Thyroid Growth Immunoglobulins

In Non-Toxic Goitre:

A Comparison Of Methods

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Abstract

Non-toxic goitre (NTG) is one of the commonest thyroid disorders world wide. This study followed, clinically and using ultrasonography, a small group of patients with NTG receiving a 6 month treatment course of sodium thyroxine and observed whether thyroxine would decrease significantly the thyroid volume or palpable size of the thyroid gland. This was not shown to be the case and no change could be demonstrated. Thyroid growth immunoglobulins (TGI) have been implicated in the development of NTG. The presence of TGI in the IgG from the subject groups was determined by their ability to stimulate proliferation in the Fischer rat thyroid cell line (FRTL5). Detection of proliferation in the FRTL5 cells was measured using 3 different methods.

The first was colourimetric and used the dye 3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), with which a significant increase in TGI was detected in the Multiple Sclerosis (MS) group when compared with the normal control (NC) and goitre groups. The second method used the incorporation, of ^{3}H-thymidine into FRTL5 cells, this revealed a significant statistical decrease in the post treatment goitre group when compared to the pre treatment goitre group. The final method, a commercially available kit, employed the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into FRTL5 cells and showed a significant reduction in the presence of TGI in the MS group when compared to the NC group. The study also revealed poor correlation

between the MTT and ^{3}H-thymidine methods. The significance of these results is discussed.

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Chapter Two **Subjects and Methods**

Abbreviations

BSA	Bovine serum albumin
BTS	Blood transfusion service
BrdU	5-bromo-2'-deoxyuridine
^{3}H-thymidine	Tritiated thymidine
CBA	Cytochemical bioassay
Ci	Curie
CFU	Colony forming units
CHO	Chinese hamster ovary
CNS	Central nervous system
Conc	Concentration
CSF	Cerebrospinal fluid
CTC	Collagenase, trypsin, heat inactivated chicken serum
DAB	Diaminobenzidine
DEAE	Diethylaminoethyl
DIT	Di-iodotyrosine
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetracetic acid
EGF	Epidermal growth factor
ESTA	Eluted stain assay
FRTL5	Fischer rat thyroid cell line
G.C.A.	Glacial acetic acid

GRI	Glasgow Royal Infirmary
HCL	Hydrochloric acid
H_2O_2	Hydrogen peroxide
HSA	Human serum albumin
Ig	Immunoglobulin
IGFI	Insulin like growth factor I
IGFII	Insulin like growth factor II
KH_2PO4	Potassium di-hydrogen orthophosphate
K_2HPO_4	di-Potassium hydrogen orthophosphate
MIA	Metaphase index assay
MIT	Mono-iodotyrosine
MNG	Multinodular goitre
MS	Multiple sclerosis
MTS	5-(3-carboxymethylphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl
MTT	3-(4,5-dimethylthyazole-2-yl)-2,5-diphenyltetrazolium
NaOH	Sodium hydroxide
NCS	Newborn calf serum
NTG	Non-toxic goitre
PB	Phosphate buffer
PBS	Phosphate buffered saline
P/S	Penicillin Streptomycin
RAIU	Radioiodine uptake

SAPU	Scottish antibody production unit
SLE	Systemic lupus erythematosis
SSPE	Sub-acute sclerosing panencephalitis
T4	Thyroxine
T3	Tri-iodothyronine
TGFβ	Transforming growth factorβ
TGI	Thyroid growth immunoglobulin
TSI	Thyroid stimulatory immunoglobulin
TPO	Thyroid peroxidase
TRH	Thyrotrophin releasing hormone
TSH	Thyroid stimulating hormone
WHO	World Health Organisation

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The work described in this thesis was carried out at the University Department of Medicine Glasgow Royal Infirmary. The detailed planning of the work and its execution were my individual responsibility and except where indicated, the work was personally performed.

