He suggested that specific immunoregulation is controlled by a network of idiotypes and anti-idiotypes that exhibit complementary V-region structures. Prior to explaining this theory, it is important to define certain terms which were introduced by Jerne. Epitope was described as the antigenic determinant or haptenic site on an antigen molecule. Paratope is the "contact" area of the antigen-binding site of the antibody molecule. Idiotope (idiotypic determinant) is the epitope of an antibody molecule. Idiotypes are complexes of idiotopes. Most idiotypes require a correct combination of heavy and light chains for their expression. Anti-idiotypes are antibodies raised against the idiotope of an immunoglobulin molecule and they can be monoclonal or polyclonal in origin (Green, Flood and Gershon, 1983; Rajewsky and Takemori, 1983).

According to Jerne's theory, when a foreign antigen enters the organism it is recognized by antibody molecules (Ab1) and the clones of B-cells carrying the receptor for this antibody are expanded. The variable region of Ab1 presents certain idiotypes that are also recognized as antigenic and are capable of triggering the production of anti-idiotypes (Ab2). This reaction does not neccessarily stop there, idiotopes of Ab2 trigger the production of anti-anti-idiotopes (Ab3). Some Ab3 molecules are capable of interacting with the epitope that originated the first response (Figure 1.2). The ultimate role of such idiotypic

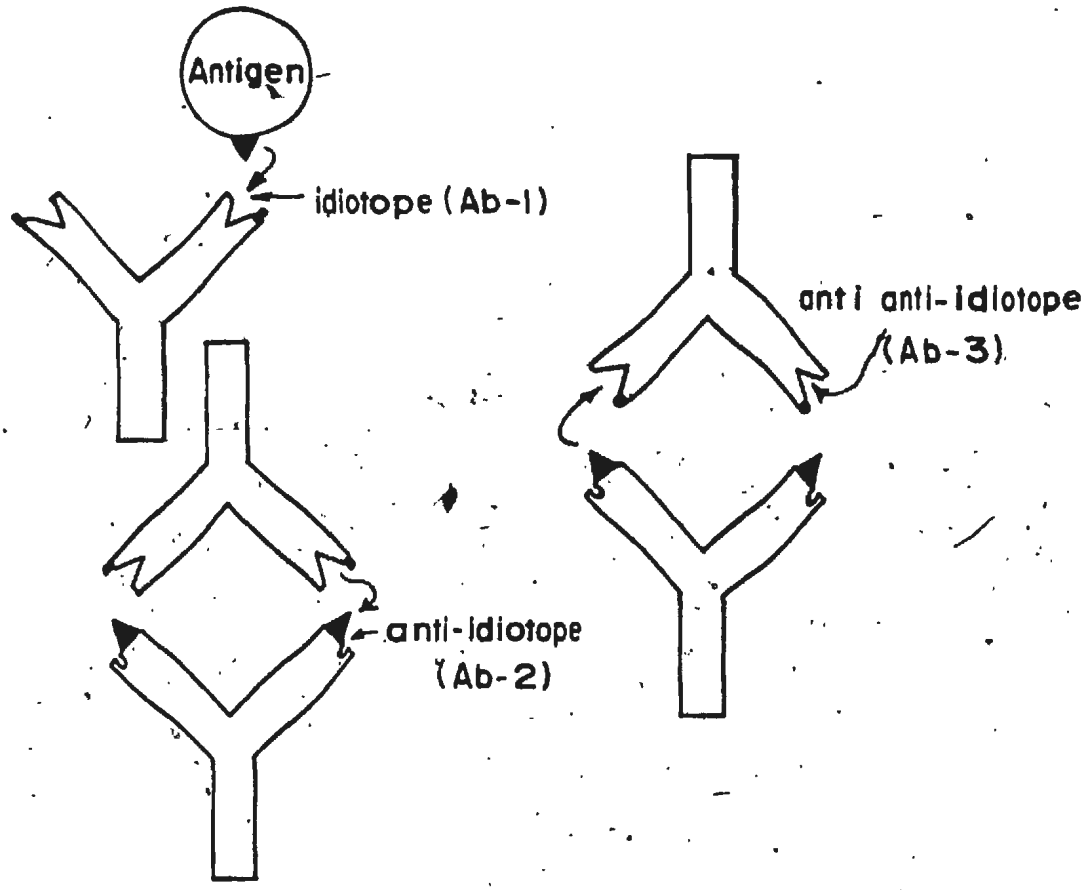


Figure 1-2: Schematic representation of the interaction of the Antigen/Idiotypic/Anti-idiotypic Networks. (Jerne, 1974)

modulation is to "down-regulate" the initial antibody response and establish a new "steady state". In this way antibodies and B-cells on whose surface these antibodies are expressed are subject to continual suppressive effects by complementary antibodies.

Antigen/idiotype and idiotype/anti-idiotype interactions are dependent on the tridimensional structures of their combining sites. If the tridimensional structure of the combining site of the idiotype "fits" the epitope as well as the anti-idiotype (Ab2), the anti-idiotype should inhibit the binding of the antigenic epitope to the idiotype. Such "anti-idiotypes" carry an internal image of the antigen. They are therefore called "internal image" anti-idiotypes.

T-cell membrane surface receptors allow these cells to recognize and respond to antigen stimulation. Several reports have shown that many of the known functional types of T-cells have idiotype-like structures on their receptors, and that these can trigger anti-idiotypic antibody responses (Janeway, Sakato and Eisen, 1975; Binz and Wigzell, 1977; Rajewsky and Eichman, 1977). These findings incorporate the whole T-cell system into the network regulation concept. Idiotypic determinants have been identified in both helper and certain classes of suppressor T-cells. Suppressor T-cells with anti-idiotypic determinants have now also been described.

B-cells seem to respond to antigen with two clusters of receptors, one that recognizes epitopes and the second active in mitogen recognition and in T-cell communication. These receptors carry idiotypic determinants and have also been identified as capable of triggering anti-idiotypic reactions (Bona, Pernis, 1984).

Many studies have clearly demonstrated the operation of immunological networks under experimental conditions. Fewer studies, however, have documented their importance in the normal regulation of the immune response.

In the last few years, evidence supporting regulation based on structural

characteristics within the immune system has become overwhelming (Rodkey, 1980). The experimental basis for the idiotypic network is well established. Support has further increased with the identification of naturally occurring network mediated regulation in several systems. It was first demonstrated that normal outbred animals have both the genetic and the biosynthetic capacity to mount auto-anti-idiotypic antibody responses specific for the individuals own previously synthesized antibody products (Rodkey, 1976). To avoid changes in antigenicity due to manipulation, a different approach was tested. This time the carbohydrate antigens of Micrococcus lysodeikticus were injected into rabbits. In a first round, anti-M.lysodeikticus antibodies (idiotypes) were detected. Several weeks later a second type of antibodies were detected, this time they were specific auto anti-idiotypic antibodies as determined by affinity chromatography (Brown and Rodkey; 1979). Other authors immunized rabbits with human serum albumin and human lactoferrin. Auto anti-idiotypic antibody producing cells were detected with both monovaleht and bivalent specifically purified and fluorochrome labeled idiotypes. The number of anti-idiotypic positive cells ranged from 0.7-44% in different animals (Jackson and Mestecky, 1979). Furthermore, after immunization with tetanus toxoid antigens, anti-idiotypic antibodies were detected in humans (Geha, 1982). It has also been shown that auto-anti-idiotypic antibodies and idiotype specific suppressor T cells can effectively suppress either idiotype synthesis by B-cells or even effector T cell functions (Rodkey, 1980).

A new and potentially useful experimental application for the principles of the "network theory" was developed by Sege and Peterson (1978). Antibodies raised in rats against insulin were affinity purified and injected into rabbits to raise anti-insulin anti-idiotypic antibodies. The expectations that these anti-idiotypic antibodies would be capable of recognizing the hormone receptor with a certain degree of specificity were tested. The anti-idiotypes were found to inhibit the binding of ^{125}I-insulin to its receptor, and to mimic insulin actions in mediating α-aminoisobutyric acid uptake by thymocytes. This was the first study to propose that anti-idiotypic antibodies could recognize a hormone receptor. This pioneering

work demonstrated the potential use of antihormone anti-idiotypic antibodies as anti-receptor specific antibodies. This initial lead, however, was not immediately followed because of the low bioactivity of the anti-idiotypes raised, and also because it was observed that normal immunoglobulin type G (IgG) was able to produce some of the insulin effects examined.

Further studies on other systems, β-adrenergic receptors (Schreiber, Couraud, Andre, Vray and Strosberg, 1980; Homcy, Rockson and Haber, 1982) and acetylcholine receptors (Wassermann, Penn, Freimuth, Treptow, Wentzel, Cleveland and Erlanger, 1982; Dwyer, Bradley, Kendrick Urquhart and Kearney, 1983) have confirmed this original concept. Applying the "network theory's" principles, a new experimental approach to the study of ligand-receptor interactions has been achieved.

1.5. OBJECTIVES

The principles introduced by the "network theory" of the immune system (Jerne, 1974) and the data accumulated demonstrating the potential use of anti-idiotypic antibodies as probes to study hormone-receptor interaction (Sege and Peterson, 1978; Schreiber, et al., 1980; Homcy, et al., 1982; Wasserman, et al., 1982; Dwyer, et al., 1983), opened new and interesting perspectives for a better understanding of some anti-receptor antibody mediated diseases.

Graves' disease represents a unique system, mainly because its etiological agent, the thyroid stimulating antibody (TSAb) is thought to be an anti-TSH receptor antibody (McKenzie and Zakarija, 1978. Kidd, et al., 1980) Despite the fact that TSAb has been found to produce biological responses similar to those of TSH, at the time that this study was initiated there was still no agreement as to whether Graves' disease antibody is a true anti-receptor antibody or is related to membrane components close to it.

It was challenging to apply the basic concepts of the network theory and

attempt to raise anti-TSH anti-idiotypic antibodies, one class of which might be "internal image" anti-idiotypic antibodies, representing "artifically raised" anti-TSH receptor antibodies. These antibodies could then, have the same properties as the naturally occuring antibodies found in Graves' disease.

This challenge was taken up by members of the Thyroid Research Laboratory at Memorial University of Newfoundland who began this study by raising suitable antibodies around December of 1979 (Islam, Pepper, Briones-Urbina and Farid, 1983). I became particularly interested in this field of research and started testing the bioactivity of the available anti-TSH anti-idiotypic antibodies. In starting this work it was difficult to foresee if such antibodies would recognize the TSH-receptor and, if they did so, to predict what type of effect would they have at this level, stimulatory or inhibitory. The first objective of this study, then, was to investigate if these antibodies could recognize the TSH receptor. If this testing was successful the next objective was to characterize the type of interaction that occurred and its consequences.

If, after careful study, it was concluded that anti-TSH anti-idiotypic antibodies were indeed capable of recognizing the receptor and also of activating receptor mediated biological responses, it would be considered appropriate to use this approach in investigating different characteristics of the hormone-receptor interaction.

TSH subunits are biologically inactive when dissociated (Pierce and Parsons, 1981). Thus it has been difficult to study their role in receptor recognition and cell activation. A novel experimental approach was considered theoretically possible to circumvent this problem. Internal image anti-idiotypic antibodies raised against antibodies specific for the α and β subunits of TSH should be capable of recognizing the TSH receptor either alone or in combination. The objective of this set of experiments was to obtain new information as to the role of each of the individual TSH subunits in interaction with their receptor.

Knowledge about the receptor structure and the mechanisms involved in the regulation of its biosynthesis would allow for a better understanding of the interaction of TSH and TSAb with the TSH receptor. The availability of antibodies capable of identifying the TSH receptor would permit the study of some of the factors involved in the control of the biosynthesis and turnover of the TSH receptor.

The aims of this study can, then, be summarized as follows:

1- To investigate if anti-TSH anti-idiotypic antibodies are capable of interacting with the TSH receptor.

2- If this interaction occurs, to study its nature, i.e. inhibitory or stimulatory.

3- If possible, to compare the biological responses produced by anti-TSH anti-idiotypic antibodies with those produced by TSH and the naturally occurring TSAb of Graves' disease.

4- To investigate the possibility of raising anti-TSH subunit specific anti-idiotypic antibodies capable of recognizing the TSH receptor.

5- To explore the possibility of using this antibody model in studying the participation of the subunits of TSH in the hormone-receptor interaction.

6- And finally, if these antibodies are demonstrated to be specific for the TSH receptor, to benefit from this characteristic to design a model that permits the study of the influence of TSH on the synthesis and turnover of its own receptor.

Chapter 2
MATERIALS AND METHODS

2.1. PREPARATION OF ANTI-TSH ANTI-IDIOTYPIC ANTIBODIES

Anti-TSH anti-idiotypic Abs were prepared as described by Islam, Pepper, Briones-Urbina and Farid (1983 b). Briefly, three 6 week old male Sprague-Dawley rats were immunized with 50 micrograms (μg) of purified hTSH(Union Chimique Belgique, Brussels, Belgium) in complete Freund's adjuvant (CFA)(Grand Island Biological Co., Grand Island, N.Y.). The animals received a booster injection, 3 weeks later, of 20 μg of hTSH in complete Freund's adjuvant (Gibco, Grand Island, N.Y.). Bleeding was carried out three weeks after the second injection. Immunoglobulins were isolated from serum and tested for their capacity to precipitate radiolabeled bTSH. Anti-TSH antibodies were purified in a TSH affinity column (TSH-Affi-gel 10)(Bio-Rad, Richmond, California)

Three New Zealand white male rabbits were immunized with 50 μg of the IgG eluted from the TSH affinity column in CFA. The animals were boosted with 30 μg of IgG after 3 weeks and bled 2 weeks after the second injection. IgG was prepared from these sera and extensively absorbed with glutaraldehyde-immobilized pooled Sprague-Dawley rat IgG (Fuchs and Sela, 1973) to remove isotypic and allotypic specific antibodies.

2.2. PREPARATION OF ANTI-TSH SUBUNIT-SPECIFIC ANTI-IDIOTYPIC ANTIBODIES

Monoclonal antibodies TS28, an IgM specific for the hTSH α subunit and GC73 an IgG specific for the hTSH β subunit were kindly provided by Dr. Jurag Ivanyi (Wellcome Research Laboratories, Beckenham, Kent, England). Briefly, these two monoclonal antibodies were prepared as described by Galfre, Milstein and Wright (1979) and Ivanyi and Davis (1980) by fusing spleen cells from Lou species rats immunized with hTSH with the Y3.Ag1.2.3. rat myeloma cell line.

In the present study four New Zealand White male rabbits were immunized with those antibodies. Two received 100 μg of IgM (TS28) and the other two 100 μg of IgG (GC73) in CFA (Gibco), injected in multiple subcutaneous sites. After one month, the rabbits were boosted every second week for 10 weeks with 50 micrograms of the specific monoclonal antibody in incomplete Freunds adjuvant (Gibco). Two weeks after the last injection the animals were exsanguinated. It is stressed that the difference in the immunization schedules in producing anti-TSH anti-idiotypic antibodies and in preparing anti-TSH subunit specific anti-idiotypic antibodies is due to the fact that the screening test that was being utilized for detecting the possible presence of anti-idiotypic antibodies (2.12) was negative when performed every two weeks. With every negative result a new booster injection was given. At the end of the fifth boost, depite the fact that our screening test was still negative, the animals were sacrificed and their serum processed for further testing. Immunoglobulins from preimmune serum as well as from immunized and control animals were obtained by precipitation at 0°C with half saturated ammonium sulphate (Heide and Schwick, 1973). The product was extensively dialysed against a buffer containing 200 mM TRIS HCl; 25 mM NaCl, pH 7.4; and passed through a rat immunoglobulin affinity column (Rat-Ig/Reacti-gel)(BioRia Laboratories, Montreal, Canada) to remove allotypic and isotypic specific antibodies.

2.3. SELECTION OF SUBJECTS

Ten patients with Graves' disease, seven patients with Hashimoto's thyroiditis and ten healthy controls were chosen to study the interaction of their Ig's with thyroid plasma membrane protein blots.

The criteria for the selection of Graves' disease patients included the presence of florid clinical features, a diffuse goiter,the absence of thyroid nodularity as verified by rectilinear scanning after the administration of ^{99}Tc, high 24h radioactive iodine uptake and elevated thyroid hormone levels as determined by radioimmunoassay. TSAb levels were not determined.

In the case of Hashimoto's thyroiditis, five of the seven patients had goiters. All of the seven patients also had high titers of anti-microsomal antibodies (1:1600 or higher) but were negative for anti-thyroglobulin antibodies in the standard haemagglutination test. All these patients were currently receiving oral thyroxine therapy and were euthyroid.

2.4. PREPARATION OF THYROID PLASMA MEMBRANES

Porcine thyroid tissue (approximately 40 g) obtained from the local abattoir was homogenized in 10 volumes of a buffer containing 10 mM TRIS HCl, 250 mM sucrose, 1 mM $MgCl_2$ and 1 mM EGTA, pH 7.4 (STEM) at 4°C with a Polytron Tissue Homogeneizer (Polytron Instruments Inc.) for a period of 2 min to cause tissue disruption. The homogenate was centrifuged at 4500 x g at 4°C for 5 min and the supernatant discarded. The pellet was resuspended in STEM and homogenization repeated for a period of 1 min. The product was filtered through a nylon mesh. The filtrate, mainly a single cell suspension on microscopic examination, was placed for 20 min in a nitrogen cell disruption bomb (Parr Instrument Co., Moline, Ill.) under constant stirring and at a pressure of 900 psi. At the end of the stabilization period rapid decompression resulted in cell rupture with an efficiency of 90-95%. The suspension was then successively centrifuged at 1000 x g and 4500 x g at 4°C for 10 minutes each time; the pellet was discarded

in both cases. The membrane fraction was recovered by centrifuging the above supernatant at 32000 x g for 30 minutes at 4°C.

Pellets containing the crude membrane fraction were resuspended in 21 ml of a buffer containing 55% sucrose in 10 mM Tris HCl, 1 mM $MgCl_2$ and 1 mM EGTA, pH 7.4 (TEM) by gentle homogenization with a glass, teflon pestle tissue grinder. The product was transferred in equal volumes into six 4 inch diameter, cellulose nitrate tubes (Beckman, Palo Alto, California). Sucrose solutions of 45%, 40%, 35% and 30% in TEM (approximately 8 ml each) were carefully layered on top of the membrane suspension. Centrifugation was carried out in a Beckman SW27 swinging rotor at 113,000 x g for 90 minutes at 4°C. Membrane bands at the interface between 30% and 35% and 35% and 40% were collected by aspiration with a Pasteur pipette and pooled. The recovered material was diluted with TEM to achieve a sucrose concentration of 250 mM and product was centrifuged at 113,000 x g for 30 minutes at 4°C. The final pellet containing the purified membranes was resuspended in STEM. Protein concentration was determined by the Folin phenol method (Lowry et al. 1951). Aliquots of 100 ul with protein contents between 400-500 ug were stored in liquid nitrogen. The plasma membrane preparations were assayed for 5'nucleotidase and NADPH cytochrome C reductase and examined under electron microscopy to assess their degree of purity. These procedures were kindly performed by Dr. N.M. Islam and the Department of Electron Microscopy, respectively, at the Health Sciences Centre, MUN, Newfoundland. Where human thyroid, plasma membranes were utilized the preparation procedure was identical. Human thyroid tissue was obtained at surgery.

2.5. DETERMINATION OF ADENYLATE CYCLASE ACTIVITY

Aliquots equivalent to forty micrograms of sucrose gradient purified thyroid plasma membrane protein were pre-incubated for ten minutes at 37°C with either b-TSH (USV Labs, Mississauga, Ontario),(100 to 250 mU/ml), anti-idiotypic

antibodies (200 and 250 μg/ml), monoclonal antibodies (250 μg/ml) or control immunoglobulins (200 and 250 μg/ml). A.C. was assayed in a final volume of 200 μl in 50 mM TRIS HCl pH 7.5 in the presence or absence of 10 uM guanosine-5'-{β'γ-imido} triphosphate (Gpp(NH)p)(Sigma Chem. Co., St. Louis, Missouri). The reaction was initiated by adding to the preincubated membranes equal volumes of an adenosine triphosphate (ATP) regenerating system containing 4 mM $MgCl_2$, 5 mM theophyllin, 4 mM ATP, 15 mM creatine phosphate (Sigma Chem. Co.) and 25 μg of rabbit muscle creatine phosphokinase (Sigma Chem. Co.)(Zor, Kaneko, Lowe, Bloom and Field, 1969). Incubation was carried out for 30 minutes at 37°C. After the incubation period was over, assay tubes were centrifuged at 32,000 x g for 15 minutes at 4°C. The supernatants were recovered and kept at 4°C for immediate cAMP determinations.

cAMP was measured using a protein binding assay (Gilman, 1970). Briefly, 10 μl of the above supernatant containing unknown amounts of cAMP were placed in 12 x 75 mm plastic culture tubes (Falcon Plastics, Oxnard, Canada) with 45 μg of equine muscle cAMP dependent protein kinase (Sigma Chemical Co.) and 10 pM ^3H cAMP (~40,000 cpm)(New England Nuclear, NEN, Boston, Mass.) The final assay volume of 200 μl was achieved by addition of a 50 mM sodium acetate buffer pH 4.0. Incubation was carried out for 2 hours at 4° C. At the end of this period 1 ml of 200 mM potassium phosphate pH 6.0 was added to each assay tube to stop the reaction. Bound and free cAMP were separated by collecting bound complexes on HATF 0.45 μm nitrocellulose filters (Millipore, Bedford, Mass.). Assay tubes and individual filter holders were washed three times with 10 ml each of cold 20 mM potassium phosphate pH 6.0 to remove unbound radioactivity. Filters were air dryed at room temperature and placed in scintillation vials with 1 ml of scintillation grade 2-ethoxyethanol (J.T. Baker Chemical Co., Phillipsbing, N.J.). After the filters were dissolved, 10 mls of Scinti-verse, a scintillation counting cocktail, (BDH Chemicals Ltd., Toronto, Canada) were added and the radioactive content was determined in a Beckman LS-330 counter (Beckman Industries). Efficiency calculated for ^3H was 64%. Counting errors between 1-5%.

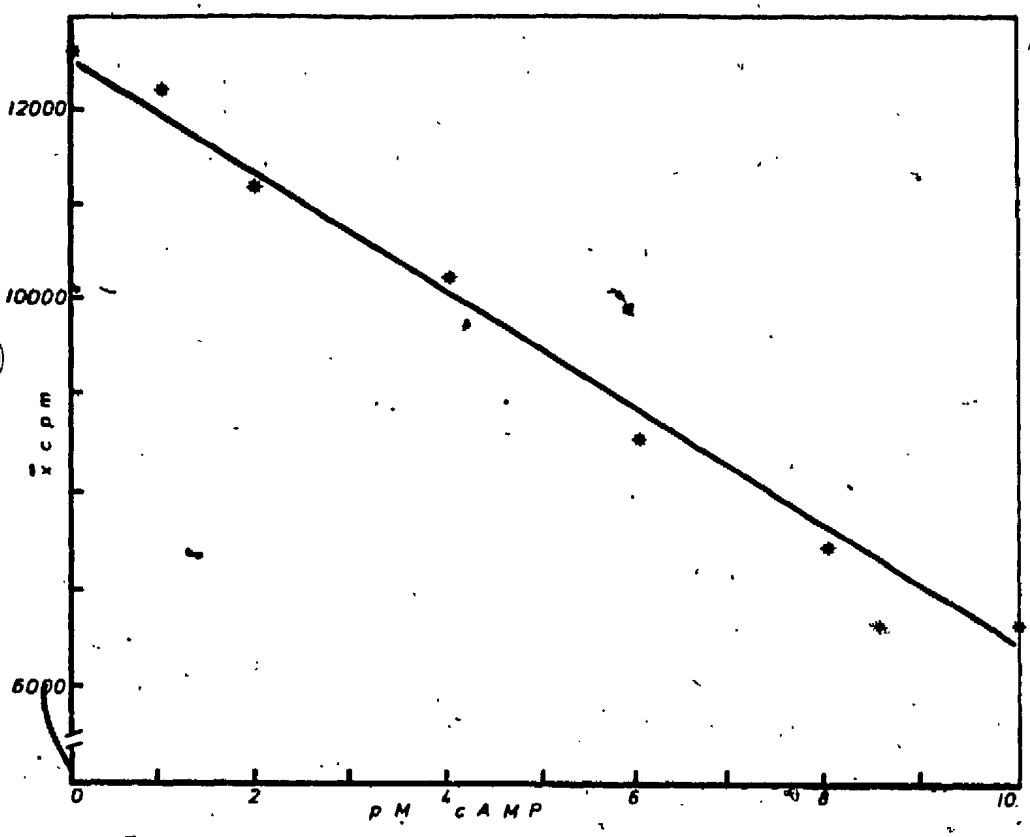
cAMP values were obtained by comparing experimental data with a contemporary standard curve prepared following similar methodology. A sample of a typical standard curve is included in figure 2.1. Known concentrations of cAMP (Sigma Chem. Co.) from 1 to 10 pM were included. Linearity of the standard plot was obtained within the ranges of 2 to 8 pM. It is important to mention that when 10 ul of the supernatant contained higher amounts of cAMP than those appropriate to compare with the linear part of the standard curve the sample was adequately diluted to achieve an adequate level and final value was later calculated accordingly.

When the effect of TSH subunit specific monoclonal antibodies or immunoglobulins from patients with Hashimoto's thyroiditis on TSH-driven A.C. was investigated, these antibodies (250 μg/ml) or normal rat or human immunoglobulins (250 μg/ml) as controls were preincubated for 10 minutes with thyroid membranes prior to the addition of TSH in one group of experiments. In other experiments test immunoglobulins were added simultaneously with TSH. The rest of the procedure continued as described above.

2.6. PREPARATION OF DISPERSED THYROID CELLS

Porcine thyroid tissue was obtained from the local abattoir and transported on ice. Fat and fibrous material were carefully removed, thyroid tissue was then sliced with a scalpel into approximately 0.5 mm fragments and washed twice in Hanks balanced salt solution (HBSS) (Flow Labs, McLean, Virginia). Five grams of tissue were transferred into a 125 ml capacity siliconized Erlenmeyer Flask and incubated with 2835 U of collagenase (Worthington Diagnostic Systems, Dublin, Va) dissolved in 10 mls of HBBS. Incubation was carried out for 30 minutes at 37°C in a shaking water bath at 120 strokes/min. in an atmosphere of 95% 0_2 and 5% $C0_2$. All subsequent incubation steps were carried out under similar conditions.

At the end of the first digestion step the supernatant was decanted and the



pM cAMP	$\bar{X}$cpm
0	12743
1	12433
2	11260
4	10127
6	8640
8	7612
10	6820

Figure 2-1: Sample of a typical cAMP standard curve.

$\bar{X}$cpm: corrected for background

TC: ~ 40,000 cpm

tissue washed twice with 50 ml HBSS. The partially digested material was further trimmed of loose fibrous material and second digestion step was started. Tissue was incubated for one hour with 3915 U of collagenase dissolved in 10 mls of HBSS. At the end of this period 10 mls of HBSS were added to terminate digestion by dilution. The product was filtered through a nylon mesh and the filtrate centrifuged at 200 x g for 10 minutes at room temperature. The cell pellet was washed twice by resuspending in 40 ml with HBSS and further centrifugation. The cells were resuspended with 40 ml HBSS containing 0.005% W/V deoxyribonuclease (Sigma Chemical Co.)and left for 10 min at room temperature. Incubation was terminated by centrifugation at 200 x g for 5 minutes. The cells were then washed with 40 ml of HBSS and pellet resuspended in Eagles' modified Minimum Essential Medium (MEM)(Flow Laboratories) containing 0.2% Na bicarbonate, 15% fetal calf serum (Flow Laboratories) and 1% antibiotic/antimycotic/kanamycin (Gibco) (MEM/FCS). Cell viability was determined at this stage with the trypan blue exclusion test and was demonstrated to be greater than 90%. Cell yield was approximately 2-4 x 10^7 cells per gram of thyroid tissue used.

Several experiments were carried out using a similar method but different balanced salt solutions. Krebs Ringer bicarbonate was used instead of HBSS but as the cell viability obtained with the latter proved to be superior, this procedure was adopted for further experimentation.

2.7. RADIO-IODINE UPTAKE BY DISPERSED THYROID CELLS

Radio-iodine uptake by dispersed thyrocytes was carried out using two methodologies. In the experiments that tested the biological activity of the TSH-anti-id, 2.5 x 10^6/ml cells in a volume of 1 ml in Krebs Ringer Bicarbonate solution were placed in 12 x 75 mm plastic tubes (Falcon Plastics, Oxnard, California) and incubated with bTSH (5, 10 and 50 mU/ml), TSH-anti-id (125-250 μg/ml) or NR-Ig (125-250 μg/ml). The incubation was carried out for periods of

10 minutes to 4 hours. At the end of those intervals 10^5 cpm of ^{131}I (NEN) were added per tube in the incubation carried out for 10 more minutes at room temperature. The tubes were then centrifuged at 200 x g for 10 minutes, the supernatant was removed and the cells were then washed twice with the Krebs Ringer Bicarbonate solution. The radioactive content of the pellet was then determined.

On later experiments a different methodology was used. This choice was made because it was observed that allowing the cells to plate and recover from the isolation procedures improved their viability. This method was used in testing the α-anti-id and β-anti-id antibodies. Dispersed thyroid cells, prepared as previously described, were allowed to plate at a concentration of 2 x 10^6/ml in MEM/FCS in 6 well tissue culture dishes (Costar, Cambridge, Mass) in a volume of 5 mls per well. Culture was carried out at 37°C in a humidified atmosphere of 95% air and 5% $C0_2$. After a 24 hour recovery period, plated cells were incubated for 4 hours with either bovine TSH (100 or 200 mU/ml), anti-idiotypic antibodies (250 or 500 μg/ml), NR-Ig (250 or 500 μg/ml) or control medium. At the end of this incubation period cells were carefully harvested by scraping with a rubber policeman. Cell number was determined with the use of a haemocytometer and the concentration adjusted to 1 x 10^6/ml in HBSS, all these were viable cells. One ml of cell suspension was allowed to react for 30 minutes at 37°C with 1 x 10^5 cpm of ^{131}I (NEN) in plastic 5 ml culture tubes (Fisher Scientific Co., Pittsburg, PA). Cells were then chilled to 4°C and washed twice with one ml of cold HBSS by centrifuging at 200 xg for 10 min and later resuspending by gentle agitation. The radioiodine content of the pellet was determined and percent uptake calculated. Counting errors were between 1-5% for the counts added and the time counted. Typical uptake values were of the order of 5-6 % per pellet. Control values were considered those obtained when the cells were incubated in control medium.

2.8. FOLLICULAR ORGANIZATION OF CULTURED THYROID CELLS

Thyroid cells were prepared as previously described, and resuspended in MEM/FCS at a concentration of 1×10^6/ml.

Culture was carried out for seven days in Lab-Tek 8 chamber tissue culture slides (Lab-tek Division, Miles Lab., Naperville, Ill). A volume of 0.4 ml of cell suspension containing 4×10^5 cells was delivered per well. Different final concentrations of either b-TSH (100 and 200 mU/ml), anti-idiotypic antibodies (250 and 500 μg proteins/ml) or control immunoglobulins (250 and 500 μg protein/ml) were included in 8 wells each to study the influence of these substances upon organization of the cultured cells. The medium was replaced once at day 4. At day 7 the Lab-Tek chamber cover and its plastic support base were carefully removed to avoid disturbance of the slide content. Slides were carefully washed by gentle immersion in phosphate buffered saline pH 7.2 and a cover slip was placed on top of plated cells.

Cultures were examined under a Zeiss Phomi II light microscope. Pictures were taken using a phase contrast 50x lens with or without a green filter and a 35 mm MPS55 (Wild) camera attachment.

For PAS stained specimens, cell monolayers were rinsed as before and fixed for 18-20 h at 4° C in glutardyaldehyde-picric-acid-acetic acid (GPA: 99% of a mixture of 1 part 25% glutarydyaldehyde (Merck Laboratories, Darmstadt, Germany) and 3 parts saturated picric acid (BDH Chemicals Ltd., Toronto, Canada) mixed with 1% of glacial acid (Fisher Scientific Limited, Fair Lawn, N.J.). Cells were then washed successively in 80%, 70% ethanol and distilled water and subsequently stained with a PAS staining kit (PAS; Harleco, EM Industries, Inc., Gibbstown, N.J.). Other samples were stained with both PAS and haemalum to obtain a better visualization of the nuclei.

2.9. RADIO-IODINATION OF THYROTROPIN AND ANTIBODY PREPARATIONS

Ten micrograms of highly purified bovine thyrotropin (gift of Dr. John Pierce, UCLA)(~ 30 IU/mg)(Table 2.1) or 25 μg/protein of different immunoglobulins (Anti-idiotypic antibodies, TSH subunit specific monoclonal antibodies and control rat or rabbit immunoglobulins) were radio-iodinated with ^{125}I using the lactoperoxidase method (Thorell and Johansson. 1971). Labelled proteins were separated from free iodine on Ultrogel AcA54 columns (Reactifs IBF, LKB Labs, France) by eluting with a buffer containing 20 mM Tris HCl, 25 mM NaCl and 0.1% bovine serum albumin (BSA), pH 7.4. ^{125}I human TSH was purchased from Nuclear Medical Laboratories (Irving, Texas), specific activity was 50 μCi/mg.

2.10. ^{125}I-bTSH BINDING TO DISPERSED THYROCYTES

Isolated thyrocytes were cultured for 24 hours after preparation at a concentration of 2 x 10^6/ml in MEM/FSC. After the recovery period was over b-TSH (USV Lab)(100 mU/ml) was added to the culture medium of two different groups of cells. Group 1 was incubated in the presence of TSH for a period of 3 hours and group 2 for a period of 15 hours. A third group did not receive TSH and was kept as control.

At the end of these incubations cells were gently collected with a rubber policeman and subsequently centrifuged at 200 x g for 5 minutes at room temperature. Cell pellets were resuspended in HBSS at a concentration of 1x 10^7 cells/ml.

Three hundred-μl of the above cell suspension (3 x 10^6 cells) were allowed to react with ~ 100,000 cpm (~ 2.5 pM) of ^{125}I-bTSH in the absence or in the presence of 50 to 500 mU/ml of native bTSH (USV Labs) for 30 minutes at 37°C in a total final volume of 500 μl in HBSS in 1.5 ml polypropylene conical bottom micro test tubes (Brinkmann Instruments, Inc., Westbury, N.Y..) At the end of

Table 2-1: Equivalences of the different TSH preparations used.

Preparation	Source	mg Protein	I.U.*
bTSH	UCLA (Pierce)	1 mg	30
bTSH	USV Labs (Thytropar)	1 mg	1**
hTSH	NIADDK	1 mg	1.5***

* International Unit: Activity compared to the International Standard: International Laboratory of Biological Standards, Holly Hill, London, England.

** Thyrotropin activity tested using the method of chick thyroid iodine depletion assay of Piotrowsky, Steelman and Koch (1953) and compared to the international standard.

*** Biopotency compared to the World Health Organization human TSH standard 68/38 from the International Laboratory of Biological Standards, Holly Hill, London, England.

the incubation, samples were centrifuged for 1 minute in a Beckman Microfuge (Beckman Instruments) at room temperature the supernatant was aspirated, and the radioactive content of the pellet determined in a Beckman Gamma-300 counter (Beckman Instruments). Calculated efficiency for the counter for ^{125}I was 74%. Counting errors were between 1-5% for the number of cpm's added and the time counted.

2.11. INTERACTION OF ^{125}I-ANTI-IDIOTYPIC ANTIBODIES WITH THYROID PLASMA MEMBRANES

For the experiments testing TSH-anti-id one hundred micrograms of sucrose gradient purified porcine thyroid plasma membrane in 50 μl of 25 mM NaCl, 20 mM TRIS-HCl pH 7.4: binding buffer (BB) were incubated for 30 minutes at 37°C with ~20,000 cpm (~5 ng/protein) of radiolabeled anti-idiotypic antibodies or radiolabeled control rabbit immunoglobulins in the absence or in the presence of either bTSH at concentrations of 40 to 200 mU/ml, native anti-idiotypic antibodies (50-200 μg/ml) or control immunoglobulins (50-200 μg/ml).

For the experiments testing the binding of radiolabeled α-anti-id and radiolabeled β-anti-id to porcine thyroid plasma membrane ~50,000 cpm (~10 ng/protein) of labeled antibodies were reacted with 100 μg of purified membrane, in similar volume of BB as above, in the absence or in the presence of 250, 500 or 1000 mU/ml of unlabeled TSH or 500 or 1000 μg/ml of unlabeled α-anti-id or β-anti-id. The final assay volume was 200 μl. After incubation, tubes were centrifuged at 32,000 x g for 10 minutes at 4°C. The supernatant was aspirated and the radioactive content of the pellet determined. Counting errors between 1-5% for the number of counts present and the time counted.

2.12. EFFECT OF ANTI-IDIOTYPIC ANTIBODIES ON THE BINDING OF RADIOLABELED TSH TO THYROID PLASMA MEMBRANE

One hundred micrograms of purified porcine thyroid plasma membrane in 50 μl of BB were incubated with ~0.5 pM of ^{125}I-bTSH (~20,000 cpm) in the absence or in the presence of different concentrations of either native bTSH (50-250 mU/ml), anti-idiotypic antibodies (250, 500 and 1000 μg/ml) or control immunoglobulins (500, 1000 μg/ml) for 30 minutes at 37 °C. The assay was carried out in BB in a final volume of 200 μl. At the end of the incubation period the samples were centrifuged at 32,000 x g for 10 minutes at 4°C. The supernatant was aspirated and the radioactive content of the pellet determined. Counting errors were between 1-5%.

2.13. BINDING OF RADIOLABELED THYROTROPIN TO TSH-SUBUNIT SPECIFIC MONOCLONAL ANTIBODIES

One hundred micrograms per milliliter of each monoclonal antibody (TS28 or GC73) were preincubated for 14 hours at 4 °C or 30 minutes at 37 °C in the absence or in the presence of either 50-200 mU/ml of bTSH or 125, 250 or 500 μg/ml of the respective anti-idiotypic antibodies. ^{125}I-TSH [bovine (bTSH)(~20,000 cpm, ~0.5 pM per tube)(~100,000 cpm/ml ~ 2.5 pM/ml)] or human (hTSH) (~20,000 cpm, ~ 0.48 pM per tube)(~100,000 cpm/ml ~ 2.4 pM/ml) was added to samples and incubated at 37 °C for 30 minutes. The final assay volume was 200 μl and the concentrations expressed represent those obtained in the final volume. In another group of experiments identical quantities of monoclonal antibodies were incubated for 30 minutes at 37 °C with radiolabeled hTSH in the presence or absence of native human TSH (1.5 IU/mg)(kindly donated by NIADDK, Baltimore,Maryland) (Table 2.1) at concentrations varying from 250 μU/ml to 25 mU/ml. All assays were carried out in BB in a final volume of 200 μl. At the end of the incubation periods bound and free TSH were separated by adding 1 mg of bovine gamma globulin in 50 μl of 1

M NaCl and 250 µl of polyethylene glycol 6000 to obtain a final concentration of 12 %, followed by centrifugation at 32,000 x g for 10 minutes at 4°C. The supernatant was aspirated and the radioactive content of the pellet determined. Counting errors for these experiments were also between 1 to 5 %.

Titration experiments were carried out to search for the ideal concentrations for polyethylene glycol to obtain an adequate precipitation of the TSH-antibody complex vs. increasing the background counts. The concentration of 12% was chosen after careful comparison between these two parameters. At higher concentrations no better precipitation was obtained but the background counts were highly increased, hence the 12% concentration was used.

2.14. INTERACTION OF RADIOLABELED MONOCLONAL ANTIBODIES WITH ANTI-IDIOTYPIC ANTIBODIES

Radiolabeled monoclonal antibodies (~50,000 cpm, ~15 ng) in 100 µl or radiolabeled control rat immunoglobulins containing mostly IgG but also IgM (N rat Ig)(~50,000 cpm, ~15ng) in 100 µl were incubated in the presence of specific anti-idiotypic antibodies (100-1000 µg/ml) in 100 µl or similar concentrations of control rabbit immunoglobulins (NR Ig) also in a volume of 100 µl for 60 minutes at 37°C. The reaction was carried out in a total final volume of 200 µl. At the end of the incubation period bound and free antibodies were separated by adding 1 mg of bovine gamma globulin in 50 µl of 1 M NaCl and 250 µl of polyethylene glycol 6000 to obtain a final concentration of 12 %. This was followed by centrifugation at 32,000 x g for 10 minutes at 4°C. The supernatant was then aspirated and the radioactive content of the pellet determined.

2.15. PORCINE THYROID PLASMA MEMBRANE PROTEIN FRACTIONATION BY SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Polyacrylamide gel electrophoresis was carried out using the discontinuos buffer system described by Laemmli (1970). Briefly, stock solutions containing 30% w/v acrylamide (Bio-Rad Labs., Richmond, California) and 0.8% w/v N-N'bis-methylene acrylamide (Bio-Rad Labs) in deionized water were prepared. Acrylamide solutions at concentrations of 7.5% and 15% (used in experiments described in 3.1.7, 3.2.1 and 3.2.2) or 5% and 15% (used in all experiments in section 3.4). were made using the stock solution and a buffer containing 0.375 M Tris-HCl pH 8.8 with 0.1% sodium dodecyl sulphate (SDS). To achieve polymerization 0.05% or 0.025% v/v respectively of tetramethylethylenediamine (Eastman Kodak Co., Rochester, New York) and 0.25% w/v of ammonium persulfate (BDH Chemicals, Toronto, Canada) were added. 13 x 14 x 0.3 cm slab linear gradient gels were prepared by using a gel gradient mixer (Hoefer Scientific Instruments , San Francisco, California). The stacking gels 5% and 4% respectively, contained 0.125 M Tris HCl, pH 6.8 and 1% SDS. The electrode buffer contained 0.025 M Tris base and 0.192 M glycine, pH 8.3 , with 0.1% SDS.

Protein samples containing 100 μg in 100 μl of sucrose gradient purified thyroid plasma membranes in a buffer with 0.0625 M Tris-HCl, pH 6.8, 3% SDS, 10% glycerol in the presence or absence of 5% β-mercaptoethanol were thoroughly mixed and placed in boiling water for 5 minutes. After the samples had cooled off, 0.01% bromophenol blue was added as tracer dye. The samples were placed in individual slots in the stacking gel and electrophoresis carried out at 200 volts until the tracer dye reached approximately 0.5 cm from the bottom of the gel, around 5 to 6 hours at 4°C. To determine the molecular weight of the resolved proteins the following standards were included: myosin (Mr=205,000)(Sigma Chem. Co.), ferritin monomer (Mr=220,000), phosphorylase-B (Mr=94,000), bovine serum albumin (Mr=67,000), carbonic anhydrase (Mr=30,000), soybean

trypsin inhibitor (Mr= 20,000) and α- lactalbumin (Mr=14,000) (Pharmacia Fine Chemicals, Piscataway, New Jersey).

2.16. ELECTROPHORETIC TRANSFER OF PROTEINS FROM SDS-PAGE ON TO NITROCELLULOSE PAPER (PROTEIN BLOTS)

The protein blotting procedure was slightly modified from that previously described (Towbin, Staehalin and Gordon, 1979; Burnette, 1981). A "sandwich" was prepared using the following successive layers: the lower side of a transfer plastic cassette (Hoefer Scientific), an electrotransfer porous polyethylene sheet, 3 mm thick (Hoefer Scientific), one sheet of blotting filter paper (BioRad Lab), a 13 x 14 cm sheet of BA-85 0.45 μm nitrocellulose paper (Schleicher and Schuell, Keene, New Hampshire). The SDS gel was carefully placed on top of the nitrocellulose and another sheet of blotting paper was added, finally the upper side of the transfer casette was replaced and secured. This procedure was carried out with the layers submerged in a buffer containing 20 mM Tris base, 150 mM glycine and 20% v/v methanol (transfer buffer) at room temperature.

The "sandwich" was then placed in the chamber of a TE 42 Transfor electrophoretic unit (Hoefer Scientific Instruments) previously filled with transfer buffer, to run at 400 mA for a period of 14 hours at 4°C. Transfer of polypeptide bands was more than 80% efficient as measured by comparing the intensity of the staining of the protein bands in the polyacrylamide gels prior to transfer and post transfer with the use of a densitometer.

2.17. INTERACTION OF TSH, HUMAN AND RABBIT IMMUNOGLOBULINS WITH THYROID PLASMA MEMBRANE PROTEIN BLOTS

To test TSH binding, thyroid plasma membrane protein blots were prepared as described previously (2.16). The paper was first washed twice with buffer containing 20 mM tris HCl, pH 7.4, at room temperature. Blocking of unreacted

sites was carried out with 3 % bovine serum albumin (BSA) in 20 mM Tris HCl, pH 7.4 (BSA-blocking). Blocked papers were incubated with bTSH (USV laboratories) at a concentration of 1 U/ml diluted in BSA-blocking for 4 hours at 4°C in a total volume of 5 ml. At the end of this incubation, papers were briefly washed and anti-hTSH antibody (raised in rabbit)(Bio-RIA Laboratories) at a dilution of 1:30,000 in a final volume of 5 ml was added and maintained for 14 hours at 4°C. Unreacted antibody was then removed by washing with several changes of 40mM Tris HCl, 200mM NaCl, pH 7.4 (TBS). A second blocking procedure was carried out using 10 % horse serum, 3 % BSA in 5 ml of TBS (Blocking reagent). Peroxidase conjugated anti-rabbit IgG (Kirkegaard and Perry Laboratories Inc., Gaithersburg, Maryland) at a dilution of 1:500 in 5 ml of blocking reagent was added and maintained for 4 hours at room temperature. After this period unbound peroxidase labeled antibody was removed by repeatedly washing with TBS. Development was carried out with 0.05% w/v 4 chloro- 1 naphthol (Aldrich Chemical Co., Milwaukee, Wis.) and 0.33% hydrogen peroxide (BDH chemicals) in 5 ml of TBS. This solution was applied and maintained for 15 minutes at room temperature.

To test antibody binding to protein blots, papers were briefly washed with TBS. Unreacted sites were blocked using blocking reagent. Test immunoglobulins; either human from patients with Hashimoto's thyroiditis or Graves' disease or rabbit (anti-idiotypic antibodies), and control immunoglobulins (normal human or rabbit) were diluted to concentrations of 1 and 2 mg/ml in blocking reagent and incubated with blotted nitrocellulose papers for 14 hours at 4°C in a total volume of 5 ml. Apart from the fact that peroxidase conjugated anti-immunoglobulins used were species-specific, all further steps were as described above.

When displacement of antibody binding by TSH was investigated, nitrocellulose paper bands were reacted in presence of bTSH as mentioned above and after briefly washing, test immunoglobulins (Hashimoto's, Graves' or anti-idiotypic immunoglobulins) were incubated for 14 hours at 4°C as previously described. All other steps were identical to those described above.

2.18. BIOSYNTHETIC LABELING OF DISPERSED THYROCYTES

Thyrocytes, prepared as previously described (2.6), were adjusted to a concentration of $2x10^6$ /ml in MEM/FCS (all viable cells) and delivered into 6 well tissue culture dishes (Costar Laboratories) in a volume of 5 ml per well, and cultured for 24 hours in a humidified atmosphere of 95 % air and 5 % CO_2 at 37°C (conditions that were maintained throughout) to allow recovery from dispersion procedures. To study the rate of synthesis of the TSH receptor and the influence of TSH upon it, b-TSH was added to the culture medium of "test" wells at different concentrations (50-500 mU/ml) for 12 hours. Control wells received no TSH. To study the biosynthetic labeling, culture medium was first removed from stimulated and control cells avoiding disturbance of the cell monolayer. The monolayer was then carefully washed with leucine free medium (Flow Laboratories), containing 5 % FCS, 0.2 % sodium bicarbonate and 1 % antibiotic/ antimycotic/ kanamycin (Gibco) (labeling medium). Subsequently 40 μCi of ^{3}H leucine (NEN) in 2 mls of labeling medium were delivered per well and maintained for periods ranging from 15 minutes to 8 hours. After labeling cells were chilled at 4°C, carefully scraped off with a rubber policeman and washed with cold HBSS for three times by resuspending and pelleting. Four samples of cells were analyzed for each period of time and for each one of the different conditions investigated. The final cell pellets were stored frozen at -70°C until analyzed.