Chapter One General Introduction

1.1 The Thyroid Gland

1.1.1 Thyroid Development: Embryonic, Anatomical and Functional (McDougall 1992a).

In the embryo the thyroid develops from the foregut and descends by a circuitous route to its normal cervical position. The first evidence of its presence occurs at around four weeks gestation as an evagination between the first and second pharyngeal pouches. This evagination lengthens to form a tube and descends inferiorly and anteriorly, passing the hyoid bone anteriorly. It loops round and behind the hyoid before continuing its descent in the neck. The lateral lobes form from two lateral buds, these fuse with the ultimobranchial body that supplies cells which become the parafollicular cells. The pathway from pharynx to anterior neck is marked by the thyroglossal duct. The tubular structure is lost and by the sixth week atrophies.

Early growth and development of the thyroid do not seem to be thyroid stimulating hormone (TSH) dependent as the pituitary does not appear to synthesize and secrete TSH until week 10 - 12.

Thyroprotein appears as early as 29 days post conception and predates the capacity to concentrate iodide. At week 11 iodide can be concentrated by the thyroid and T4 synthesized. Thyroid hormones condition late phase skeletal maturation and influence late pre-natal lung maturation. They

are required for normal development of brain and intellect (Larsen and Ingbar 1992a).

Foetal thyroid tissue grows roughly in parallel with body weight, weighing approximately 0.2 g at 20 - 25 weeks (Spencer and Banever 1970). Immediately after birth there is a sharp rise in TSH. This is thought to provide heat for the new-born.

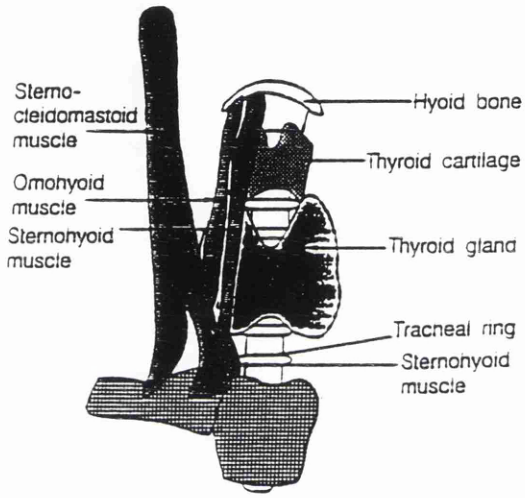


Figure 1.1 Diagram of The Thyroid (in relation to the trachea, neck muscles and manubrium from McDougall 1992).

1.1.2 Physiology of the Thyroid Gland (Larsen and Ingbar 1992a)

The thyroid gland is located anteriorly in the neck below the thyroid cartilage. It weighs approximately 20 grammes in the normal healthy adult and has huge potential for growth, some goitres can weigh hundreds

of grammes. The thyroid gland is bilobed, resembling two halves of a large plum, connected by the isthmus and situated on either side of the trachea (Figure 1.1) Each lobe measures approximately 2.0 - 2.5 x 2.0 x 4.0 mm, but the right lobe tends to be larger and even more highly vascularised than the left. The thyroid filters 4.0 - 6.0 mls/min/g of blood. The kidney by comparison filters 3.0 mls/min/g. Each lobe is fixed by connective tissue to the lateral aspects of the trachea, the upper margin of the isthmus lying just below the cricoid cartilage. Thyroid tissue is composed of thyroid follicular cells, the thyrocytes, which make up 70 % of the total cells. The remainder is comprised of some 20 % endothelial cells and 10 % fibroblasts (Dumont et al. 1991). These are arranged in spherical structures called follicles and are supported by mesenchymal tissue and cells (Klinck et al. 1970). Figure 1.2 shows the follicle and follicular cell structure.

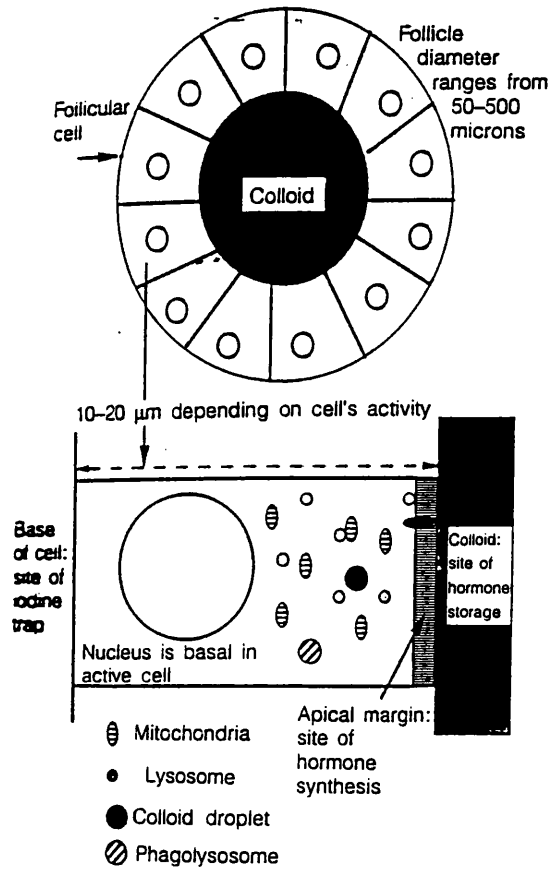


Figure 1.2 Diagram showing the follicle and follicular cell from McDougall (1992).

Physiological enlargement of the thyroid occurs at puberty, (especially in females), during pregnancy and at the menopause and in some normal females it is visible to the naked eye.

The parathyroid glands are situated on or beneath the posterior surface of the thyroid lobes. Calcitonin secreting parafollicular cells are located at the periphery of the follicles.

1.1.3 Endocrine Function

As an endocrine gland the thyroid produces and secretes two physiologically important hormones into the blood stream, thyroxine (T4) and tri-iodothyronine (T3). Both are stored in the gland follicles loosely coupled to thyroglobulin. Release is regulated by TSH from the pituitary gland. Dietary iodine, is preferentially extracted from the blood stream by thyroid follicular cells, the rate of which is also regulated by TSH secretion (McDougall 1992b). It is transported across a basal membrane into the thyroid cell in a process involving active transport which concentrates iodide against a chemical and electrical gradient (Wolff 1964). There is accumulation within the cell and movement across it to the apical membrane. It is thought that the iodide is oxidised at or near the apical membrane by peroxidase in the presence of hydrogen peroxide (Strum and Karnovsky 1971). It then couples with the tyrosine residues of thyroglobulin forming mono-iodotyrosine (MIT) and di-iodotyrosine (DIT). One molecule each of MIT and DIT combine to form T3, T4 is formed from the coupling of 2 DIT molecules. Hydrolysis of thyroglobulin results in the release of T3, T4, MIT, DIT and amino acids (Dunn and Dunn 1982). Microsomal iodotyrosine dehalogenase de-iodinates the MIT and DIT and the iodide is then reused in the follicle (McDougall 1992b).

In the circulation the vast majority of T4 and T3 are bound to thyroxine binding globulin, thyroxine binding prealbumin and albumin which renders them metabolically inactive (Oppenheimer et al. 1968, Robins et

al. 1978). Only about 0.4 % of T3 and 0.04 % of T4 circulate in the unbound (free) state. In peripheral tissues free T3 exerts its influence instantly on all body cells. T4 requires conversion to T3 and is therefore slower acting (Braverman et al. 1970, Sterling et al. 1970, Balsam and Ingbar 1979). These thyroid hormones regulate oxygen consumption of all body cells and tissues and are essential for normal growth and development, both physical and mental (Felig et al. 1987). Thyroxine is more abundant than T3, but T3 is more important at the cellular level (Schwartz et al. 1971).

TSH is a glycoprotein, with a molecular weight of 28,000 daltons and is synthesised by the thyrotropes of the anterior pituitary (Pierce 1971, Pierce and Parsons 1981). It has a half life in the circulation of approximately 1 hour and is metabolised mainly in the liver and kidneys (McDougall 1992b). T3 and T4 receptors can be found on the thyrotrophes of the anterior pituitary and have around 10 - 20 times greater affinity for T3 than T4 (De Groot et al. 1989). TSH has a distinct circadian rhythm, it is secreted episodically and in individually variable patterns (Week and Gundersen 1978). It also shows a diurnal variation, peaking at around midnight to 3 or 4 am with a nadir at about 11 am to 12 noon. The secretion of TSH is pulsatile with peaks every 10 minutes (Greenspan et al. 1986).