To investigate the turnover rate of the receptor, four groups of thyrocytes were cultured under similar conditions as those described above. Groups 1 and 2 received TSH enriched medium (100 mU/ml) for 12 hours prior to labeling, the other two were incubated in control medium. Biosynthetic labeling was carried out as before, with 40 μCi of ^{3}H- leucine in labeling medium for a period of 3 hours, recognized from previous experiments to be the optimal labeling time. Groups 2 and 3 were labeled in the presence of bTSH (100 mU/ml), while groups 1 and 4 were labeled in the absence of TSH. After 3 hours the radioactive medium

was removed. The monolayers were carefully washed with 5 mls of MEM/FCS at room temperature. Five ml of leucine containing MEM/FCS warmed at 37°C were then dispensed per well to start the cold chase which was continued for different periods of time (from 15 minutes to 96 hours). Group 4 received TSH (100 mU/ml) during the cold chase while the other groups were chased in absence of TSH. At the end of the cold chase periods the cells were chilled to 4°C, scraped, washed three times with chilled HBSS by resuspending and pelleting and the final pellet was stored frozen at - 70 °C until analyzed.

2.19. IDENTIFICATION OF RADIOLABELED RECEPTOR BANDS

Pellets of previously biosynthetically labeled thyrocytes (2.18) were lysed with 100 µl of 1.5 % SDS in 62.5 mM Tris HCl, pH 6.8 and 10 % glycerol. Samples were vigorously vortexed and placed in boiling water for 5 minutes followed by centrifugation at 32,000 x g for 10 minutes at room temperature to remove any non-dissolved material. Protein content was determined using a trichloro acetic acid (TCA) precipitation method, with which SDS is known not to interfere (Schaffner and Weissman, 1973). One hundred µl of the cell lysate, containing approximately 200 µg of protein, were resolved on 5-15 % slab linear gradient gel, using the discontinuous buffer system of Laemmli previously described (2.15). Proteins on SDS-PAGE were electrotransferred onto nitrocellulose paper as described above (2.16).

Using the enzyme-linked immuno-binding assay described before, protein blots were incubated with anti-idiotypic antibodies known to be capable of recognizing the TSH holoreceptor protein band as well as several breakdown products. The polypeptide band corresponding to the TSH holoreceptor and other unrelated bands were carefully cut. Nitrocellulose pieces were placed in scintillation vials and 1 ml of scintillation grade 2- ethoxyethanol (T.J. Baker Chemical Co.) was added. When the papers were dissolved, 10 mls of ScintiVerse universal scintillation cocktail (Fisher Scientific Limited, Fair Lawn, N.J.) were added and the radioactive content of the samples determined.

2.20. DETERMINATION OF THE ^{3}H-LEUCINE POOL IN BIOSYNTHETICALLY LABELED THYROCYTES

Dispersed thyroid cells at a concentration of 2 x 10^6 /ml were allowed to plate in 6 well tissue culture dishes (Costar Labs.) for 24 hours in a volume of 5 mls/well in MEM/FCS. Subsequently they were incubated for 12 hours in either bTSH (100 mU/ml) containing medium (group 1), or in control medium (groups 2 and 3). At the end of this period medium was removed from all three groups and the cell monolayers were carefully washed with labeling medium. Subsequently the cells were incubated with 40 μCi of ^{3}H leucine in 2 mls of labeling medium per well for a period of 3 hours. Groups 1 and 2 were labeled in the presence of bTSH (100 mU/ml) and group 3 was labeled in control medium. Four replicates were studied in each group.

When labelling was over, cells were chilled at 4°C, carefully scraped from the dishes and washed with 5 mls of chilled phosphate buffered saline (PBS) (Gibco) three times by resuspending and pelleting. The final pellet was resuspended in 0.1 ml of PBS and 0.4 ml of 1 N NaOH was added, the samples were mixed by vortexing and then heated at 56°C for 10 minutes. Subsequently 0.1 ml of a solution containing 2.5 mg/ml of bovine serum albumin (BSA) in water and 2 mls of 10 % TCA were added to each sample, the solutions mixed by vortexing and incubated for 15 minutes at 4°C. When incubation was over the samples were centrifuged at 2000 x g for 10 minutes at room temperature and the supernatants, containing the free amino acids, saved. The pH of the supernatants was adjusted to 2.2 using 3 N LiOH. One ml of each of the samples was dispensed into a 5 ml plastic culture tube and 1 ml of lithium citrate buffer pH 2.2 was added to stabilize the pH. The leucine content was determined using a Beckman 121 Amino acid analyzer (Beckman Co.). The column eluate was collected every 2 minutes (2.5 ml fractions) and later counted to determine the radioactivity of the leucine peak. The radioactive leucine contents of the 3 groups of cells investigated were calculated and compared. The analysis was kindly performed by Mr. D. Hall of the Biochemistry Department at Memorial University of Newfoundland.

2.21. PREPARATION OF THYROGLOBULIN PROTEIN BLOTS

Protein blots were prepared by first resolving 15 μg of commercially available porcine thyroglobulin (Sigma Chemical Co.) by polyacrylamide gel electrophoresis under non-reduced conditions as previously described (2.15). The protein bands were later transferred on to nitrocellulose paper under similar conditions as those used to prepare protein blots of thyroid plasma membranes (2.16).

2.22. ANALYSIS OF THE DATA

Where statistical comparisons were made, the nonparametric Mann-Whitney U test was applied (Siegel, 1956). The p value was considered significant at values equal to or less than 0.05. Binding affinities were detemined by Scatchard analysis (Scatchard, 1949).

Chapter 3
RESULTS AND DISCUSSION

3.1. ANTI-TSH ANTI-IDIOTYPIC ANTIBODIES

3.1.1. Displacement of binding of ^{125}I-bTSH to thyroid plasma membranes by anti-TSH anti-idiotypic antibodies

Anti-TSH anti-ids raised as previously described (2.1) were tested for their capacity to inhibit ^{125}I-bTSH from binding to thyroid plasma membranes. These tests were used as an initial screen to detect any competitive activity that these antibodies may have with TSH at the TSH receptor level.

Preliminary experiments studied the interaction of different concentrations of the porcine thyroid plasma membrane (50-1000 μg/ml) with increasing amounts of radiolabeled TSH (50,000- 5'000,000 cpm/ml). At 500 μg/ml with the addition of 100,000 cpm/ml approximately 11% of the radioactivity was bound. A dose response increasing in the amount bound was obtained up to 250,000 cpm with a maximal binding of approximately 14%. Higher cpms only increased the background counts. Higher amounts of membrane produced a relative increase in the number of counts bound but the overall percentage of binding did not increase (Bako et al. personal communication).

Sera from 2 out of 3 rabbits immunized with anti-TSH antibodies (Id-IgG) were found to be able to inhibit ^{125}I-bTSH from binding to both porcine and human thyroid plasma membranes under similar experimental conditions (2.12). Porcine thyroid membrane bound 11% of the ^{125}I-bTSH added, while human membrane

bound 24% of the ^{125}I-bTSH added. TSH-anti-id caused a dose dependant inhibition of this binding. At a dose of 200 µg/ml, 38% of the radioactivity bound to human thyroid plasma membranes was displaced, 52% of the radioactivity bound to porcine membranes was displaced, NRIg at the same dose only displaced 17% of the radioactivity bound to porcine thyroid membranes. It was therefore observed, that there was adequate dose dependent inhibition of ^{125}I-bTSH binding by TSH-anti-id in both porcine and human thyroid plasma membranes. Being as porcine membranes were more readily available this system was utilized for further experiments. (Fig 3.1).

Scatchard analysis of these interactions yielded a curvilinear plot (Islam et al., 1983 b). This appears to suggest either that there is more than one binding site occupied by the "Internal Image" antibodies or that there are antibodies that bind to the receptor with different degrees of affinity. It is also important to point out that the amount of antibody preparation required to produce up to 52% displacement of the radioactive label bound to the porcine thyroid plasma membrane is rather large. This is most likely indicative of the small number of molecules that truly represent the "Internal Image" type antibodies with the capability of binding to the receptor and displacing its natural ligand. As the antibody preparations were polyclonal in origin, it was expected that there should be antibody molecules directed to other antigenic epitopes on the TSH molecule different from the binding site specific for TSH. Other experimental studies using anti-idiotypic antibodies have also encountered that only a small percentage of antibody molecules in the preparation were found to be "internal image" antibodies (Strosberg, Couraud and Schreiber, 1981).

The K_D of the high affinity site to which TSH-anti-id binds was assigned a value of 7.0×10^{-9}M. This value is similar to that of ^{125}I-bTSH (1.3×10^{-10}M) suggesting that the fraction of immunoglobulin bound to the membrane is likely attached to the TSH-R.

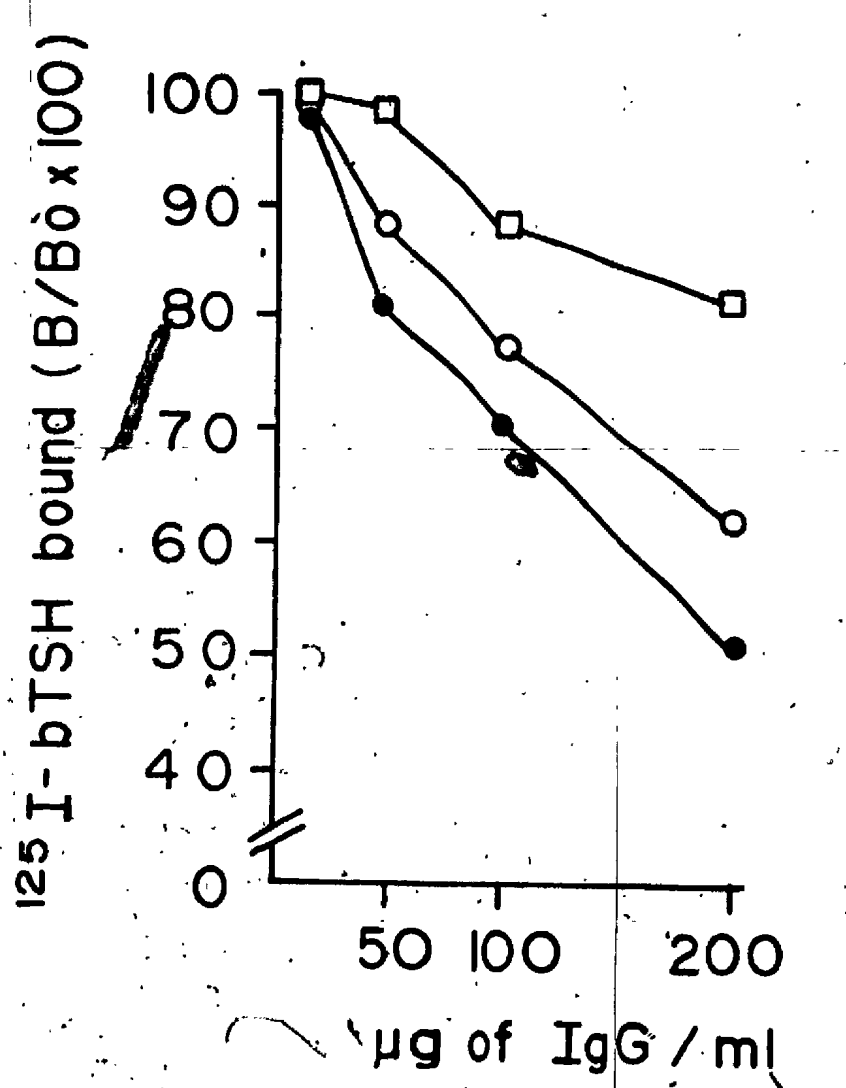


Figure 3-1: Inhibition of ^{125}I-bTSH binding to thyroid plasma membranes by TSH-anti-id.

(□) NR-Ig on porcine membranes (preimmune serum).

(O) TSH-anti-id on human membranes.

(●) TSH-anti-id on porcine membranes.

Bo = ^{125}I-bTSH counts bound to thyroid membrane in absence of TSH-anti-id.

B = ^{125}I-bTSH counts bound to thyroid membrane at a given dose of TSH-anti-id.

N = 3 (3 parallels for each time).

The 2 sera causing inhibition of ^{125}I-bTSH binding to thyroid plasma membranes were pooled and used for further experiments.

3.1.2. Interactions between idiotypic and anti-idiotypic antibodies

Next, it was imperative to establish the antigen specificity of the competition between the first antibody an anti-TSH (Id-IgG) with the second antibody an anti-anti-TSH (TSH-anti-id).

The interaction of Id-IgG with radiolabeled TSH-anti-id was investigated. Approximately 30-35 μg of Id-IgG were able to precipitate more than 90% (Mean: 94%, SD 2.1) of the radiolabeled anti-id (20,000 CPM $\sim$5 μg). Subsequently the influence of TSH-anti-id on the interaction of Id-IgG with TSH were tested. Id-IgG at a dose of 30 μg bound 50-65% of the ^{125}I-bTSH (10,000 cpm) added to the system. It was rather surprising that up to 30 μg of Id-IgG were required to precipitate approximately 50-65% of the ^{125}I-bTSH. The preliminary experiments had demonstrated that 5 μg precipitated approximately 17%, 10 μg 23%, 20 μg approximately 36% while 40 μg only precipitated 71% of the radiolabeled TSH (Islam, M.N., personal communication). Linearity in the dose response curve was observed up to 30 μg. Several explanations can be thought of for requiring these high doses of antibodies. It could represent incomplete precipitation of antibody complexes with the method used, low affinity of the Id-IgG for TSH or low numbers of actual anti-TSH molecules in the preparations used. As these experiments were more designed to observe the behavior of these different preparations in competitive protein binding assays that to establish a mol to mol interaction of the antibodies this matter was not pursued further. For further experiments 30 μg of Id-IgG were used, this amount precipitated 50% of the radiolabeled material, range that is acceptable to compare displacement curves in competitive protein binding assays (Work and Work, 1978).

Varying amounts of TSH-anti-id inhibited this binding in a dose dependant manner. At 10 μg 23% (SD 1.5) of the radiolabeled TSH bound to Id-IgG was

displaced. At a dose of 20 μg 55% (SD 2.8), at 50 μg 75% (SD 3.5) and at 100 μg 88% (SD 4.1) of the bound radiolabeled TSH had been displaced by TSH-anti-id. On the contrary NR-Ig at similar doses displaced a maximun of 10% of the radioactivity bound.

To exclude the possibility that some of the effects produced by TSH anti-id on the binding of ^{125}I-bTSH to Id-IgG were due to its capacity to bind TSH (anti-TSH antibody instead of anti-idiotypic antibody), the binding of TSH-anti-id to radiolabeled TSH was studied. TSH-anti-id was unable to bind ^{125}I-bTSH or ^{125}I-hTSH to any significant degree over a wide range of concentrations. Up to 1000 μg/ml of TSH anti-id were tested. This concentration resulted in the precipitation of only 11% (SD 3) of the ^{125}I-bTSH (20,000 CPM ~0.5 pM) added. Blank tubes in which radiolabeled hormone and buffer alone were added counted 8% (SD 4) of the radioactivity added, representing the background counts. NR-Ig precipitated similar percentage of counts as TSH-anti-id.

These experiments demonstrate that TSH-anti-id and Id-IgG bind to form complexes that are precipitated by PEG. TSH-anti-id is capable of inhibiting the binding of radiolabeled TSH to the Id-IgG but the binding of this antibody to radiolabeled TSH is minimal and not more significant than that obtained with NR-Ig or simple background counts. From these results one can conclude that the effects of TSH-anti-id on the binding of radiolabeled TSH to the Id-IgG are most likely due to the presence of anti-TSH activity but to the presence of true "internal image" anti-idiotypic antibodies.

3.1.3. Binding of TSH-anti-id to thyroid plasma membranes

The direct binding of radiolabeled TSH-anti-id (20,000 cpm ~5 μg) with 100 μg of crude thyroid plasma membranes was studied as described above (2.11). Preliminary experiments tested the binding of increasing amounts of radiolabelled TSH-anti-id with 100 μg of thyroid plasma membrane, amounts of 30,000 cpm or higher only increased the background counts while the percentage of binding did

not increase past 50% (Bako, G., personal communication). An average of 4% (SD 0.7)of the radioactive ^{125}I-TSH-anti-id added was found bound to the crude thyroid membrane preparation. This low binding was not surprising considering that only a small percentage of the antibody preparation used likely represented "internal image" anti-idiotypic antibodies capable of recognizing the TSH receptor in the membrane preparation. Unlabeled bTSH in doses of 40-200 mU/ml were added to the system described above. The hormone caused a dose dependent inhibition of this binding. At 160 mU/ml 64% (SD 5.3) of the radioactive TSH-anti-id was displaced.

Unlabelled TSH anti-id in doses of 50-200 μg/ml and NR-Ig in similar doses were used to investigate saturability of binding. Up to 70% (Mean: 65.8, SD 3.2) of the ^{125}I-TSH-anti-id bound to thyroid plasma membranes was displaced by unlabeled TSH-anti-id at a dose of 200 μg/ml. By contrast only 18% (SD 2) of the counts were displaced when 200 μg/ml of unlabeled NR-Ig were added to the system. The difference between these two values was statistically significant ($p < 0.05$). These results suggest specificity and saturability of the binding of TSH-anti-id to thyroid plasma membrane.

3.1.4. Effects of TSH anti-id on adenylate cyclase activation

The next group of experiments was designed to establish the biological effects of these antibodies after their interaction with the TSH receptor (TSH-R). It is well documented that TSH mediates many of its actions through the activation of AC with the subsequent generation of cAMP from ATP (Zor et al, 1969; Saltiel et al, 1981).

The effect of TSH-anti-id on AC activation was measured and compared to that of TSH. The limited capacity of bTSH to stimulate AC on the porcine thyroid plasma membrane has been previously described (Verrier, Planells and Lisstzky, 1977). It was found, however, that in the presence of GTP or its non hydrolyzable analogue guanosine 5'(β-γ-imido) triphosphate (Gpp[NH]p) the activation of AC was quite significant (Saltiel et al., 1981).

The activity of bTSH on the stimulation of AC was measured in the presence of GTP and Gpp[NH]p at 37°C. The addition of 10 μM GTP or 10 μM Gpp[NH]p to the cAMP generation system (2.5) produced a 9% stimulation from the basal activity of the porcine thyroid plasma membrane bound AC. The addition of 25 mU/ml of bTSH increased this stimulation to 20%, at 50 mU/ml 40% and at 100 mU/ml the maximal stimulation was obtained with 45% from the basal activity. For these experiments the basal activity varied between 134-165 pM cAMP/mg protein/minute. Some of the variations in the basal activities appeared to be related to the use of different batches of porcine thyroid plasma membrane. The different results were always reproduceable within the same batch of membrane and the trends of stimulation or inhibition were constant on repeated experiments.

The influence of TSH-anti-id upon porcine thyroid plasma membrane bound AC was studied at different temperatures in the presence of Gpp[NH]p (2.5). At 30°C TSH-anti-id (200μg/ml) caused a 9% stimulation of the AC basal activity. On the contrary NR-Ig (200μg/ml) caused a 12% inhibition of the basal activity. By comparison TSH (100 mU/ml) produced a 60% stimulation ($p < 0.05$) (Fig 3.2). At 37°C 200 μg/ml of TSH-anti-id produced a 21% stimulation from the basal activity ($p < 0.05$). NR-Ig (200 μg/ml) caused a 10% inhibition. As mentioned above 100 mU/ml of bTSH produced a maximal stimulation of 45% from basal ($p < 0.05$)(Fig 3,2).

Studies on AC activation demonstrate that TSH-anti-id like TSH is capable of stimulating porcine membrane bound AC. These findings suggest that this TSH-anti-id not only recognizes and interacts with the TSH-R but also initiates "TSH-like" signals, indicating that these antibodies are agonists at the receptor level.

Because of the modest increases in AC generated it was considered necessary to study the effect of TSH-anti-id on two physiological functions thought to be mediated by cAMP. The first set of experiments explored the effect of TSH-anti-id upon iodide uptake by dispersed thyrocytes and the second tested the influence of these antibodies on the organization of cultured thyroid epithelial cells into follicular structures.

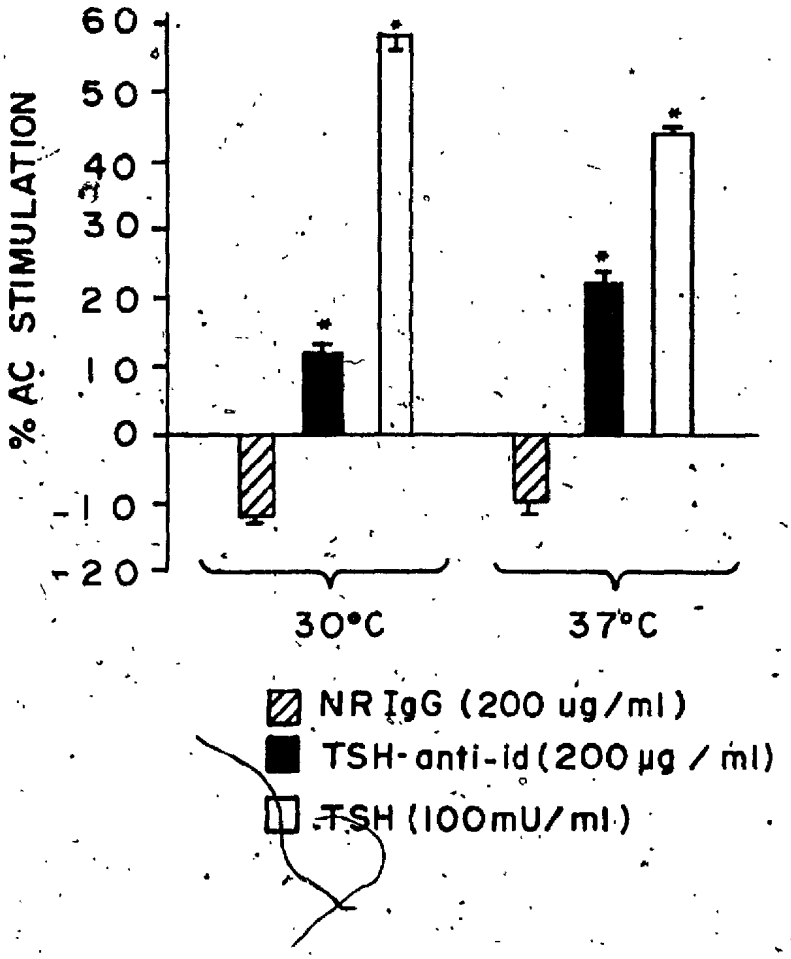


Figure 3-2: Effects of TSH, TSH-anti-id and NR-Ig on the guanyl nucleotide dependent AC activation at 30°C and 37°C.

*: p 0.05 from basal.

N = 6 : ($\bar{X}$ of 6 experiments, 2 parallels each).

3.1.5. Effects of TSH-anti-id on the iodide uptake by thyroid epithelial cells

Iodide uptake by dispersed thyrocytes was studied at different time intervals. Thyrocytes were incubated with ^{131}NaI (1x 10^5 cpm/ml) in the presence of TSH-anti-id, bTSH, NR-Ig and control medium for 10 minutes and 4 hours (2.7). When incubated in the presence of either TSH (5, 10 or 50 mU/ml) or TSH-anti-id (125 or 250 μg/ml) for 10 minutes the percentage of ^{131}I taken up by thyroid cells was lower than that obtained in the presence of NR-Ig or in control medium. Incubation for 4 hours on the other hand stimulated the uptake of ^{131}I in both TSH and TSH-anti-id incubated cells to a significantly higher degree than that caused by NR-Ig.

TSH in concentrations of 5, 10 and 50 mU/ml were used to investigate dose effect on ^{131}I uptake by thyrocytes. At 10 mU/ml ^{131}I uptake was increased from the control value of 4.2% to 7.1%. At 50 mU/ml the uptake was of 6.8%. TSH-anti-id at a dose of 125 μg/ml produced an uptake of 6.5%, while at a dose of 250 μg/ml the uptake was 5.3%. NR-Ig (125 μg/ml) produced an uptake of 3.9% and at 250 μg/ml 4.3%. Both of these latter values were within the levels of uptake found in cells incubated only in control medium (Table 3.1).

It is noticeable that a lower concentration of TSH-anti-id (125 μg/ml) produced a higher stimulation in uptake than a higher concentration (250 μg/ml). Lower concentrations, however, were not tested. No obvious explanation for these results can be offered from the experiments performed.

The effect of TSH on iodide uptake by the thyroid gland has been shown to be biphasic, with an initial increase in the efflux rate followed by an acceleration in the influx rate (Halmi, et al., 1960; Williams and Malayan, 1975). Lower ^{131} NaI uptake at 10 min in cells studied in the presence of TSH and TSH-anti-id than in cells studied in the presence of control medium appears to indicate decreased influx on the radioactive material into the first two groups of cells. It could be

Table 3-1: Effect of TSH, TSH-anti-id and NR-Ig on the radio-iodine uptake by porcine thyrocytes after 4 hours of incubation.

		^{131}I uptake (%) ± SEM	Stimulation (%)
Control		4.2 ± 0.06	100
bTSH	5	4.57 ± 0.07	109
(mU/ml)	10	7.05 ± 0.03	168*
	50	6.75 ± 0.04	161*
NR-Ig	125	3.88 ± 0.03	92
(ug/ml)	250	4.28 ± 0.02	102
TSH-anti-id	125	6.54 ± 0.03	156*
(ug/ml)	250	5.33 ± 0.03	127

*: p 0.05 from basal values or compared to those obtained in presence of NR-Ig. (Mann Whitney U Test).

N = 6 (2 parallels per experiment)

2.5×10^6 cells/tube in 1 ml

cpm added: 10^5 ^{131}I

speculated that this is due to the efflux of iodine occurring in the TSH and TSH-anti-id stimulated cells. Studies to determine the efflux of iodine from the cells, however, were not performed. The results obtained in these experiments therefore suggest that TSH-anti-id appears to induce similar changes in iodide uptake kinetics in the cultured thyrocyte system studied as does TSH itself.

3.1.6. Effects of TSH-anti-id on the organization of thyroid epithelial cells in culture

The capacity of cultured thyroid cells to organize into follicular structures when in the presence of TSH has been previously demonstrated by several workers (Kerkoff et al., 1964; Fayet et al., 1970; Kondo et al, 1980). This response is thought to be mediated by cAMP (Lissitzky, Fayet, Giraud, Verrier and Torresani, 1971; Bourke, Carseldine, Ferris, Huxham and Manley, 1981).

Thyrocytes cultured either in the presence of TSH-anti-id or TSH (2.8) were capable of organizing into follicles. By contrast cells cultured in the presence of NR-Ig or in control medium formed a uniform monolayer in which the cells adopted a slightly elongated shape, somewhat similar to that exhibited by fibroblasts. Follicular structures started to form at 3 or 4 days of culture and were quite well organized at day 7 in TSH stimulated cells. Thyrocytes cultured with TSH-anti-id started to organize into follicles between days 4 and 5. At day 7 the structures were clearly recognizable as follicles but they were observed to be noticeably smaller than those obtained with TSH. Day 7 follicles were comparable to those obtained at day 5 or 6 with TSH by simple observation, no specific method for quantification was utilized (Fig 3.3).

3.1.7. Interaction of TSH-anti-id with protein blots of thyroid plasma membrane

The final set of experiments done with TSH-anti-id investigated the interaction of these antibodies with protein blots of purified thyroid plasma membrane (2.17). It has been previously determined that the TSH holoreceptor is an Mr~ 200,000 glycoprotein heterocomplex (Islam and Farid, 1985; Bako et al., 1985).

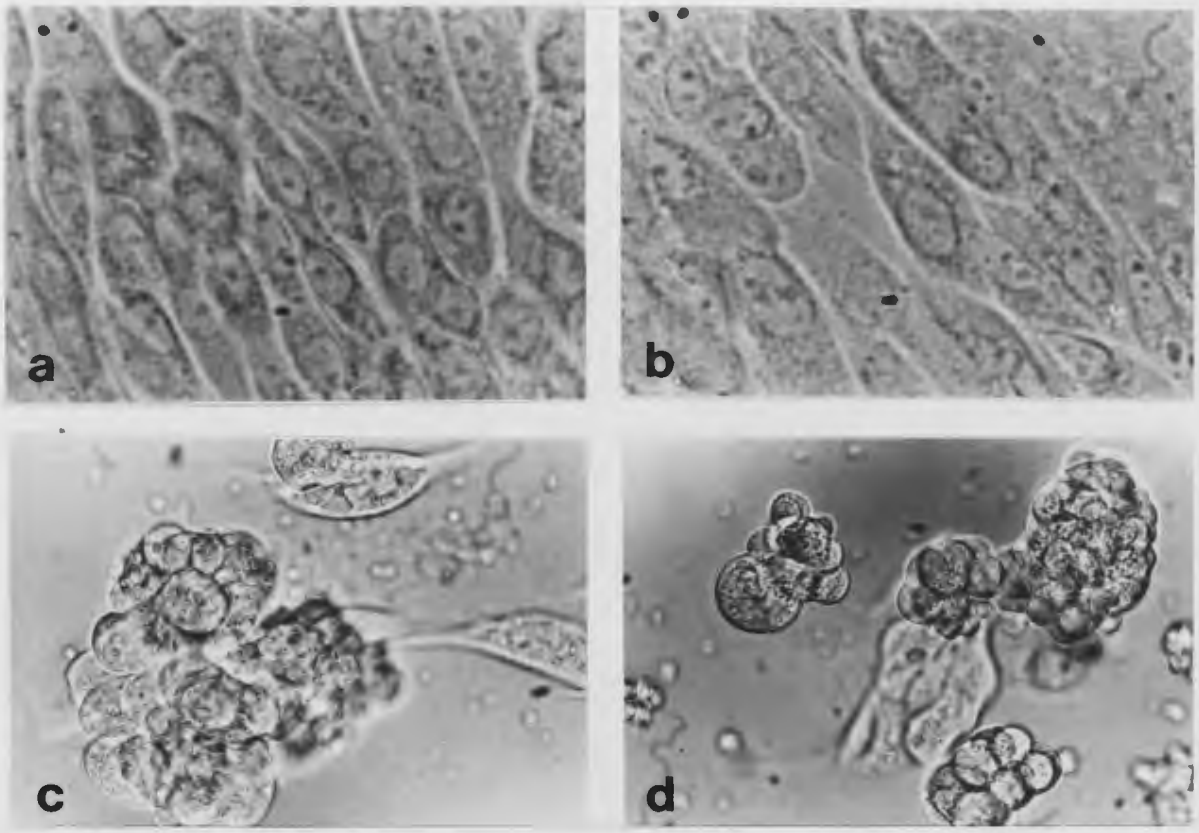


Figure 3-3: Effect of TSH, TSH-anti-id and NR-Ig upon the organization of thyrocytes after 7 days in culture

a: Control medium.

b: NR-Ig.

c: TSH

d: TSH-anti-id.

Bovine TSH produced a positive reaction in protein blots of thyroid plasma membrane resolved under non-reducing conditions at the level of the Mr~ 197,000. When reducing conditions were used this band was lost and none of the reduced components appeared to interact with TSH. This suggested the need for the integrity of the Mr~ 197,000 receptor band for TSH binding (Figure 3.4, lane 2).

Using the same detection method, it was observed that the TSH-anti-id produced a very strong positive reaction at the level of the Mr~ 197,000 protein band (Fig 3.4, lane 3). Most of the reaction disappeared when the protein blots were preincubated with TSH (Fig 3.4, lane 4), whereas when the blots were preincubated with insulin (Fig 3.4, lane 5) or human chorionic gonadotrophin (HCG)(Figure 3.4, lane 6) the intensity of the band was not reduced. These findings demonstrated the specificity of the Mr~ 197,000 for TSH and TSH-anti-id. NR-Ig reacted with several lower Mr bands but was negative for the Mr~197,000 band (Fig-3.4, lane 7). The binding to these bands was not displaced by any of the above mentioned hormones. These results add more evidence to support the hypothesis that TSH-anti-id recognizes and interacts with the TSH-R.

3.1.8. Discussion of the experiments investigating anti-TSH anti-idiotypic antibodies

In these groups of experiments it was demonstrated that it is possible to produce anti-idiotypic antibodies functionally similar to the hormone used in producing the first antibody. The TSH-anti-id described here represents a TSH "internal image" anti-idiotype. Furthermore, it acts as an agonist at the TSH-R level and is capable of mimicking several of the TSH's physiological actions. It is tempting to compare the bioactivity of TSH-anti-id. to that of the spontaneously occurring thyroid stimulating antibody of Graves' Disease (TSAb: used here as a generic term to encompass antibodies detected by receptor modulation assays as well as the cAMP generation assays). So far, in all the tests performed in vitro with TSH-anti-id the biological actions attributed to the TSAb have been reproduced.

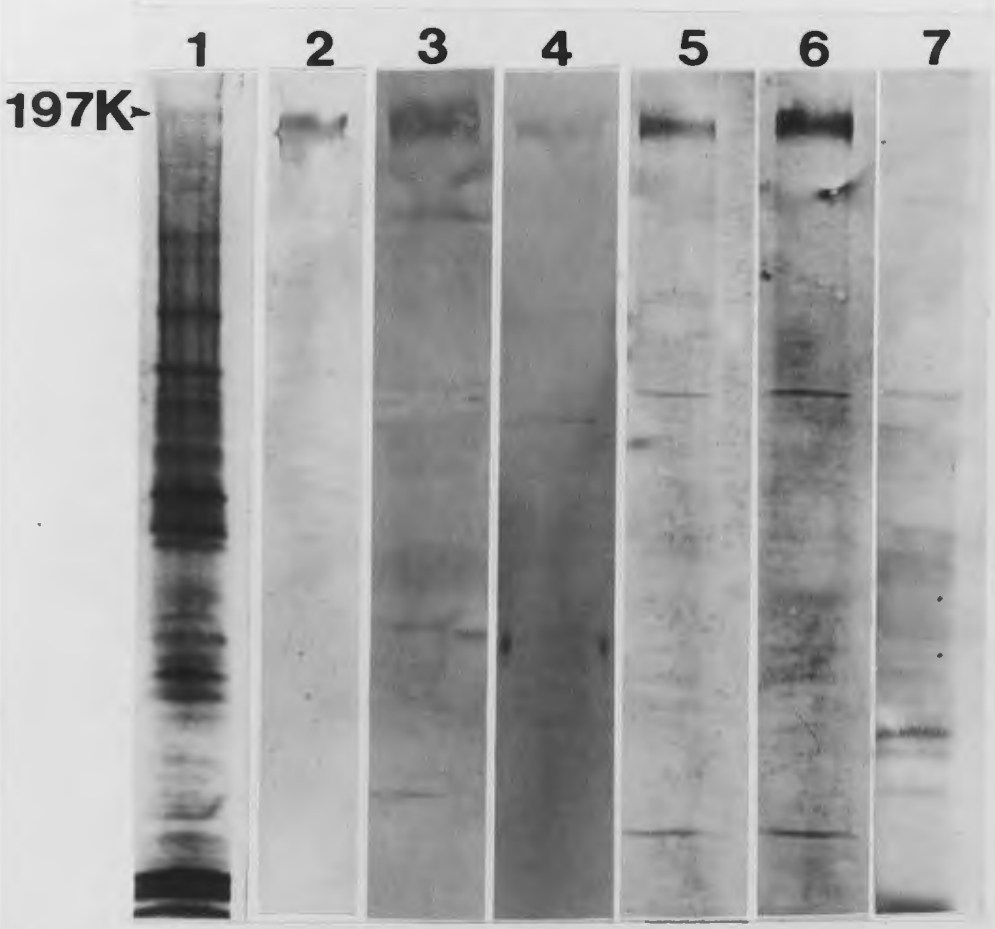


Figure 3-4: Interaction of TSH and TSH-anti-id with thyroid plasma membrane protein blots.

1: Coomassie blue stained electrotransferred protein bands. 2: bTSH (1 U/ml). 3: TSH-anti-id (1 mg/ml). 4: TSH-anti-id in TSH pretreated protein blots. 5: TSH-anti-id in insulin pre-treated protein blots. 6: TSH-anti-id in HCG pretreated protein blots. 7: NR-Ig (1 mg/ml). Positive bands at the $M_r \sim$ 197,000 are seen in lanes 2, 3, 5 and 6.

On the basis of these data we proposed a new hypothesis suggesting the anti-idiotypic origin of the TSAb, in at least some patients with Graves' Disease (Islam et al., 1983b; Farid et al., 1983). Most of the evidence found in the literature suggesting that TSAbs are anti-receptor antibodies or directed to protein moeieties close to it was based on indirect evidence (McKenzie and Zakarija, 1978). TSH-anti-id being a "mirror image" of TSH would be expected to mediate all of its "TSH-like" actions only through binding to the receptor. Interaction with other membrane moeieties should not occur with this type of antibodies. Biological response would then be expected to be highly specific.

On the contrary, if TSAb is raised against the TSH-R it is likely that other membrane components, close to and independant of the receptor, would be expected to be represented in the immunological response. The antibodies interacting exclusively with the receptor will be responsible for the specific biological responses. Antibodies raised against other membrane components may account for some unexpected experimental results obtained when testing the activity of immunoglobulins of patients with Graves' Disease. IgGs derived from patients with Graves' Disease can in some cases produce "TSH-like" activity in assays dependent on AC stimulation but not necessarily on receptor modulation assays or vice versa (Zakarija and McKenzie, 1980; Atkison, Mc Gregor, Kendal-Taylor, Peterson and Rees-Smith, 1982). Antibodies to membrane moeieties other than the receptor are, however, not the only possible explanation for these apparently conflicting results. The sensitivity and specificity of the different detection systems, interaction with other components in the sera, like normal immunoglobulins, and the varied uniformity of the antibody preparations can all contribute to these sometimes contradictory results. It is worthwhile to comment that all the explanations offered to date remain theoretical. The full understanding of the interaction of the different antibodies with the TSH-R is still lacking.

Because of the complexity of the immune response, however, one has to be very

cautious in relating the pathogenesis of Graves' Disease to the occurence of anti-TSH-anti-idiotypic antibodies. To further strengthen this hypothesis, it would be important to directly demonstrate the natural occurence of anti-TSH antibodies (idiotypes), as well as of anti-TSH-anti- idiotypic antibodies (anti-idiotypes) in patients with Graves' Disease, as well as to establish the correlation between anti-idiotypes and the activity of the disease. Ig fractions which bind ^{131}I-bTSH with high affinity and low capacity were described in both healthy controls and in patients with Graves' Disease (Biro, 1981). It could be argued that these antibodies represent naturally occuring anti-TSH antibodies. Also in other study Igs that bound radiolabeled TSH with high capacity and low affinity were found in 38% of the patients with Graves' Disease tested. These Igs also inhibited the TSH driven AC activation in a dose dependent manner (Biro, 1982). Other groups have also described the presence of auto-antibodies with high affinity for TSH in the sera of thyrotoxic patients (Beall and Kruger, 1983; Akamizu, Ishii, Ishihaka, Ikekubo and Imura, 1984). The relevance of the presence of anti-TSH auto-antibodies in some patients with Graves' Disease is still unclear. The presence of these TSH binding Igs could be explained in several ways based on the principles of the "network theory" of the immune system. Thus, TSAb may be an anti-TSH anti-idiotypic antibody (Ab 2), which developed against naturally occuring anti-TSH antibodies (Ab 1) found in the sera of these patients. Ab 1 would bind TSH. It is possible that Ab 2 triggers the production of anti-anti-idiotypes (Ab 3) some of which would be able to bind TSH (Farid et al., 1983). On the contrary, it can also be argued that if TSAb is an antibody raised against the receptor, it could promote the development of anti-idiotypic antibodies some of which would be able to bind to TSH.

Indirect evidence for the participation of idiotypic/ anti-idiotypic networks in the regaining of immunological competence in Graves' Disease can be considered because of the disappearence of circulating immune complexes that follows effective treatment of the disease with anti-thyroid drugs (Van Der Heide, Daha, Bolk, Bessemaker, De Bruin, Goslings, Van Es and Querido, 1980). Evidence for

the direct participation of idiotypic and anti-idiotypic antibodies in the actual initiation of the disease process, however, will remain more difficult to accumulate. The possibility that TSAb is an anti-TSH anti-idiotypic antibody seems valid. Further studies to demonstrate the presence of spontaneously occurring idiotypes and anti-idiotypes in patients with Graves' Disease, as well as the measurement of their fluctuations with disease remission and relapse will be necessary to substantiate or disprove this hypothesis.

Important observations that deserve to be mentioned in the discussion of these first group of experiments deal with the production of the antibodies used. The presentation of antigens is thought to be of extreme importance in the response that the laboratory animal will produce to both the initial antigen as well as the Ab1. Wigzell, Binz, Frischknecht, Peterson and Sege (1978) tested a great number of adjuvants and concluded that Freund's complete adjuvant was the adjuvant of choice to raise anti-idiotypic antibodies. Hence, the use of this agent in this work. The choice of the laboratory animals is also of great importance. Balb/c mice, for example, have been found to be bTSH non-responders (J. Ivanyi, personal communication). Rats, on the contrary, were found to be good responders to TSH. In this work the choice of Sprague-Dawley rats to raise the antibodies was, however, fortuitous. The time of sample collection was also of extreme importance. The difference between having an agonistic or an antagonistic antibody resided on the choice of an appropriate time to collect the sample material. Many anti-idiotypic responses appear to be transient likely due to the reciprocal increase of Ab3 (Kelsoe and Cernig, 1979; Couraud, Lu and Strosberg, 1983). This problem was circumvented by multiple sampling around the 2-3 week period after the last booster injections when the maximal response of Ab2 appeared to be present.

In summary it was possible to produce anti-TSH-anti-idiotypic antibodies with agonistic activity at the TSH-R. The production of these antibodies required careful choice of adjuvants, laboratory animals and multiple sample testing to determine optimal time for specimen collections.

3.2. INTERACTION OF SPONTANEOUSLY OCCURRING ANTI-THYROID AUTO-ANTIBODIES WITH THYROID PLASMA MEMBRANE PROTEIN BLOTS

3.2.1. Interaction of IgG from patients with Graves' Disease on protein blots of thyroid plasma membrane

As described above, both TSH and TSH-anti-id interact with the Mr~197,000 on protein blots of thyroid plasma membrane (3.1.7). This protein band is believed to correspond to the TSH holoreceptor (Islam, Briones-Urbina, Bako and Farid, 1983c; Islam and Farid, 1985). It was thus of interest to investigate whether immunoglobulins from patients with active Graves' Disease would react in similar fashion under similar experimental conditions.

Igs from 10 patients with active Graves' Disease were obtained by precipitation at 0°C with half saturated ammonium sulphate (Heide and Schwick, 1973). These were tested at a concentration of 1 mg/ml of protein. All 10 samples produced positive reactions in the enzyme-linked immunobinding assay used (2.17) at the level of the Mr~ 197,000 protein band (Fig 3.5, lanes 3 and 4). Interaction of the antibody was observed with protein blots of human (Fig 3.5, lane 4) and porcine (Fig 3:5, lane 5) thyroid plasma membranes under non-reducing conditions. The reaction was, however, consistently stronger with human than with porcine thyroid plasma membranes. This finding indicates a degree of antibody specificity for the human TSH receptor, and implies that the human TSH receptor is organized in a similar fashion to that of the porcine receptor.

The binding of the antibodies to this band disappeared when protein blots were pretreated with bTSH (Fig 3.5, lane 5). Under reducing conditions the Mr~ 197,000 was no longer visualized with Graves' Ig nor were either of the receptor components, suggesting again that the integrity of the receptor is necessary both for TSH and TSAb binding.

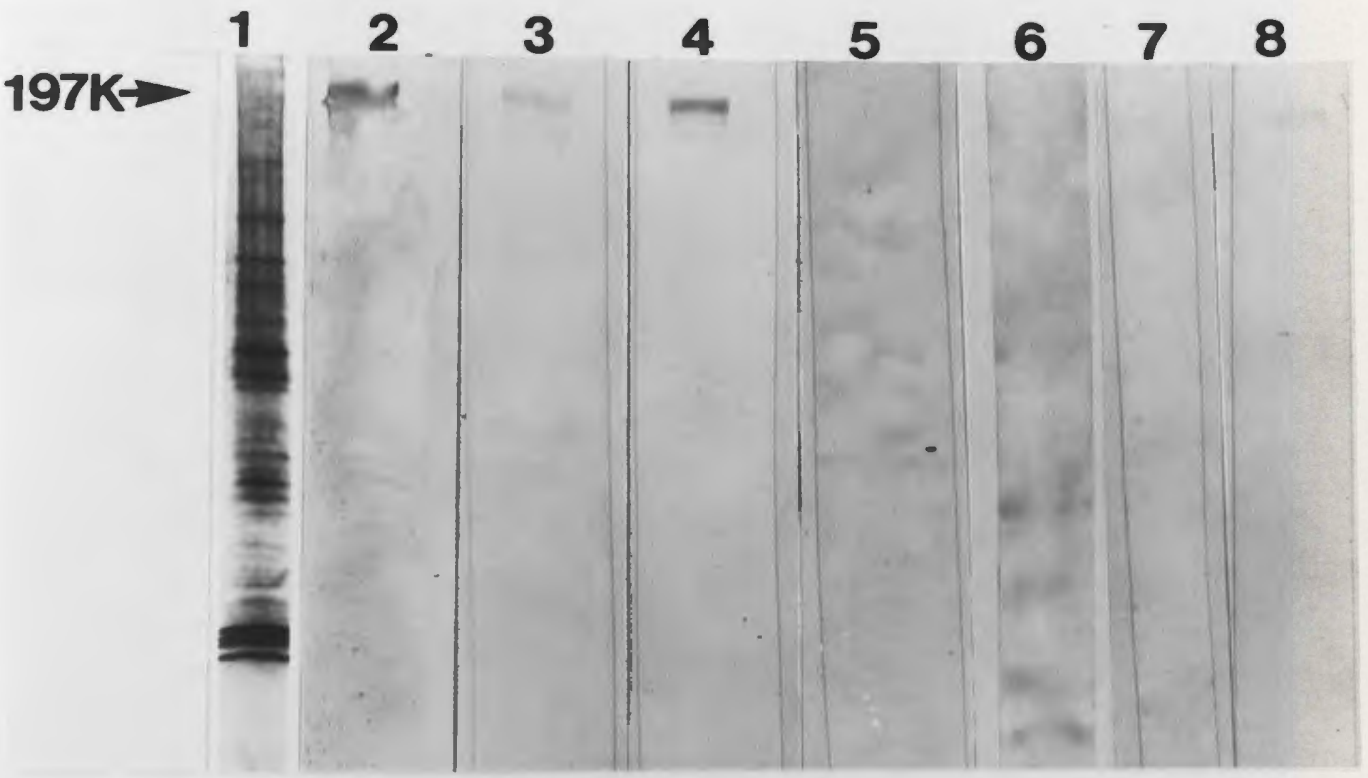


Figure 3-5: Interaction of TSH, Graves' Ig and Hashimoto's Ig with thyroid plasma membrane protein blots under non-reduced conditions.