The synthesis and secretion of TSH is stimulated by thyrotrophin releasing hormone (TRH) from the hypothalamus in response to low levels of circulating thyroid hormones (Burgus et al. 1970, Patel et al. 1972, Sterling and Lazarus 1977). Elevated levels of T3 and T4 in the

blood suppress the production of TSH by negative feedback to both the hypothalamus and the pituitary (figure 1.3). The thyroid is also capable of self regulation which is thought to involve the homeostasis of stored thyroid hormone, although this is less important than the TSH control mechanism (Ingbar 1972). The hypothalamus is also sensitive to less well defined influences i.e. weather, seasons, temperature and temperament.

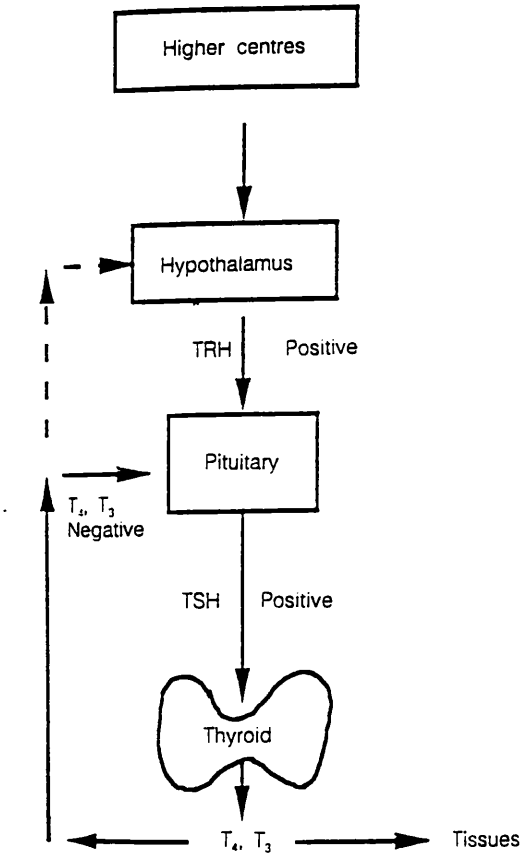


Figure 1.3 Simplified diagram of the control of thyroid function from McDougall (1992).

1.2 Goitre Development

1.2.1 Aetiological Factors

There are many causes of thyroid gland enlargement (goitre) including:-

Inflammation

Autoimmunity

Neoplastic disease

Iodine deficiency

Inherited and acquired dyshormonogenesis

Goitrogens

Puberty

Pregnancy

Smoking (Ericsson and Lindgarde 1991)

In 1928 Chesney et al. provided the first evidence that foodstuffs contained goitrogenic substances by demonstrating the development of goitres in rabbits fed on cabbage (genus brassica). Since then other vegetables have been found to possess goitrogenic properties. Over the last 20 years it has been shown that thiocyanate and isothiocyanate in these cruciferae were the actual goitrogens (Podoba et al. 1973, Ermans et al. 1980, Delange and Ahluwalia 1983, Eltom et al. 1984, Gaitan 1989). Thiocyanate acts by blocking the iodide pump. This is the mechanism which concentrates serum iodide 25 - 50 times (Gmelin and Virtanen 1960).