1: Coomassie blue stained electrotransferred protein bands. 2: TSH (1 U/ml). 3: Graves' Ig (1 mg/ml) in porcine thyroid membrane blots. 4: Graves' Ig in human thyroid membrane protein blots. 5: Graves' Ig in blots pretreated with TSH. 6: Hashimoto's Ig from sera with anti-microsomal antigen titre of 1/25,600 (1 mg/ml). 7: Normal human Ig (1 mg/ml). 8: Normal human Ig (1 mg/ml). Positive bands at the $M_r \sim$ 197,000 holoreceptor band are seen in lanes: 2, 3, 4 and a faint positive in 8.

Nine out of 10 control Igs tested were negative for this band in both human and porcine thyroid membrane protein blots (Fig 3.5, lane 7).However 1 of them exhibited a faintly positive band (Fig 3.5, lane 8). The thyroid function of the control individual positive for this assay was normal for up to 18 months after the time of testing. What was interesting is that this person was involved in work dealing with the isolation of the TSH-R. This incidental finding suggests that the presence of autoantibodies alone is not enough to cause autoimmune disease; derangement at other levels of the immune control systems appear to be required for this to occur.

The anti-receptor nature of TSAb has long been postulated based on the testing of TSAb's TSH-like activities (McKenzie and Zakarija, 1978; Kidd et al., 1980; Onaya et al., 1973; Rees-Smith and Hall, 1974; Endo et al., 1978). The demonstration that the TSAb binds the Mr~197,000 TSH holoreceptor protein band in the assay system investigated, provides the first evidence to support the notion that TSAb is a true anti-receptor antibody.

3.2.2. Interaction of Igs from patients with Hashimoto's Thyroiditis on protein blots of thyroid plasma membrane

It was also of interest to determine how Igs from patients with HT reacted with the Mr~197,000 holoreceptor protein. A first group of samples corresponding to 3 patients was tested using Ig at a concentration of 1mg/ml; all the reactions were negative (Fig 3.5, lane 6 shows a typical reaction of a serum with anti-microsomal antibody titre of 1/25,600) The Ig concentration was then increased to 1.5 and 2 mg/ml. At 2 mg/ml bands of various degrees of intensity were observed at the Mr~197,000 level in all 7 patients tested (Fig 3.6, lanes 1, 3, 5, 7 and 9). In contrast to what was observed with Graves' Igs, however, when the blots were pretreated with TSH the bands persisted (Fig 3.6, lanes 2, 4, 6, 8 and 10). It appears then, that the domain to which Hashimoto's Ig binds in the TSH-R is not very closely associated with the TSH binding site.

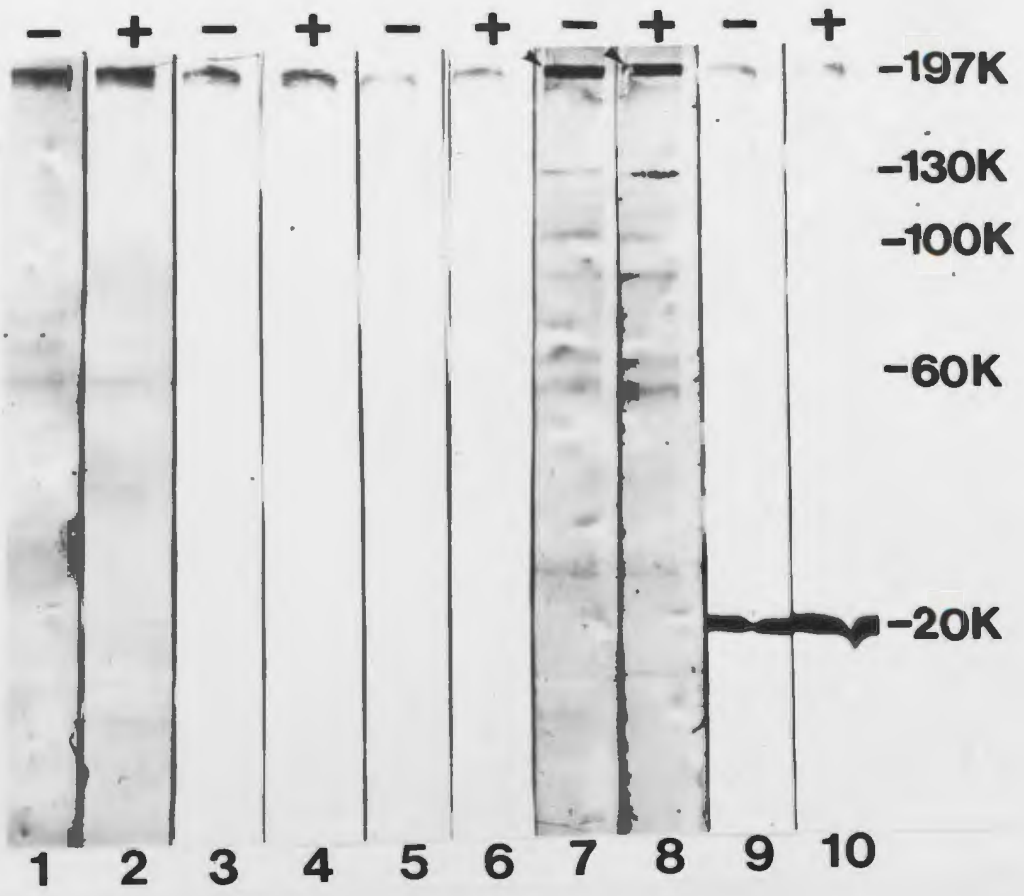


Figure 3-6: Interaction of Igs from 5 patients with Hashimoto's thyroiditis (2 mg/ml) with thyroid plasma membrance protein blots under non-reduced conditions.

(-): no pretreatment of the blots with TSH. (+): blots pretrated with TSH. Positive bands are seen in all lanes at the $M_r \sim$ 197,000. The arrows in lanes 7 and 8 indicate the $M_r \sim$ 210,000 band.

Two patients exhibited a second positive band in the high molecular weight region. This band corresponds to the Mr~210,000 (Fig 3.6, lanes 7 and 8). Another patient exhibited a very prominent band at the Mr~20,000 level (Fig 3.6, lanes 9 and 10). The nature of the Mr~ 20,000 peptide is currently unknown. The characteristics of the Mr~ 210,000 was further explored and will be explained later. Seven control Igs were negative for both bands at similar concentrations (Fig 3.7).

Under reducing conditions the Mr~ 197,000 was no longer visible as in previous experiments when TSH (3.1.7), TSH-anti-id (3.1.7) and TSAb (3.2.1) were tested. This suggests that for the binding of HT Igs the integrity of the receptor is also required. Under reducing conditions the Mr~210,000 migrated with a mobility consistent with an Mr~ 180,000.

A number of reports in the literature raised the possibility that sera from patients with Hashimoto's Thyroiditis (HT) may contain antibodies directed against the TSH-R or plasma membrane determinants closely associated with the receptor (Endo et al., 1978). Some of these Igs have an array of activities on AC. Some workers have found them to be stimulatory, others inhibitory, while others have found them to have no activity what so ever (Bliddal, Bech, Feldt-Rasmussen, Thomsen, Ryder, Hansen, Sierback and Friis, 1982). Endo, Borges, Amir and Ingbar (1982) compared a method of preparing receptor purified Graves' Disease specific immunoglobulins using guinea pig adipocyte membranes with the method using human thyroid membranes. They found that their purified Igs were able to bind to both human and guinea pig thyroid membranes and that this binding was exclusively displaced by Graves' Disease IgG but not by Hashimoto's Thyroiditis IgG. The binding of the receptor purified Igs prepared with the methods using human thyroid membranes, however, were inhibited by both Graves' and Hashimoto's IgG. This lead the authors to believe that human thyroid membranes appear to contain antigens complementary to both Graves' and Hashimoto's disease antibodies, while guinea pig adipocyte cells contain

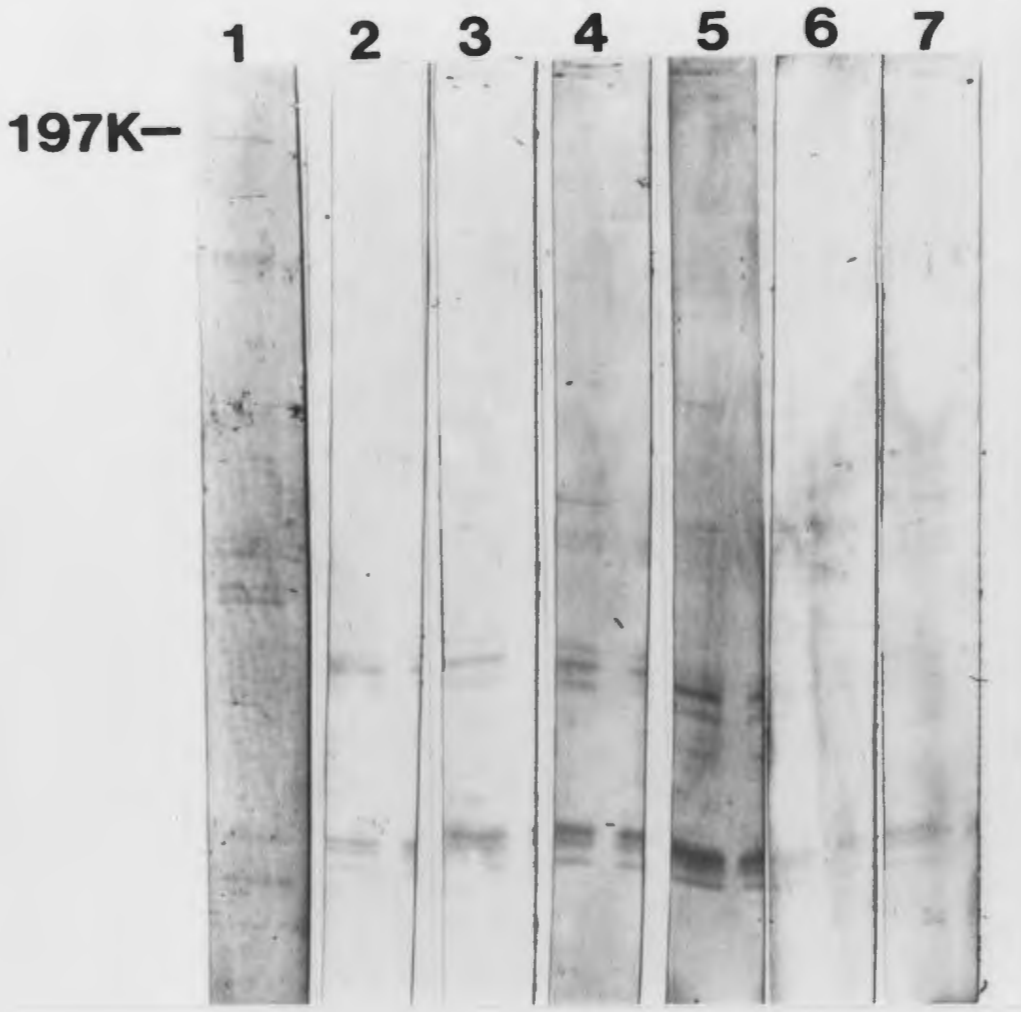


Figure 3-7: Interaction of Igs from 7 normal human controls (2 mg/ml) with thyroid plasma membrance protein blots, under non-reducing conditions.

The $M_r \sim$ 197,000 and the $M_r \sim$ 210,000 are negative.

antigens complementary only to Graves' disease antibodies. Moreover, unlabeled TSH was able to partially displace the purified Graves' disease Igs bound to the thyroid membranes suggesting that at least part of the binding of these Igs is at the level of the TSH-R. It remained unclear, however, if the thyroid membrane antigens complementary for Hashimoto's Thyroiditis antibodies were part of the TSH-R or not. Other authors (Tao and Kriss, 1982) have suggested that the antibodies found in Hashimoto's Thyroiditis may be part of a group of heterogeneous antibodies that are able to bind antigens in thyroid membrane preparations containing TSH receptors. The binding sites, however, were thought to represent antigenic areas separate from the TSH-R. This mostly because of the inability of TSH to interfere with the binding of these antibodies.

With this background the experiments performed became of major interest. The results have demonstrated several facts. The first establishes that all 7 patients with HT tested have anti-TSH-R antibodies. These antibodies however, appear to be "weaker" than those found in cases of Graves' Disease. It appears reasonable to suggest that the anti-TSH-R antibodies found in HT and those found in Graves' Disease are directed to different epitopes in the TSH-R. This contention is sustained on the grounds that contrary to Graves' Igs the positivity obtained with HT Igs was not inhibited with the pretreatment of the protein blots with bTSH.

3.2.3. Characterization of the Mr~ 210,000 protein band

As mentioned above (3.2.2), 2 out of the 7 Igs from patients with HT tested, showed a positive band on thyroid plasma membrane protein blots at the level of the Mr~ 210,000. Since the composition of this band was unknown the possibility that it may constitute a thyroglobulin breakdown product was considered. The odds against this possibility were that all the patients with HT chosen for this study were negative for anti-thyroglobulin antibodies in conventional clinical tests and that all the membrane preparations used had been separated on sucrose gradients expecting to obtain a purified product. Despite this, it was still deemed necessary to test this possibility.

Porcine thyroglobulin protein blots were prepared under non-reduced conditions (2.21). A high titer rabbit polyclonal anti-human thyroglobulin antibody (kindly donated by Dr. I. Senciall, Faculty of Medicine, MUN, Nfld.) was used as positive control in the enzyme-linked immuno-binding assay described elsewhere (2.17). Anti-thyroglobulin antibody at a concentration of 750 μg/ml interacted with bands at the level of the Mr$\sim$ 210,000 and the Mr$\sim$ 180,000 as well as other lower molecular weight breakdown products in protein blots of both thyroglobulin (Fig 3.8, lane 1) and plasma membrane (Fig 3.8, lane 2). The intensity of the reaction was lower with protein blots of plasma membrane. The Igs from the two patients that were positive for the Mr$\sim$ 210,000 band were allowed to interact with thyroglobulin protein blots. These Igs at a concentration of 2 mg/ml exhibited a positive reaction with proteins of Mr$\sim$ 210,000 and Mr$\sim$ 180,000 (Fig 3.8, lane 3). In protein blots of membrane the Mr$\sim$ 210,000 as well as new band at the level of the Mr$\sim$197,000 were seen (Fig 3.8, lane 4). Igs from patients with Graves' Disease at both 1 mg/ml and 2 mg/ml were negative in the thyroglobulin protein blots (Fig 3.8, lane 5). In protein blots of thyroid plasma membrane 1 mg/ml of Graves' Ig was enough to produce a positive reaction at the level of the Mr$\sim$ 197,000. No significant difference was observed with Graves' Ig at a concentration of 2 mg/ml (Fig 3.8, lane 6).

These results suggested in the first place that sera from patients with Hashimoto's thyroiditis which were negative for anti-thyroglobulin antibodies in conventional heamagglutination tests can give positive responses in the enzyme-linked immuno-binding assay, demonstrating the greater sensitivity of the method. Secondly, it was shown that the porcine thyroid plasma membrane preparations produced in our laboratory by sucrose gradient purification contain several thyroglobulin breakdown products. When resolved by linear gradient SDS-PAGE at concentrations of 5 to 15 % under non reducing conditions the major thyroglobulin bands appear at the Mr$\sim$ 210,000 and Mr$\sim$ 180,000. These bands were easily detected with anti-thyroglobulin antibodies. It is important to mention that the native molecule of Mr$\sim$660,000 and its Mr$\sim$330,000 subunits will not enter the polyacylamide gel at the concentrations used.

Figure 3-8: Examples of interaction of anti-thyroglobulin antibodies, Hashimoto's Ig and Graves' Ig on porcine thyroglobulin and thyroid plasma membrane protein blots.

1: Anti-thyroglobulin antibody (750 ug/ml) on thyroglobulin blot. 2: Anti-thyroglobulin antibody (750 ug/ml) on plasma membrane blot. 3: Hashimoto's Ig (2 mg/ml) on thyroglobulin blot. 4: Hashimoto's Ig (2 mg/ml) on plasma membrane blot. 5: Graves' Ig (1 mg/ml) on thyroglobulin blot. 6: Graves' Ig (1 mg/ml) on plasma membrane blot. Graves' IgG 2 mg/ml has similar effects as those observed with 1 mg/ml.

3.3. TSH-SUBUNIT SPECIFIC ANTI-IDIOTYPIC ANTIBODIES

The experiments to be described next involve the production of TSH-subunit specific anti-idiotypic antibodies with the objective of investigating the role of the subunits of TSH in the hormone-receptor interaction. The first part will describe the characteristics of the subunit specific monoclonal antibodies used in raising anti-idiotypic antibodies. The second part will delineate the characteristics of the idiotypic/ anti-idiotypic interactions. The last section will deal with the biological activity of the anti-idiotypes.

3.3.1. Characteristics of the anti-TSH subunit specific monoclonal antibodies

The monoclonal antibodies TS28 and GC73 were received from the Wellcome Research Laboratories (Poole, England). They were produced as described in the reference quoted above (2.2).

The next section will describe the investigations done in our laboratory to characterize the interaction of these antibodies with TSH and its actions on TSH mediated receptor events.

3.3.1.1. Binding of TSH-subunit specific monoclonal antibodies to human and bovine TSH

Binding of both radiolabeled bTSH and hTSH to monoclonal antibodies TS28 and GC73 was tested. Dose response curves performed for these experiments used initially increasing amounts of antibody with constant amounts of radiolabelled bTSH (100,000 opm/ml ~2.5 pM/ml) or hTSH (100,000 cpm/ml ~ 2.4 pM/ml). Up to 200 µg/ml were reacted with bTSH with no appreciable binding being obtained. With hTSH TS28 bound a maximun of 58% (SD 7) and GC73 47% (SD 5) of the radiolabel added. Next, 100 µg/ml of each antibody were reacted with increasing amounts of radiolabeled material (50,000- 1'000,000 cpm/ml). The maximal binding was obtained with 200,000 cpm, being 73% (SD 7) for TS28 and

69% (SD 3) for GC73. Increasing doses just caused increase in the background activity with minimal increase in the percentage of binding. It is again possible that the large concentrations of antibody required depend on incomplete precipitation of the monoclonal antibodies/ TSH complexes but this was not further explored as the results demonstrated reproduceability. The usefulness of polyethylene glycol in separating free from antibody bound polypeptides has long been recognized (Desbuquois and Aurbach, 1971). It could be argued that the concentrations used in these experiments might have been too low, as the smaller the complex the higher the concentration required to produce an adequate separation of the free from the antibody-bound radiolabeled TSH. The most quoted figure is 15%. In the preliminary experiments performed, however, concentrations of 12% produced as efficient a precipitation of the complexes as 15%. A 15% concentration on the contrary caused a marked increase in the background counts.

Both monoclonals exhibited high specificity for hTSH. Unlabeled hTSH was used to try to competitively displace the binding of the monoclonals to the labeled hTSH. One hundred μg/ml of each antibody were reacted with 100,000 cpm ~2.4 pM/ml of ^{125}I-hTSH. The counts bound to TS28 in the absence of cold TSH constituted 50% of those added and for GC73 46%. Cold TSH displaced this radioactivity in a dose dependant manner. With TS28 the maximum displacement was obtained at a concentration of 2.5 mU/ml of hTSH. At this dose more than 95% of the radioactivity bound was displaced. With GC73 a dose of TSH approximately 10 times higher was required to cause displacement of the radioactivity. Maximum displacement was obtained at 30 mU/ml where approximately 90% of the radioactivity was displaced (Table 3.2).

To test the specificity of the binding of hTSH to the monoclonal antibodies HCG and insulin were also used in competition assays. HCG and insulin at doses of 2.5, 5.0, 12.5, 25.0 and 50.0 IU/ml were used in displacement experiments. The

Table 3-2: Interaction of TS28 + GC73 with labeled hTSH and displacement by unlabeled hTSH.

	cpm$\bar{X}$	Binding % ± S.D.
^{125}I-hTSH (TC)*	19,000	
+ TS28 (100 ug/ml)	9381	49.4 ± 1
+ TS28 (100 ug/ml) + hTSH (0.5 mU/ml)	5455	28.7 ± 3
+ TS28 (100 ug/ml) + hTSH (2.5 mU/ml)	758	3.9 ± 1
+ TS28 (100 ug/ml) + hTSH (5 mU/ml)	778	4.0 ± 2
^{125}I-hTSH (TC)*	18,000	
+ GC73 (100 ug/ml)	8259	45.9 ± 1
+ GC73 (100 ug/ml) + hTSH (15.0 mU/ml)	6613	36.7 ± 2
+ GC73 (100 ug/ml) + hTSH (20.0 mU/ml)	4095	22.8 ± 2
+ GC73 (100 ug/ml) + hTSH (30.0 mU/ml)	996	5.5 ± 1

* Total counts.

N = 5 : (2 parallels each).

binding of radiolabeled hTSH to TS28 was displaced by HCG in a dose dependent manner. At 25 IU/ml 95% of the radioactivity bound was displaced. By contrast HCG up to a dose of 50 U/ml had no effect on the binding of GC73 to the ^{125}I-hTSH (Table 3.3) The study of the effects of hLH and hFSH on the binding of hTSH to TS28 and GC73 were not repeated in our laboratory. The information received from the laboratory that produced these monoclonal antibodies indicated that hFSH and hLH have no effect on the binding of hTSH to GC73. Both hormones, however, were capable of displacing the binding of hTSH to TS28 (J. Ivanyi, personal communication). Insulin had no effect on the binding of either of the antibodies at the concentrations used.

It is known that all glycoprotein hormones, TSH, LH, FSH and HCG share a structurally similar alpha subunit, while their specificity resides on the beta subunit (Pierce and Parsons, 1981). After analyzing the results obtained it was considered that the displacement of radioactive TSH binding to TS28 by all glycoprotein hormones may imply that the binding site for this antibody is localized in the alpha subunit of those hormones. ^{125}I-hTSH binding to GC73 was exclusively displaced by hTSH as would be expected for a monoclonal antibody specific for the beta subunit of TSH.

3.3.1.2. Effect of TS28 and GC73 on the binding of ^{125}I-bTSH to thyroid plasma membranes

In an attempt to establish the TSH site to which these monoclonal antibodies are directed, their effect on the binding of radiolabeled bTSH to thyroid plasma membranes was studied. It is important to point out that bTSH and not hTSH was used for these experiments. Despite initial expectations that the use of hTSH might be indispensable, initial screening studies produced promising results with the use of bTSH. Considering that bTSH was easier to obtain commercially this product was used for further experiments. ^{125}I-bTSH (~20,000 cpm ~0.5 pM) was reacted with 100 μg of sucrose gradient purified porcine thyroid plasma membrane. Fourteen percent of the radioactivity added was bound. TS28 at

Table 3-3: Effect of HCG on the binding of ^{125}I-hTSH to TS28 and GC73

	cpmX	Binding %
^{125}I-hTSH (TC)	15780	
+ TS28 (100 ug/ml)	9286	58.9
+ TS28 (100 ug/ml) + HCG (2.5 U/ml)	8635	54.7
+ TS28 (100 ug/ml) + HCG (5.0 U/ml)	5964	37.8
+ TS28 (100 ug/ml) + HCG (12.5 U/ml)	1400	8.9
+ TS28 (100 ug/ml) + HCG (25.0 U/ml)	503	3.2
^{125}I-hTSH (TC)	15540	
+ GC73 (100 ug/ml)	7045	45.3
+ GC73 (100 ug/ml) + HCG (12.5 U/ml)	6601	42.5
+ GC73 (100 ug/ml) + HCG (25.0 U/ml)	6703	43.1
+ GC73 (100 ug/ml) + HCG (50.0 U/ml)	7338	47.2

N = 6 (parallels)

TC = Total counts

doses of 250 μg/ml and 500 μg/ml were added and the percent of ^{125}I-bTSH binding was 12.2% (SD 2) and 12.8% (SD 1.8) respectively. GC73 at similar doses (250 and 500 μg/ml) were also tested. When 250 μg/ml of GC73 were added the percent of ^{125}I-bTSH binding was 8.8 % (SD 1.5) and at 500μg/ml 6.8% (SD 1.4). Thus, the monoclonal GC73 was able to displace 50 % of the radioactivity bound to thyroid plasma membranes while TS28 had no effect on this assay.