Although it is now a rare event, sporadic goitres have developed as a result of dietary idiosyncrasy or medication (Vought et al. 1972, Braverman 1986). Iodine was sometimes used to treat various pathologies such as tuberculosis, leprosy, syphilitic and mycotic lesions where the immune mechanism is known to play a major role (Braverman 1986). The mechanism by which iodine does this is unknown, however it has been proposed that it acts by increasing the immune response (Manari and Venturi 1986). Potassium iodide is contained in some expectorants. Prescription of iodopyrine for asthma has resulted in as many as 30 % of recipients developing goitres. Other potent medicinal sources include amiodarone, multivitamins and some dyes used to colour pills and foodstuffs (Braverman 1986). In Japan endemic goitre affects 10 % of the coastal population of Hokkaido due to high consumption of seaweed (Gaitan 1988). Iodine administration during pregnancy may result in an infant with goitre, as can administration of radiographic contrast media. Lithium carbonate used in the treatment of manic depressive psychosis, has also been well described in goitre development (Schou et al. 1968). In the rat it has been shown that lithium acts by blocking the resorption of thyroglobulin (Leppuluoto et al. 1973). Soya bean factors have a similar mode of action (Van Wyk et al. 1959). Soya bean formula, prior to being enriched with iodine, has lead to goitre in infants by enhancing faecal loss of hormone. The pesticide dithiocarbamate and propylenethiourea, its metabolite and degradation product, have been shown to be both teratogenic and goitrogenic in the rat (Vitari et al. 1985), but as yet there has been no human evidence of this.

1.3 Non-Toxic Goitre

Goitres, from the Latin, guttur, meaning throat, were reputedly known in China as early as 2700 BC. Amongst other Italians of the Renaissance period, Leonardo da Vinci (1452-1519), described the thyroid gland. In 1619 Fabricius ab Acquapendente (1537-1634) recognised that goitres arose from the thyroid. In 1776 Albrecht von Haller (1708-1777) of Berne described the thyroid as a ductless gland.

1.3.1 Definition of Non-Toxic Goitre

Non-toxic goitres (NTG) are also known as simple, sporadic or euthyroid goitre. The most widely adopted criteria for goitre definition are those of Perez et al. (1960). Non-toxic goitre is incapable of precise definition. The term is used exclusively to describe abnormal thyroid enlargement not associated with hyper or hypothyroidism and not as a result of an inflammatory or neoplastic process (Larsen and Ingbar 1992c). The modified WHO definition is that of a slowly developing diffuse or nodular enlargement of the thyroid gland, resulting from excessive replication of epithelial cells with subsequent generation of new follicles of widely differing structure and function (Studer and Rameli 1982).

1.3.2 Clinical Aspects

Simple goitres are not painful, rarely interfere with swallowing or breathing and are quite often discovered accidentally. They can be disfiguring when multinodular and haemorrhage into them can cause pain and swelling. Clothing around the neck can feel tight and rarely compression symptoms may affect the trachea, oesophagus and laryngeal nerves. As a result of thyroid enlargement there can be dysphagia, choking, inspiratory stridor and venous engorgement.

1.3.3 Diagnosis

It is important to investigate any enlargement of the thyroid gland if only to allay patient fears. On examination the normal thyroid gland can be felt to be smooth and firm. A thyroid gland whose lateral lobes have a volume greater than the phalanges of the thumb of the examiner is said to be goitrous (Querido et al. 1974).

In NTG serum T3, T4 and TSH are all within the normal range, (for our lab the reference ranges are T3; 0.9 - 2.8 nmol/L, T4; 55 - 144 nmol/L, and TSH; <5.0 mU/L). This ultrasensitive TSH assay can detect levels of TSH down to 0.05 mU/L. The T3/T4 ratio is often increased perhaps reflecting defective iodination of thyroglobulin (Ermans 1969, Rapoport et al. 1972, Studer et al. 1974). Serum thyroglobulin is increased (Pezzino et al. 1978). Radiolabelled iodine uptake is increased possibly due to mild iodine deficiency and a biosynthetic defect. In long-standing