3.3.1.3. Effect of TS28 and GC73 on the TSH mediated thyroid plasma membrane bound AC activation

Continuing with the search for the TSH site to which the monoclonal antibodies TS28 and GC73 bind, their effect on the TSH mediated AC activation on porcine thyroid plasma membrane was studied. This was investigated using bTSH at 37°C in the presence of 10 μM Gpp[NH]p as described above (2.5). TSH (200 mU/ml) resulted in a 82% stimulation of the thyroid plasma membrane bound AC activity compared to basal activity ($p \leq 0.05$). At concentrations of 250 μg/ml TS28 produced a 10% stimulation, GC73 an 8% stimulation and normal rat Ig (N-rat-Ig) a 15.6% stimulation above basal.

When TS28 or GC73 were added for a preincubation period of 10 minutes prior to the addition of bTSH, these antibodies caused inhibition of the expected activation mediated by bTSH. TS28 produced a 40% inhibition of the stimulated TSH activation. GC73 a 51% inhibition. N-rat-Ig by contrast caused only an 18% inhibition of stimulated AC activity (Table 3.4 a)

Surprisingly when either TS28 or GC73 were added simultaneusly with TSH, the TSH mediated AC activation was further enhanced. TSH produced a 59% stimulation of AC activity as compared to basal. TS28 (250μg/ml and TSH (200mU/ml) caused a 128% stimulation and GC73 (250 μg/ml) and TSH (200mU/ml) a 118% stimulation (Table 3.4 b). The results obtained with N-rat-Ig were no different than those obtained in preincubation experiments.

Table 3-4: Effect of monoclonal antibodies TS28 and GC73 on AC activity stimulated by TSH added: a: after or b: at the same time as monoclonals.

	Concentration	cAMP (pM/mg/min) ± S.D.	Stimulation (%) basal	Stimulation relative to TSH stimulation (%)
a	Basal	134 ± 44	100	
	TSH (200 mU/ml)	244 ± 57	182.0*	100
	TS28 (250 ug/ml)	148 ± 30	110.0	60.6**
	GC73 (250 ug/ml)	145 ± 14	108.0	59.5**
	NR-Ig (250 ug/ml)	155 ± 9	115.6	63.5**
	TSH + TS28	147 ± 30	109.7	60.2**
	TSH + GC73	121 ± 9	90.3	49.5**
	TSH + N-rat-Ig	200 ± 18	149.0*	82
b	Basal	142 ± 7	100	
	TSH (200 mU/ml)	225 ± 15	159.0*	100
	TS28 (250 ug/ml)	175 ± 3	123.0	77.8
	GC73 (250 ug/ml)	190 ± 12	133.8	84.0
	TSH + TS28	325 ± 2	228.0*	144.4*
	TSH + GC73	310 ± 18	218.0*	137.8*
	TSH + N-rat-Ig	154 ± 11		68.5

N = 6 (average of 6 parallels)

* $p < 0.05$ stimulation from basal (Mann Whitney U Test).

** $p < 0.05$ inhibition from TSH stimulated value (Mann Whitney U Test).

3.3.1.4. Discussion of the experiments investigating the effects of TS28 and GC73 on the binding of radiolabeled TSH to thyroid plasma membranes and on the TSH-mediated AC activation

Before interpreting the results obtained from the experiments investigating the effect of TS28 and GC73 on the binding of ^{125}I-bTSH to thyroid plasma membranes (3.3.1.2) and on TSH mediated AC activation (3.3.1.3) it is important to stress several points. Bovine TSH and not hTSH was used to test the effects of the monoclonals on these 2 experimental systems. Also, neither of the two ^{125}I labeled monoclonals, TS28 or GC73, showed specific binding to sucrose gradient purified thyroid plasma membrane. A maximum of ~50,000 cpm of ^{125}I antibodies was added to 100 μg of purified thyroid plasma membrane and binding was less than 2% in both cases.

Against this background it is reasonable to suggest that upon interaction with the receptor, bTSH undergoes conformational modifications such as to expose epitopes which are now recognized by the monoclonal antibodies which are otherwise highly specific for hTSH in solution.

That GC73 added at the same time as the ligand can inhibit the binding of ^{125}I-bTSH to thyroid plasma membranes seems to indicate that this antibody reacts with an epitope probably β subunit related which becomes exposed upon binding to the receptor. The lack of interference in this assay by TS28 suggests either that binding dependent conformational changes in bTSH does not result in a epitope recognizable by TS28 or, else that if such a site is generated, it is not relevant to the TSH binding.

The interference by both antibodies with the TSH-driven AC activation demonstrates that both epitopes bound may have important functions in activating signal delivery. Interestingly, when TS28 or GC73 were preincubated in the presence of the membrane preparations before the addition of TSH they inhibited the TSH-driven AC activation. In contrast, when they were added

simultaneously with bTSH they further enhanced the hormone's effects. The augmentation in TSH-driven AC activity caused by the simultaneous addition of either monoclonal antibody may be interpreted as the occurrence of a "cross-linking" by the monoclonals of the receptor bound bTSH permitting receptor aggregation and clustering as has been shown for other receptors (Schechter, Hernaez, Schlessinger and Cuatrecasas, 1979; Schlessinger, 1980).

The incubation of the monoclonals at 37°C before adding TSH may have allowed them to adopt an "active conformation", such that they promptly bind to bTSH as soon as it binds to the receptor and exposes epitopes recognized by these monoclonals. The fact that radiolabeled monoclonals were unable to bind porcine thyroid membranes in any significant quantity as compared to N-rat-Ig allows one to exclude the possibility that the monoclonals recognize specific epitopes at the level of the TSH receptor.

These studies suggest that upon binding to the receptor TSH appears to undergo considerable conformational changes. These changes appear to be essential for the induction of adenylate cyclase activation. It also appears that some hormone domains are more important that others for receptor binding. These data were crucial in suggesting the use of these monoclonals to raise anti-idiotypic antibodies that might be expected to represent "internal images" of the TSH subunit sites that interact with the receptor.

3.3.2. Properties of the interactions between TSH-subunit specific idiotypic/anti-idiotypic antibodies

Immunoglobulins obtained from the rabbits immunized with either TS28 or GC73 were each tested alone and in all possible combinations. Only one combination exhibited biological activity. This corresponded to Igs from one rabbit immunized with TS28 (α-anti-id) and one rabbit immunized with GC73 (β-anti-id). These two Igs in combination were used in the experiments to be described.

3.3.2.1. Binding of anti-idiotypic antibodies with TS28 and GC73

The anti-idiotypic antibody preparations were tested for binding to the complementary monoclonal antibodies. α-anti-id bound 31.6% (SD 2.1) of the ^{125}I-TS28 (~50,000 cpm). β-anti-id bound 58.6% (SD 16.0) of the ^{125}I-GC73 (~50,000 cpm). NR-Ig used as control bound only 18% of the radioactivity in the case of ^{125}I-TS28 and 8% of the radioactivity in the case of ^{125}I-GC73. Radiolabeled N-rat-Ig bound 20.5 (SD 3.5) of the α-anti-id and 14.4 (SD 4.7) of the β-anti-id added (Table 3.5).

3.3.2.2. Effect of the anti-idiotypes on the binding of ^{125}I-hTSH to TS28 and GC73

To establish if α-anti-id and β-anti-id Ig preparations comprise "internal image" antibodies their effect on the binding of ^{125}I-hTSH to TS28 and GC73 was studied. α-anti-id in concentrations of up to 500 μg/ml was unable to inhibit the binding of the radiolabeled ligand to TS28 or GC73. By contrast β-anti- id displaced the binding of ^{125}I-hTSH to GC73 in a dose dependent manner. At a dose of 500 μg/ml 48.2% of the label was displaced. β-anti-id had no effect in displacing radiolabeled ligand bound to TS28 (Table 3.6). These findings suggest that only β-anti-id represents a true "internal image" anti-idiotype.

3.3.3. Bioactivity of TSH-subunit specific anti- idiotypic antibodies

3.3.3.1. Effect of anti-ids on the binding of ^{125}I-bTSH to porcine thyroid plasma membranes

Neither α-anti-id nor β-anti-id nor their combinations were capable of inhibiting the binding of ^{125}I-bTSH to porcine thyroid plasma membranes above that caused by NR-Ig. Porcine thyroid plasma membrane bound 14% (SD 1.3) of the added ^{125}I-bTSH (~20,000 cpm ~0.5 pM). Igs from the rabbits immunized with GC73 (250 and 500 μg/ml) and of those immunized with TS28 (250 and 500 μg/ml) were used for competition assays. Neither of the four Igs altered the binding of the ^{125}I-bTSH to the membrane preparations. NR-Ig (250 and 500 μg/ml) was also

Table 3-5: Interaction of monoclonal antibodies TS28 and GC73 with anti-idiotypes.

	CPM $\bar{X}$	Binding (%) ± S.D.
^{125}I-TS28 (TC)*	45000	
+ α-anti-id (500 ug/ml)	14219	31.6 ± 2.1
+ NR-Ig (500 ug/ml)	8110	18.0 ± 5.1
^{125}I-GC73 (TC)*	54000	
+ β-anti-id (500 ug/ml)	31646	58.6 ± 16.0
+ NR-Ig (500 ug/ml)	4209	7.8 ± 3.1
^{125}I-N-rat Ig (TC)*	49125	
+ α-anti-id (500 ug/ml)	10086	20.5 ± 3.5
+ β-anti-id (500 ug/ml)	7087	14.4 ± 4.7

*: Total counts; Percent of binding from total counts.

N = 6 ; (2 parallels for each experiment)

Table 3-6: Effects of α-anti-id and β-anti-id on the binding of ^{125}I-hTSH to monoclonal antibodies TS28 and GC73.

	Binding (%) ± SEM	Inhibition (%)
^{125}I-hTSH + TS28 (100 ug/ml)	65 ± 0.5	
+ TS28 + α-anti-id (125 ug/ml)	71 ± 0.5	
+ TS28 + α-anti-id (250 ug/ml)	70 ± 0.5	
+ TS28 + α-anti-id (500 ug/ml)	71 ± 1.5	
^{125}I-hTSH + GC 73 (100 ug/ml)	63 ± 2	
+ GC73 + β-anti-id (125 ug/ml)	59 ± 1	6
+ GC73 + β-anti-id (250 ug/ml)	54 ± 1	14
+ GC73 + β-anti-id (500 ug/ml)	32 ± 0.5	48*

*: p 0.05 compared to total bound in absence of anti-idiotypes (Mann Whitney U Test).

N = 6 (2 parallels each)

unable to cause displacement of the radioactivity bound. Four possible combinations between antibodies raised against TS28 and those raised against GC73 (equimolar mixtures for final concentrations of 250 and 500 μg/ml) were also unable to displace ^{125}I-bTSH from binding to porcine thyroid plasma membranes. ^{125}I-bTSH binding in the presence of the α-anti-id (500 μg/ml) was 14% (SD 1.5). In the presence of β-anti-id (500 μg/ml) the binding was 14.7% (SD 1.7). With NR-Ig (500 μg/ml) the binding was comparable at 13.7% (SD 2.3).

3.3.3.2. Binding of radiolabeled α-anti-id and radiolabeled β-anti-id to porcine thyroid plasma membranes

Examination of the binding of ^{125}I labeled α and β-anti-ids to porcine thyroid plasma membrane was done using 100 μg of purified porcine thyroid plasma membranes and ~50,000 cpm of the labeled antibodies (2.11). Thyroid plasma membrane bound 3.1% of the ^{125}I-α-anti-id. This binding was displaced in a dose dependent manner by both unlabeled bTSH and unlabeled α-anti-id. bTSH (500 mU/ml) displaced 35% of the radioactivity bound (Fig 3.9). Unlabeled α-anti-id (1 mg/ml) displaced 41% of the label bound (Fig 3.10).

Using ^{125}I-β-anti-id 2.4 % bound to thyroid plasma membranes. Both unlabeled bTSH and unlabeled β-anti-id produced dose dependent displacement of the labeled ligand. bTSH (500 mU/ml) displaced 34% (Fig 3.9) and unlabeled β-anti-id (1 mg/ml) 66% of the radioactivity bound (Fig 3.10).

In other experiments equal amounts of α-anti-id and β-anti-id were added in the same test tube to achieve an amount of radioactivity equal to that obtained with each antibody alone (~50,000 cpm). The binding of the antibodies to thyroid plasma membrane was additive; 5.2% of the radioactive antibodies bound to thyroid plasma membrane preparations. Forty percent of the radioactivity was displaced by 500 mU/ml of bTSH (Fig 3.9). In experiments done in the same batch of porcine thyroid plasma membrane 1.7% of ^{125}I-NR-Ig (~50,000 cpm) was found to bind and native bTSH displaced only 8% of the radioactivity bound (Fig 3.9).

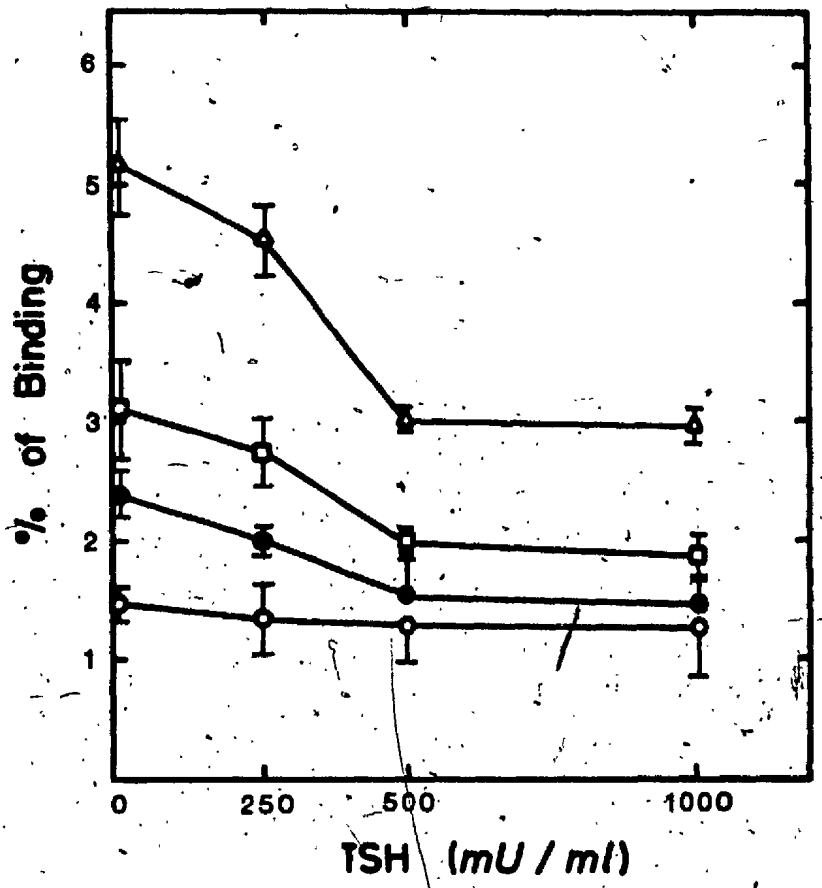


Figure 3-9: Binding of ^{125}I-α-anti-id and ^{125}I-β-anti-id to thyroid plasma membranes and displacement by bTSH.

(Δ) ^{125}I-α-anti-id + ^{125}I-β-anti-id. (□) ^{125}I-α-anti-id.

(●) ^{125}I-β-anti-id. (O) ^{125}I-NR-Ig

Bars represent ± S.D. from mean.

N = 5 (2 parallels each)

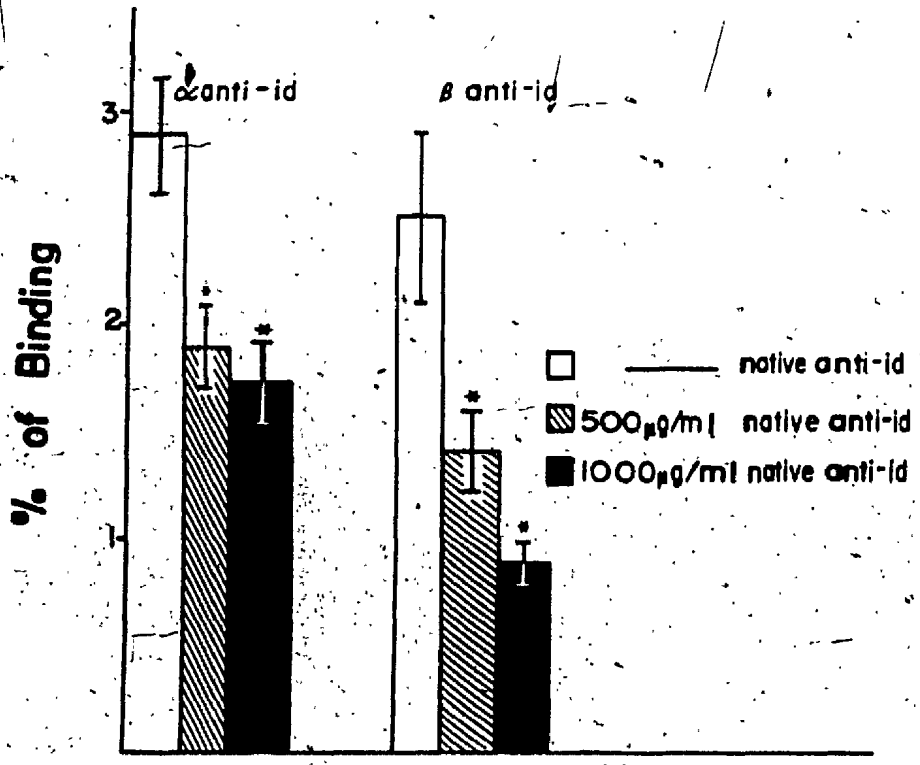


Figure 3-10: Inhibition of ^{125}I-α-anti-id and ^{125}I- β-anti-id binding to porcine thyroid plasma membranes, by the corresponding native anti-id.

*: $p < 0.05$ compared to the total bound in absence of native anti-id. (Mann Whitney U Test).

N = 5; Bars represent ± S.D. from mean.

These results show that α and β-anti-ids bind to thyroid plasma membranes. The displacement of this binding by native TSH suggests specificity for its receptor. The fact that the binding of the two antibodies appears to be independent when tested in combination suggests the presence of two distinct binding sites. Finally, as shown in figure 3.9, it can be stated that the binding of ^{125}I-NR-Ig to plasma membranes is lower than the residual bound anti-ids even in the presence of large amounts of native bTSH. This suggests either that part of the anti-ids are bound to membrane sites other than those binding TSH or more likely that a fraction of the antibodies have bound irreversibly. The latter possibility is strengthened by the fact that the residual amount of bound radioactivity is higher in the tests in which the combination of antibodies was used.

3.3.3.3. Effect of α-anti-id and β-anti-id on the thyroid plasma membrane-bound AC activity

To determine the biological activity of these antibodies, their effect on the activity of the thyroid plasma membrane bound AC was investigated at 37°C in the presence of 10 μM Gpp[NH]p (2.5). When 250 μg/ml of α-anti-id or β-anti-id were tested alone they caused inhibition of the basal activity of AC; 5.8% in the case of α-anti-id and 7.5% for β-anti-id (Fig 3.11 shows the results of a typical experiment). When both antibodies were tested in equimolar combinations (125 μg/ml of each) the mixture produced a marked enhancement of the AC activity, 90% stimulation was observed (Fig 3.11). This value was equivalent to that induced by 200 mU/ml of bTSH. bTSH (250 mU/ml) produced a 101 % stimulation from basal (Fig 3.11). NR-Ig (250 μg/ml), used as control, caused an approximately 55 % inhibition from the basal value.

These results suggest a cooperative interaction between these two antibodies in promoting AC activation, in that either of them alone, depressed the AC activity but in combination they were able to enhance it much more than additively. Seemingly the binding of one anti-id increases the affinity of the other for specific sites on the thyroid plasma membrane.

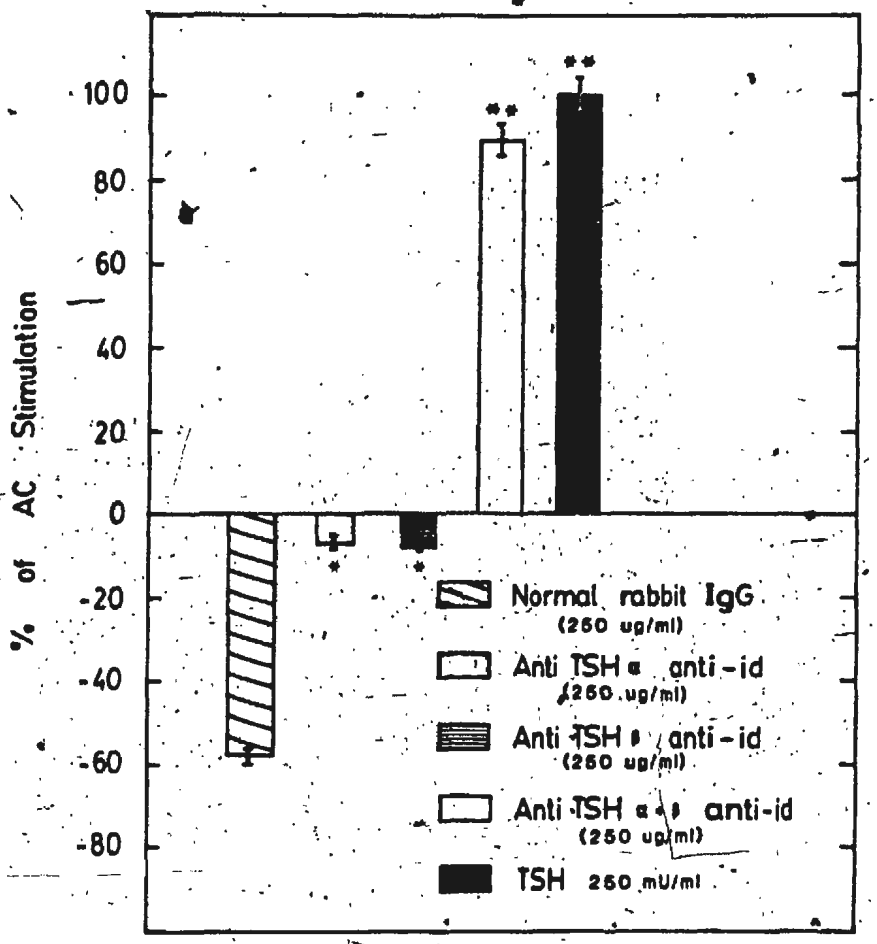


Figure 3-11: Effect of TSH, α-anti-id, β-anti-id and NR-Ig on the Gpp(NH)p dependent AC activation.

*: $p < 0.05$ compared to NR-Ig.

**: $p < 0.05$ compared to basal.

N = 6.

3.3.3.4. Effect of α and β-anti-ids on the iodide uptake by dispersed thyroid epithelial cells

Radioactive iodide uptake by thyroid epithelial cells was measured after these cells were incubated for 4 hours with either bTSH, α or β-anti-ids or NR-Ig and then in the presence of ^{131}NaI for 30 minutes. The time of 4 hours was chosen because of the results obtained in previous experiments (3.1.5), which demonstrated optimal uptake after this incubation period. α-anti-id in concentrations of up to 500 μg/ml had no effect on stimulating radiolabeled iodide uptake. β-anti-id in similar concentrations produced a 7.8% uptake. α-anti-id plus β-anti-id (250 μg/ml of each) produced a 8.33% uptake. NR-Ig (500 μg/ml) had no effect. bTSH (100 mU/ml) produced a 10% uptake which was not improved when 200 mU/ml of bTSH were used (Table 3.7)

3.3.3.5. Effect of α-anti-id and β-anti-id on the organization of cultured thyroid epithelial cells

The effect of the anti-idiotypes on the organization of cultured thyrocytes was studied over a period of 7 days. The cells cultured in the presence of α-anti-id (250 μg/ml) or NR-Ig (250 μg/ml) grew as uniform monolayers. β-anti-id (250 μg/ml), however promoted the organization of small follicular structures. The addition to the culture medium of equal amounts of α-anti-id and β-anti-id (125 μg/ml of each) promoted the organization of larger follicular structures comparable but consistently smaller than those obtained when cells were cultured with 100 mU/ml of TSH (Fig 3.12). This information was obtained by simple observation. No specific method to measure the sizes of the follicular structures was deviced. The follicular structures formed were active in producing colloid by day 7 in culture as is demonstrated by the abundance of PAS positive material contained in the central area of the newly formed follicles (Fig 3.13).

Table 3-7: Effect of α-anti-id, β-anti-id and NR-Ig on the radio-iodine uptake by porcine thyrocytes after 4 hours of incubation.

Concentrations		^{131}I uptake (%) ± SEM	Stimulation (%)
Control		5.3 ± 0.5	100
TSH	100	10.0 ± 1	187.6*
mU/ml	200	10.6 ± 0.8	198.9*
α-anti-id	250	5.9 ± 0.3	110.7
ug/ml	500	5.9 ± 0.2	110.7
β-anti-id	250	6.1 ± 0.5	113.9
ug/ml	500	7.8 ± 0.4	146.1
α-anti-id	250	8.1 ± 1.3	152.7*
+ β-anti-id	500	8.3 ± 0.2	156.2*
NRIg	250	6.7 ± 0.3	126.2
	500	6.0 ± 0.1	113.1

*: p 0.05 from basal or compared to those obtained in presence of NR-Ig (Mann Whitney U Test).

N = 4 (2 parallels each)

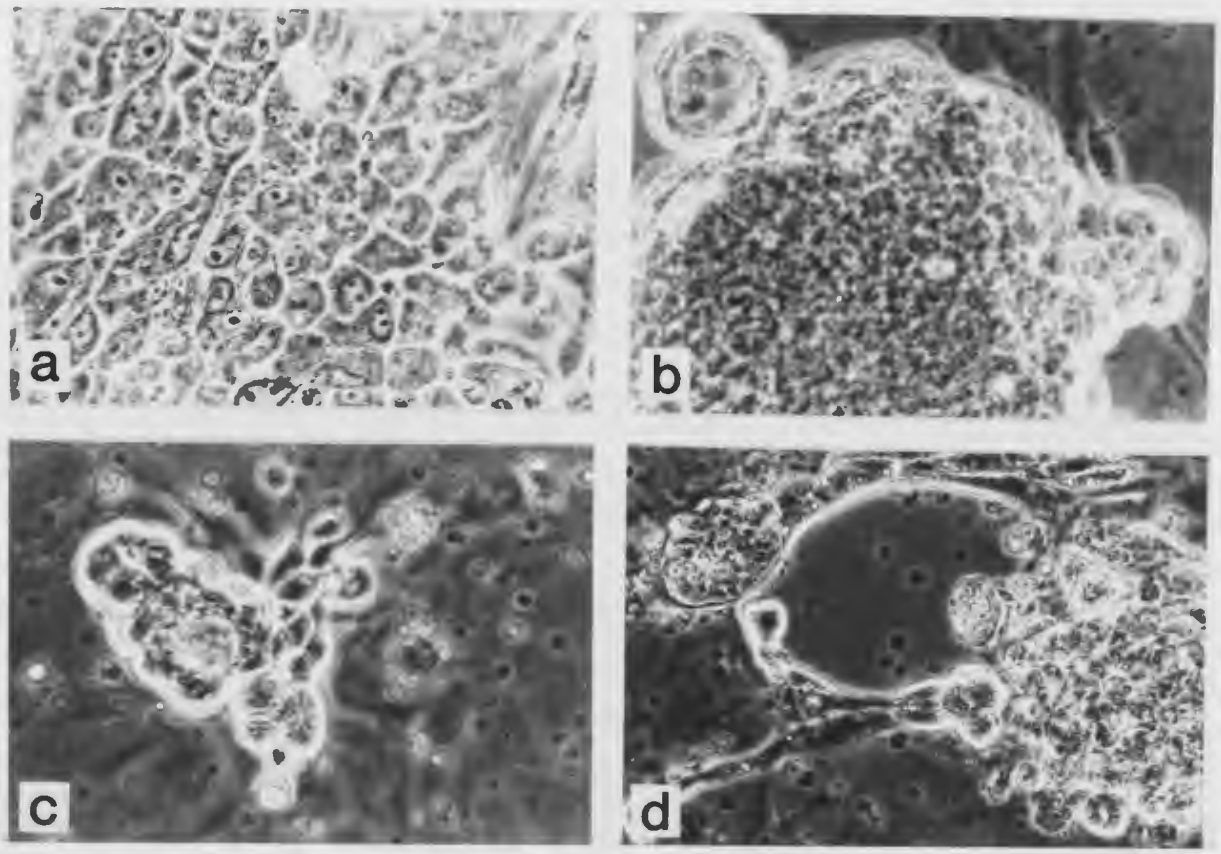


Figure 3-12: Effect of TSH and TSH subunit specific anti-idiotypic antibodies on the organization of thyrocytes after 7 days in culture.

a: α-anti-id (similar picture was obtained with NR-Ig). b: TSH. c: β-anti-id. d: α-anti-id + β-anti-id. Follicular structures are observed in b and d. Smaller follicular formation can be observed in c.

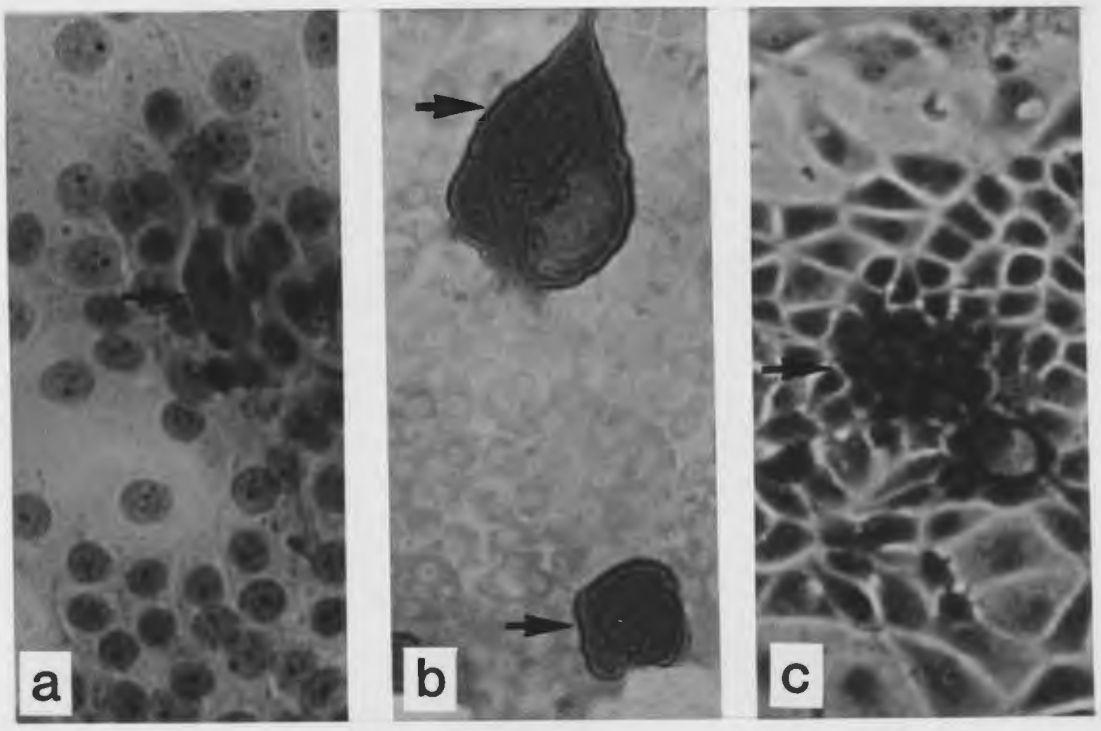


Figure 3-13: PAS and PAS-haemalum staining of thyrocytes cultured in the presence of TSH for seven days.

a: PAS-haemalum. b: PAS. c: PAS-haemalum.

Arrows indicate the PAS positive colloid in a and the follicular structures in b and c. (Staining and pictures kindly performed by Dr. G.E. Fahraeus-van Ree, Thyroid Research Laboratory, M.U.N., Nfld.).

3.3.3.6. Interaction of α-anti-id and β-anti-id with protein blots of thyroid plasma membranes

The final experiment carried out with α and β-anti-ids investigated the interaction of individual antibodies or their combinations with protein blots of porcine thyroid plasma membrane, to test their capacity to bind to the immobilized receptor band. It was observed that neither α-anti-id (1 mg/ml) nor β-anti-id (1 mg/ml) alone, produced positive reactions at the Mr~ 197,000 band level (Fig 3.14, lanes 3 and 4). The combination of equal amounts of both antibodies (500 μg/ml of each), however, produced a clearly positive band at the Mr ~ 197,000 holoreceptor protein band (Fig 3.14, lane 5). It appears that the binding of the individual anti-ids to this band was of relatively low affinity and they could have been dissociated with the high salt concentration present in the buffer used (200 mM NaCl). The combination of the two anti-idiotypes appeared to have enhanced each others affinity for the receptor in keeping with their cooperative interaction. It is implicit in this explanation that the immobilized receptor is in some way capable of undergoing certain type of conformational changes upon interaction with one antibody to increase the affinity of binding to the site recognized by the second antibody. It could also be possible that the two antibodies are capable to interact with each other in a certain fashion that provides a conformation with a higher affinity for the receptor.

3.3.4. Discussion of the results investigating the bioactivity of the Alpha-anti-id and the Beta-anti-id antibodies

The findings described suggest that these antibodies deliver two separate but cooperative signals upon binding to the receptor. These are likely necessary for the conformational changes which trigger the receptor mediated events. That β-anti-id transmits the hormone specific signals is suggested by the fact that it exhibited some biological activity on its own in some of the situations examined. By contrast α-anti-id does not seem to have such an effect, but appears to increase the binding activity of β-anti-id. This can be achieved by modulation of

the TSH-R such as to enhance the binding affinity for β-anti-id. Another possibility is that α-anti-id modulates β-anti-id to produce a conformation recognizable to the TSH receptor. This function appears to require certain specific structure because other antibodies tested were unable to trigger similar activation as did this mixture of anti-idiotypes. The latter could even explain why a "non-internal image" anti-idiotype is capable of enhancing the "internal image" anti-idiotype in producing its biological activities.

Another important observation is the functional dissociation between the inability of these antibodies to inhibit ^{125}I-bTSH binding to thyroid plasma membranes and their positive influence on AC activation. These results are consistent with the segregation of each function to different receptor domains. This proposal is substantiated by the finding that some monoclonal antibodies raised against the TSH-R inhibited ^{125}I-bTSH binding but were unable to induce AC activation, while others had the opposite effect (Valente, Vitti, Yavin, Yavin, Rotella, Grollman, Toccafondi and Kohn, 1982).

In summary the anti-TSH subunit specific anti-idiotypic antibodies represent in combination, TSH-R specific agonistic antibodies, capable of binding and of activating post binding events. The results obtained suggests that the anti-idiotypic antibody approach described here represents a good model for the study of the roles TSH subunits may have in the hormone-receptor interaction. The data obtained suggests that the β subunit has a particularly important role in specific signal delivery. The α subunit may function as a stabilizer which maintains the appropriate subunit conformation to allow optimal interaction with the receptor.

3.4. BIOSYNTHESIS OF THE TSH RECEPTOR

The mixture of α-anti-id and β-anti-id described above (3.3) was shown to be specific for the TSH-R. As shown above (3.3.3.6), the combination of these antibodies interacted with the Mr~ 197,000 TSH holoreceptor band on sucrose gradient purified porcine thyroid plasma membrane protein blots using the enzyme linked immuno-binding technique previously described (2.17). This property was to be used, therefore, to detect the TSH-R in a system designed to study the rate of receptor synthesis and its turnover in biosynthetically labeled thyrocytes in vitro (2.18).

3.4.1. Interaction of the mixture of Alpha-anti-id and Beta-anti-id with protein blots of porcine thyrocyte whole cell lysate

The usefulness of this system to study the synthesis and turnover of the receptor was investigated first by testing the ability of the mixture of α-anti-id and β-anti-id to identify specific receptor related protein bands on protein blots of thyrocyte whole cell lysate. When this experiment was carried out, the result of the interaction of these antibodies with the whole cell lysate protein blots showed a great number of positive bands (Fig 3.15, lane 2). This finding was in sharp contrast to the almost "single band" positive reaction obtained when purified plasma membrane was used (Fig 3.14, lane 5). Several of these bands were also positive when NR-Ig was used in the system indicating their non-specific nature. It was considered possible that the positive responses observed in some of these bands may be generated by naturally occurring antibodies against different intracellular components found in the control rabbit serum.

For a definitive identification of the receptor related bands among this great number of unrelated proteins, inhibition studies were done. Protein blots were preincubated with either bTSH (1 U/ml), HCG (1000 U/ml) or insulin (10 U/ml) prior to the addition of the antibody mixture. Control experiments consistently showed a faintly positive but still easily identifiable band at the Mr~ 197,000

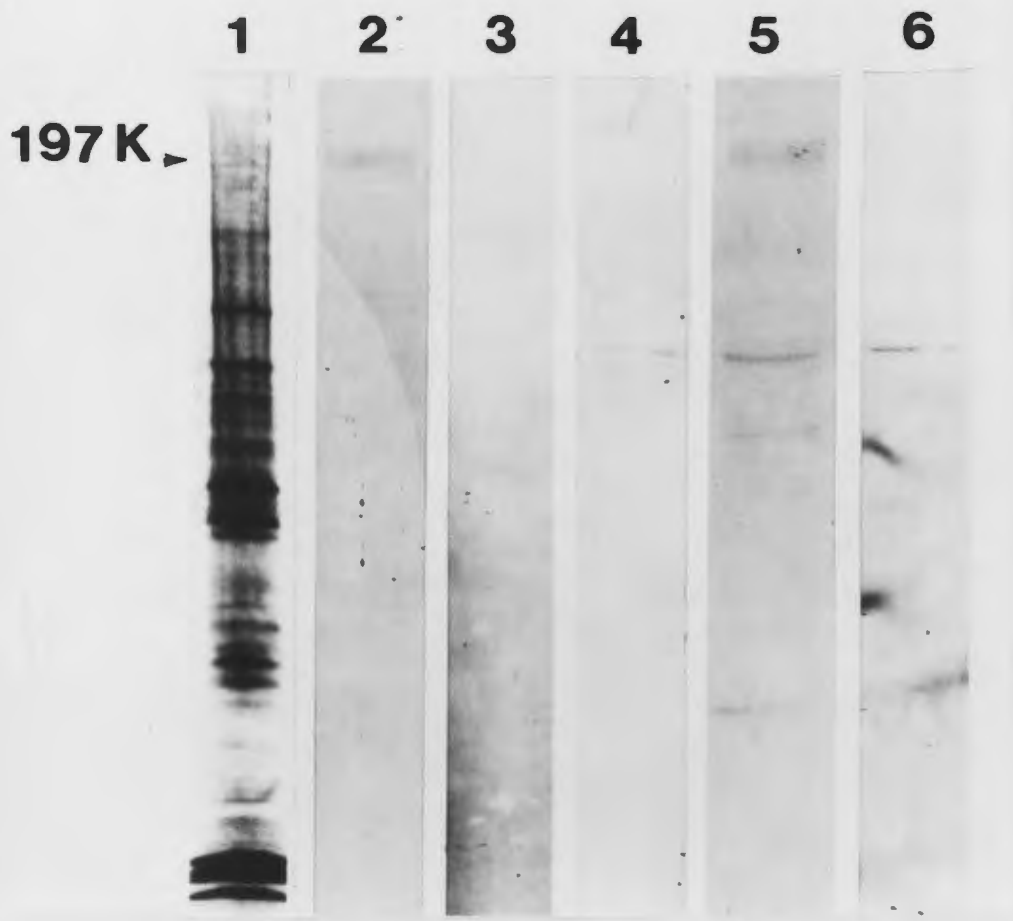


Figure 3-14: Interaction of TSH, TSH subunit specific anti-idiotypic antibodies and NR-Ig wih thyroid plasma membrane protein blots.

1: Coomassie blue stained protein bands. 2: TSH (1 U/ml). 3: α-anti-id (1 mg/ml). 4: β-anti-id (1 mg/ml). 5: α-anti-id + β-anti-id (1 mg/ml). 6: NR-Ig (1 mg/ml). Note the positive bands at the $M_r \sim$ 197,000 in lanes 2 and 5.

level (Fig 3.15, lane 2). This band completely disappeared in papers pre-treated with bTSH (Fig 3.15, lane 3), HCG partially diminished its intensity (Fig 3.15, lane 5) and insulin had no effect (Fig 3.15, lane 4). Other bands at the level of the Mr~105,000 and Mr~70,000 were also displaceable with bTSH suggesting that they were also related to the receptor proteins (Fig 3.15, lanes 2 and 3). Their appearence, however, was inconsistent in that they were present in some experiments while they were absent in others. Also a great number of unrelated bands localized in the surrounding areas made detection as well as determination of the radioactive content unreliable. These reasons precluded their use for further experiments. Under these circumstances only the holoreceptor band was considered suitable to use in the studies of the synthesis and turnover of the TSH-R.

3.4.2. Synthesis of the TSH-R

In initial experiments the rate of synthesis of the TSH-R was explored in thyrocytes cultured in the presence of TSH (100 mU/ml) for 12 hours prior to labeling and in thyrocytes cultured in the absence of TSH. These two groups of cells were later biosynthetically labeled with medium containing [^{3}H]-leucine and processed as previously described (2.18). The TSH-R band was identified as described in 2.19. Basal levels of [^{3}H]-leucine incorporation were considered as the level of radioactivity detected in the receptor band at "0 time" incubation, i.e. cells chilled at 4°C as soon as the [^{3}H]-leucine was added. Within 30 minutes of the addition of the [^{3}H]-leucine supplemented medium a significant amount of labeled receptor was detected in both groups of cells. The maximum level of stimulation of receptor labeling was detected after 3 hours of incubation with [^{3}H]-leucine enriched medium in TSH stimulated cells. The control cells achieved a similar level of receptor labeling but this result was not achieved until the fifth hour of incubation with the radioactive medium (Fig 3.16 shows an experiment with 4 parallels. When the experiment was repeated similar results were obtained.) Approximately one hour after the maximal labeling levels were achieved, a steady decline in the percentage of specific labeling was observed in

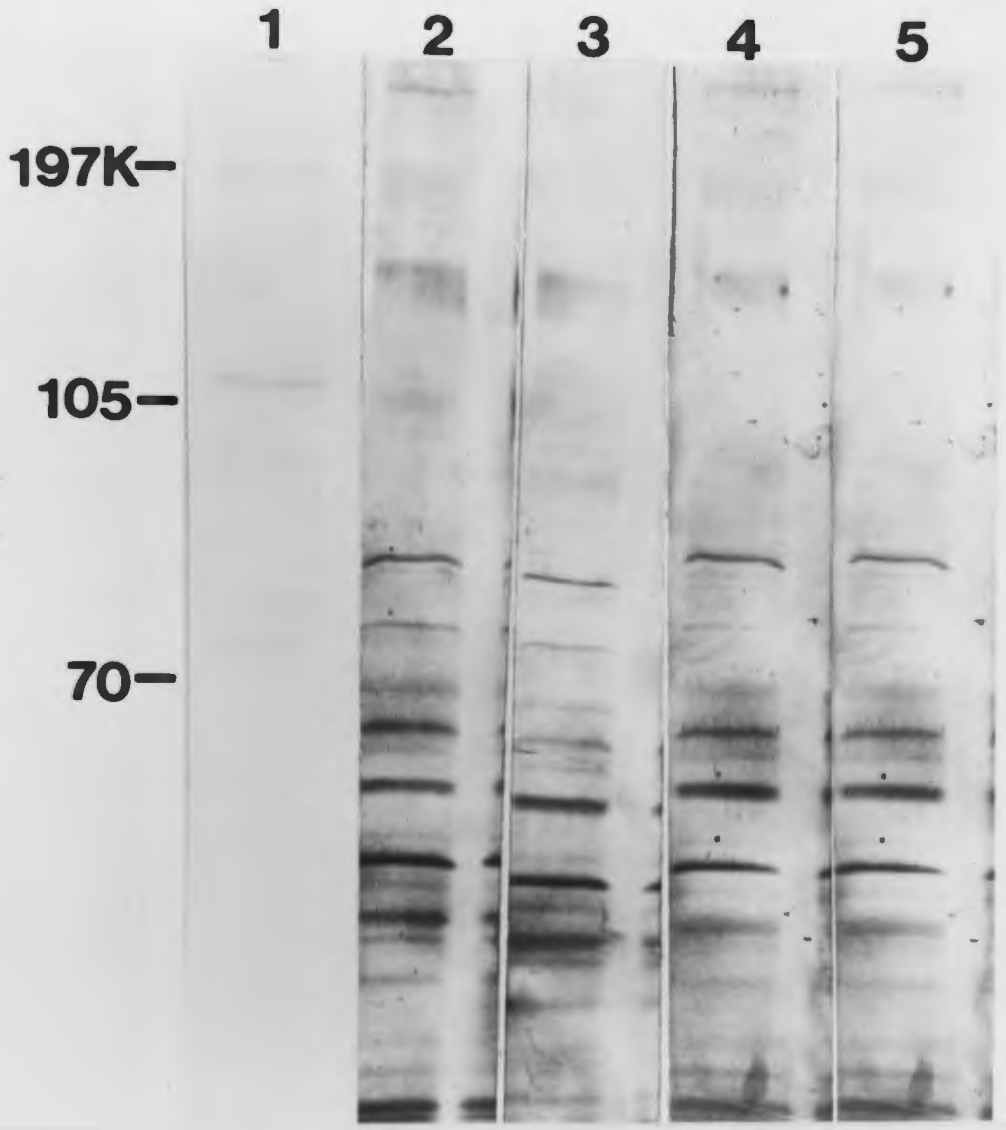


Figure 3-15: Interaction of TSH and TSH subunit specific anti-idiotypic antibodies with thyrocyte whole cell lysate protein blots.

1: TSH. 2: α-anti-id + β-anti-id. 3: α-anti-id + β-anti-id in blots pretreated with TSH. 4: α-anti-id + β-anti-id in blots pretreated with insulin. 5: α-anti-id + β-anti-id in blots pretreated with HCG. Positive bands at the $M_r \sim$ 197,000 are seen in lanes 1, 2, 4 and 5.

both groups of cells and this continued up to 8 hours the longest period of observation (Fig 3.16).

Some important features of figure 3.16 deserve comment. A "left shift" of the curve representing the rate of receptor synthesis in TSH stimulated cells when compared to non-stimulated cells is seen. This suggests that TSH promotes an acceleration of the rate of synthesis of its receptor. The most significant difference between the 2 curves is obtained at 3 hours of labeling. With this in mind the cells used in all other experiments investigating the rates of synthesis as well as the turnover were labeled for 3 hours.

3.4.2.1. Time dependent effects of TSH on the synthesis of the TSH-R

The effects on receptor synthesis of the addition of TSH to the culture medium for different time intervals was next examined. It was observed that when TSH (100mU/ml) was added for only the period of labeling (3 hours) the rate of receptor synthesis was no different from that of controls (Fig 3.17). However, when TSH was present for 12 hours prior to labeling as well as during labeling (a total of 15 hours) the rate of receptor synthesis was clearly accelerated (Fig 3.17). Even though the lag time kinetics for this response was not studied in detail the results obtained in these experiments suggest that for acceleration of receptor synthesis to occur, it is necessary for TSH to be in contact with the thyrocytes for a relatively prolonged period of time.

3.4.2.2. Effects of different doses of TSH on the synthesis rate of the TSH-R

The effects of different doses of TSH on receptor synthesis were next studied to determine if the rate of synthesis was dose dependent. The maximal acceleration was obtained with 100 mU/ml of TSH, 250 mU/ml initially produced an accentuated shift of the synthesis rate curve towards the left, but a premature falling off was observed. At 500 mU/ml the response obtained was almost identical to that obtained at 50 mU/ml (Fig 3.18). The results shown represent the mean of four samples at each point, the standard deviations are not included

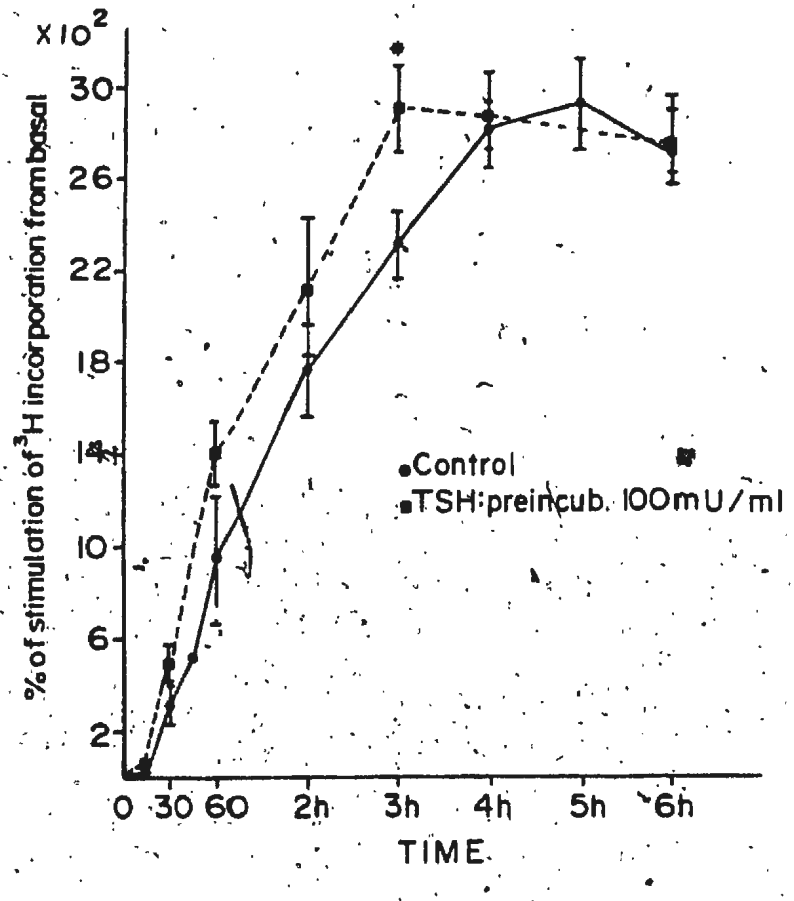


Figure 3-16: Effect of TSH (12 hours of preincubation) on the synthesis rate of the TSH-R.

Receptor synthesis starts at 30 minutes in both groups. The maximal labeling is achieved at 3 hours in TSH simulated cells, and at 5 hours in control cells. Note the "left shift" of the synthesis rate curve in TSH stimulated cells.

N = 4; *: $p < 0.032$ compared to the value of control cells (Mann Whitney U Test); bars represent ± S.D. from mean.

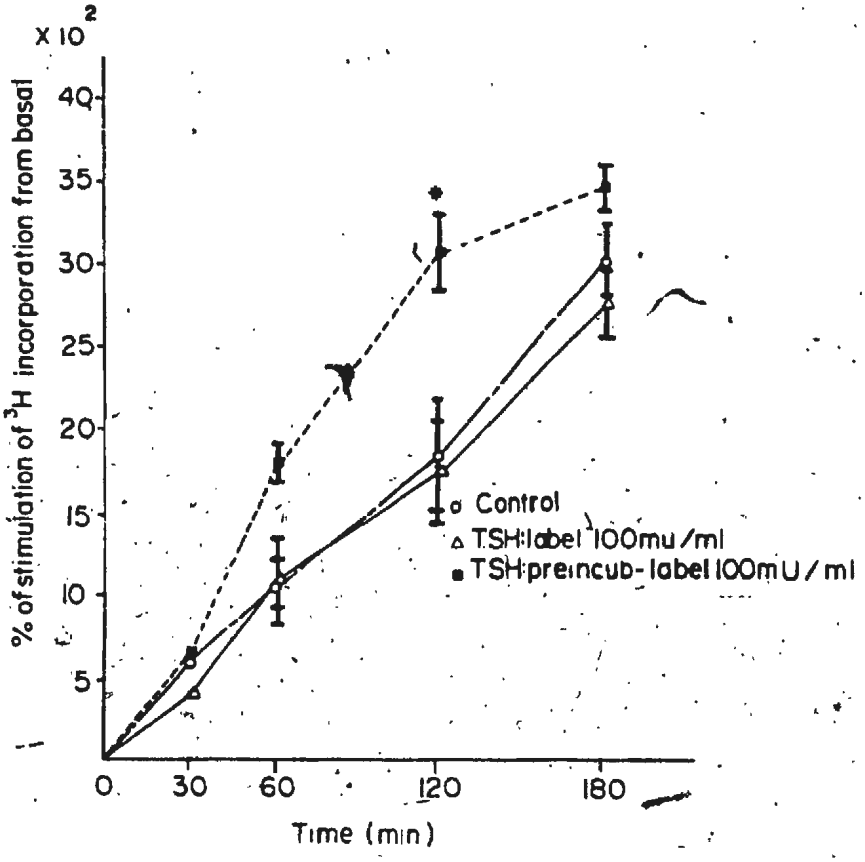


Figure 3-17: Effect of short term TSH stimulation (3 hours) vs. medium term stimulation (15 hours) on the TSH-R synthesis rate. Medium term stimulation accelerates the rate of synthesis, short term stimulation has no effect. N = 4; *: $p < 0.05$ as compared to control values at similar times (Mann Whitney U Test); bars represent S.D. from mean.

due to the closeness of the different curves. Similar results were obtained in repeated experiments. Increasing doses of TSH appear to stimulate acceleration of the receptor synthesis up to 100 mU/ml. Past this dose the responses are diminished, i.e. there is a non-linear dose responsiveness for TSH on receptor synthesis.

3.4.2.3. Determination of stable and free radioactive leucine pools in cultured thyrocytes

Because TSH stimulates amino acid metabolism in thyroid cells (Dummont and Vassart, 1978) it is possible that the leucine pool in TSH stimulated cells might be different from that of control cells. To investigate this possibility the total leucine content as well as the free leucine content were measured in cells stimulated with TSH and in control cells (2.20). Two groups of TSH stimulated cells were studied. One group received TSH for 3 hours (during labeling) and others received TSH for 15 hours (12 hours prior to labeling and during labeling), to duplicate the conditions used previously (3.4.2, 3.4.2.1, 3.4.2.2). The total leucine content in control and TSH stimulated cells was slightly higher in TSH stimulated cells than in control cells. This difference however did not achieve statistical significance ($p>0.5$)(Table 3.8). The levels of free leucine were slightly lower in cells that had been stimulated with TSH for 15 hours when compared to those that were stimulated for 3 hours. The difference, again, was not found to be statistically significant ($p>0.5$) (Table 3.8) The results verify that the observed acceleration of receptor synthesis is real and not an artifact due to an alteration of the stable leucine pool in TSH stimulated cells.

3.4.3. Turnover of the TSH receptor

Pulse-chase experiments were done to study the turnover rate of the TSH-R. Thyrocytes were first biosynthetically labeled for 3 hours followed by a cold medium chase for varying periods of time. The effects of TSH stimulation on the turnover rate was carried out by the addition of 100 mU/ml of the hormone for various time intervals and at different stages of the procedure as described above

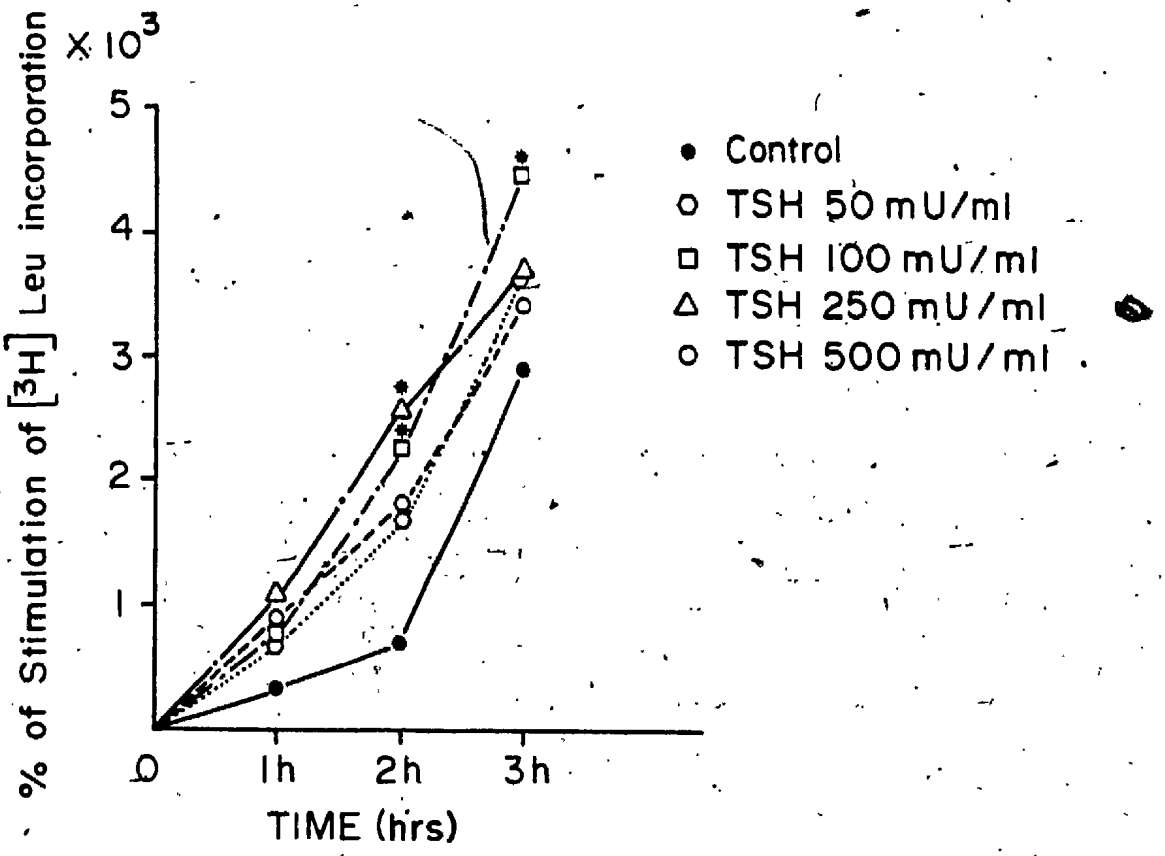


Figure 3-18: Effect of different doses of TSH on the synthesis rate of the TSH R. Responses to increasing doses of TSH indicate that the optimal does to obtain a high value at ± 3 h is 100 mU/ml.

N = 4; *: $p < 0.05$ as compared to control values at similar times (Mann Whitney U Test).

Table 3-8: Thyrocyte leucine pool.

	Total leucine content umol/10^7 cells ± S.D.	Free leucine pool pmol/ml ± S.D.
Control	993 ± 67	235 ± 10
TSH (3 hours)	1060 ± 33	193 ± 45
TSH (15 hours)	1147 ± 25	246 ± 6

(2.18). The data obtained from these experiments were fitted into exponential curves. The correlation index was calculated between 0.9 to 0.98 for the 4 curves obtained. The half lifes were calculated at the 50% point of the linear regression curve.

In control cells (cultured in the absence of TSH) the receptor had a calculated half life of 240 minutes (Fig 3.19 a).When TSH was added either during the 12 hours prior to labeling, 12 hours prior to labeling and labeling, or throughout the procedure (prior and during labeling and during the chase) the half life of the receptor was increased to a calculated mean value of 360 minutes (Fig 3.19 b,c and d). The presence of TSH in the medium appeared to contribute to prolonging the half life of the receptor but the length of the time for which the hormone was present did not appear to have any influence on the receptor's half life. It is likely that the prolongation of the half life of the TSH-R in the presence of TSH can at least be partially explained by the achievement of stable hormone-receptor complexes as has been previously described for the TSH receptor in isolated membranes (Brennan et al., 1980).

All the findings described so far regarding the synthesis and turnover of the TSH-R suggest a positive influence of TSH. These results seem to contradict most of the reports in the literature that suggest that TSH stimulation leads to receptor "down regulation" (Lissitzky, et al, 1973; Witte and McKenzie, 1981; Rapoport and Adams, 1976). These authors have measured receptor numbers as number of binding sites available for ^{125}I-TSH binding thus excluding all occupied receptors. The method used in the present studies can detect all the immunologically competent receptors that resolve at the Mr$\sim$ 197,000 protein band. This should include receptors that were occupied by hormone as well as some partially synthesized receptors that are immunologically identifiable. It is possible then, that the apparent difference in results are likely due to the different detection systems and that one group of findings does not necessarily contradict the other. To substantiate this contention the binding of radiolabeled TSH to viable

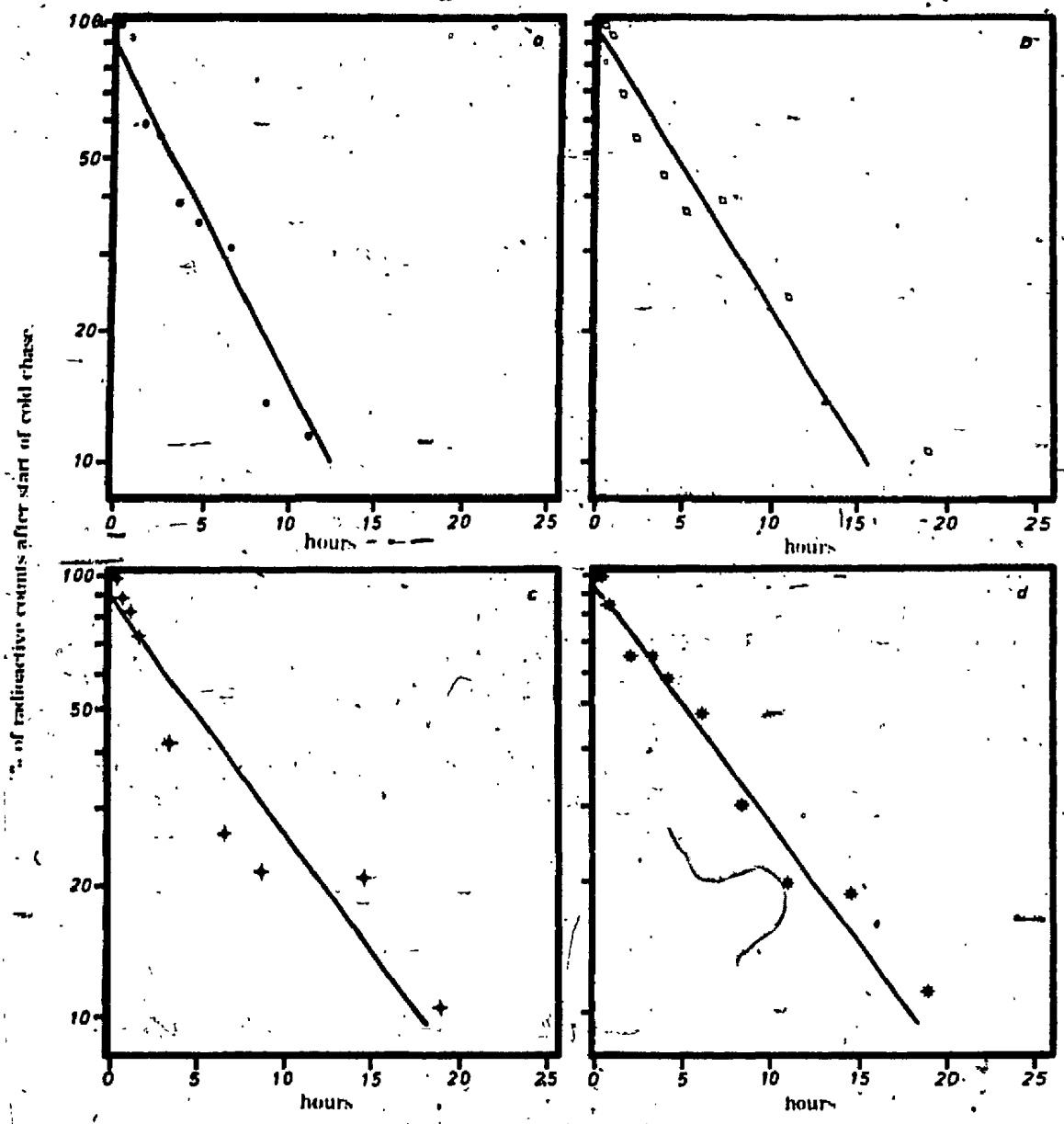


Figure 3-19: Turnover of the TSH-R

a: no TSH. b: TSH for preincubation.

c: TSH for preincubation and labeling. d: TSH for preincubation, labeling and chase.

100%: Maximal incorporation of (^{3}H) leucine into receptor protein after 3h of biosynthetic labeling;curve fitting: linear regression using a program that computes the least square fit of n pairs of data points.

Half life (t1/2) = value of x at y=50 from x=0

thyrocytes, prepared and treated in conditions similar to those in previous experiments (3.4.2, 3.4.3) was studied.

3.4.4. ^{125}I-bTSH binding to dispersed thyrocytes cultured in presence and absence of TSH

The binding of ^{125}I-bTSH to thyrocytes cultured in the presence of TSH for various time intervals as well as cultured in the absence of TSH was carried out.

Preliminary experiments were first carried out in cells cultured in the absence of TSH. $3X10^6$ cells were allowed to react with different amounts of labeled bTSH in the absence of unlabeled TSH. The following results were obtained: $3X10^6$ cells reacting with 50,000 cpm (~1.25 pM of ^{125}I-bTSH) bound a mean of 5750 cpm. The same number of cells and 100,000 cpm bound a mean of 14,930 cpm. At 150,000 cpm the mean bound was 16,780 cpm and at 200,000 a mean of 17,320 cpm bound to the cells added. These results represent the mean of three experiments. With these results in mind it was decided to procede using 100,000 cpm for the subsequent displacement studies.

In these experiments somewhat high doses of unlabeled TSH were required for the displacement of the radiolabeled ligand. During preliminary experiments lower doses of unlabeled TSH were used for displacement. At doses of 10 and 25 mU/ml only minimal displacement of the radiolabeled TSH was observed. At higher doses, however, as one can see from the results in table 3.9 there was a dose-response displacement to up to 250 mU/ml of unlabeled TSH, this indirectly demonstrating the specificity of the interaction between the thyrocytes and the radioligand.

In this system binding sites were found to be "down regulated" in TSH stimulated cells. The percentage of binding was diminished by 45 to 63% in cells cultured in the presence of TSH for 3 hours as compared to the binding obtained in control cells (Table 3.9). In cells cultured in the presence of TSH for 15 hours

binding was decreased by 20 to 54 % as compared to control cells (Table 3.9). The difference in binding between the two groups of TSH stimulated cells and control cells were found to be statistically significant ($p < 0.05$). No difference, however, was observed between the 2 TSH stimulated groups (Table 3.9).

It appears possible that this apparent reduction in binding is due to irreversible receptor occupancy. This proposition is also supported by results obtained in a study that investigated binding of radiolabeled TSH to membranes prepared from thyroid slices previously incubated in the presence or absence of TSH. It was observed that almost all the binding sites which were lost due to preincubation with TSH could be recovered by washing the membranes with 0.5 M NaCl to remove previously bound native TSH (Witte and McKenzie, 1981), at least up to a period of incubation of 14 hours. After that time it appeared that the complex had become irreversibly bound. It was impossible to test a similar experiment in our system due to obvious effects that the high concentration of NaCl or other methods used to dissociate bound hormone would have on the viability of the cells used.

3.4.5. Summary of the experiments on TSH-R biosynthesis

These experiments have provided evidence that TSH has a positive influence on the synthesis and turnover of its receptor in isolated porcine thyrocytes in vitro. These findings might prove important in explaining certain physiological responses of the thyroid gland when increased levels of circulating TSH or TSAb are present in individuals with either TSH producing tumors or Graves' Disease respectively. A positive influence of these stimulators on receptor biosynthesis can perhaps be anticipated when the known result of gland overstimulation is hyperthyroidism. This work reports a first step towards the understanding of this complicated subject. Obviously more work will be required before definite answers can be provided.

Table 3-9: Displacement by native TSH of ^{125}I-bTSH binding to dispersed thyrocytes cultured in the presence and absence of TSH.

	Control		TSH 3h (100 mU/ml)		TSH 15h (100 mU/ml)	
TSH mU/ml	cpm $\bar{X}$	binding %	cpm $\bar{X}$	binding %	cpm $\bar{X}$	binding %
0	14360	14.8	5320	5.5*	6610	6.8*
50	7398	7.7	4080	4.2*	6100	6.3
100	4272	4.4	3311	3.4	5076	5.3
250	2692	2.8	2822	2.9	4022	4.2
500	1941	2.0	1220	1.3	1562	1.6

TC: 96754 cpm

*: $p < 0.05$ (significant decrease in % binding as compared to % binding in control cells)

Control: cells cultured in absence of TSH

TSH 3h: cells cultured in presence of TSH for 3h.

TSH 15h: cells cultured in presence of TSH for 15h.

N = 6 (parallels)

cpm$\bar{X}$: corrected for background binding.

Chapter 4
CONCLUSIONS

1- Anti-idiotypic antibodies were raised to anti-TSH and to anti-TSH subunit specific monoclonal antibodies. Both of these types of anti-idiotypic antibodies were demonstrated to be agonists at the level of the TSH receptor. It is possible to produce anti-idiotypic antibodies with TSH-like activity.

2- These anti-idiotypic antibodies were useful in the detection of the receptor protein under specific experimental conditions.

3- The usefulness of anti-idiotypic antibodies in studying different characteristics of the hormone-receptor interaction has also been demonstrated. The study of the respective roles of the subunits of TSH in the hormone-receptor interaction was previously found to be difficult because of the known lack of activity of the dissociated subunits of the hormone. Anti-idiotypic antibodies raised against monoclonal antibodies specific for each subunit of TSH provided a useful model to study this interaction.

4- Studies with TSH-subunit specific anti-idiotypic antibodies favor important roles for both subunits of TSH in the hormone-receptor interaction, β subunit as transmitter of the hormone specific signals and α subunit as a stabilizer of the hormone-receptor interaction.

5- In the studies involving the interaction of spontaneously occurring anti-thyroid antibodies and protein blots of thyroid plasma membranes it was seen that Igs from patients with Graves' Disease and Hashimoto's thyroiditis both bind

an Mr~ 197,000 protein band, considered to be the TSH holoreceptor. The binding by Graves' Disease Igs is displaceable by TSH. The binding by HT Igs is not displaceable by TSH. These findings suggest that these 2 types of antibodies interact with the TSH receptor, but that the epitopes involved appear to be at different levels in the receptor.

6- Studies done on the synthesis and turnover of the TSH-R suggest that TSH has a definite role in the regulation of its own receptor. The hormone accelerated the synthesis of its own receptor and prolonged its half life. These results may be important in interpreting the onset of hyperthyroidism in cases of exceeding amounts of circulating TSH or the presence of TSAb. However, further experimentation is required.

7- Many factors appear to be of importance when raising anti-idiotypic antibodies. The choice of appropriate adjuvants, appropriate laboratory animals for immunization, time of sample collections and possible assays used to monitor the bioactivity of the products, are all of great significance. Some animals appear to be better that others in producing antibody materials. Rats appeared to be better than mice as TSH respondants. The time of sample collection is also very important. The difference between having an agonist antibody instead of an antagonistic antibody can depend only on the time of sample collection. So far, all these parameters were determined by simple observation. The adequate timing for sample collection was determined by multiple sampling.

8- A problem that one might envision which could be of particular importance is the limited quantity of antibodies produced by the immunized animal. Obviously, because of the polyclonal nature of the antibodies each new batch of antibodies produced will require extensive testing of their bioactivity before they can be utilized in other experimental situations.

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