multinodular goitre (MNG) functional autonomy may be reflected by a decreased or absent response to TRH. It has not been possible to demonstrate any correlation between TSH levels and goitre development (Delange et al. 1971). The factors that lead to goitrous hypothyroidism can also lead to NTG, hence the tendency to hypothyroidism. With time NTG's may become multinodular displaying varying degrees of autonomy, ranging from clinically euthyroid to thyrotoxic (Studer 1982). Diffuse NTG can resemble Graves' or Hashimoto's disease. If the Graves' disease is not actively toxic and there is no ocular involvement differentiation is difficult, but the presence of autoantibodies to the thyrotropin receptor is diagnostic of autoimmune disease, being present in ~80 % of patients with active Graves' disease (Larsen and Ingbar 1992b). The thyroid hyperfunction seen in Graves' disease results from the binding of these abnormal immunoglobulins to the thyroid plasma membrane, where they activate adenylate cyclase and induce thyroid growth. This results in an increase in vascularity and rate of hormone production and secretion. The immunoglobulins have not yet been differentiated by chemical or immunological means and demonstration of their presence is based on assessing the capacity of IgG to stimulate adenylate cyclase or increase the concentration of cyclic AMP in human thyroid slices or membrane preparations, they are referred to as thyroid stimulatory immunoglobulin (TSI) (Smith et al. 1988). In Hashimoto's thyroiditis, where the gland is usually more firm and irregular, an increase in antithyroid antibodies distinguishes it from NTG. Carcinoma can also resemble NTG and is diagnosed by biopsy (fine needle).

1.3.4 Incidence and Epidemiology

In the United Kingdom distribution of thyroid size in the general population is positively skewed with more high values (Koutras 1972). Solitary thyroid nodules can be palpated in around 4 - 7 % of the general population (Van Herle et al. 1982, Christensen et al. 1984, Rojeski and Gharib 1985, Gharib et al. 1987). Post mortem and ultrasound studies have shown them in about 50 % of individuals aged up to 50 (Horlocker et al. 1986, Burrow 1989). Goitre prevalence of >10 % is usually taken as the demarcation line between areas of endemic iodine deficiency and sporadic goitre (Lamberg 1991). Endemic goitre is not a problem in the United Kingdom.

In sporadic goitre iodine intake and autoimmune thyroiditis are the major determinants with environmental factors and heredity occasionally contributing (Podoba et al. 1973, Delange 1974). Goitres can develop at pregnancy or puberty, but the pathogenesis of these events is unknown and the goitre may regress or remain. There is an increased frequency of NTG in menopausal women, with a female/male ratio of 4:1 (Kilpatrick et al. 1963). This increase in prevalence is also augmented with age (Rojeski and Gharib 1985).

1.4 Aetiology of Non-Toxic Goitre

Non-toxic goitre is thyroid enlargement with no pathological explanation, no detectable disturbance of thyroid function and no identifiable extrinsic goitrogenic factor. There is no concrete evidence to suggest that simple goitre is related to hereditary factors nor to a defect in thyroid hormone biosynthesis, which could be at any point from iodine trapping to the release of iodothyronines.

1.4.1 The Role of TSH

The major element controlling thyroid growth in vivo is TSH (Dumont 1971). The traditional theory suggests that NTG develops in response to any of several factors that impair thyroid efficiency in the manufacture of adequate quantities of hormones. When this happens hypersecretion of TSH leads to stimulation of thyroid growth and increase in activity of the processes concerned with hormone biosynthesis that are capable of responding. As a result of increase in thyroid mass and unit functional activity a normal rate of hormone secretion is restored and the patient rendered eumetabolic but goitrous.

The concept that it may be an intrinsic inborn abnormality is not supported by the finding that TSH is seldom elevated in patients with non-toxic goitre (Toft et al. 1976). In the non-iodine deficient Whickham study, TSH and mean thyroid hormone levels were not found to be significantly different between goitrous and non-goitrous members of the

community (Tunbridge et al. 1977).

These findings may be explained thus; in hypophysectomysed rats, the response of thyroid weight to standard doses of TSH is augmented by previous thyroid iodine depletion (Brady 1968). Hence any factor impairing normal iodine usage may lead to gradual development of goitre in response to normal TSH concentrations, as an increase in TSH concentration may be small and significant but not detectable. Also the original goitrogenic stimulus may no longer be present at the time of study. The residual normal TSH concentration maintains but has not initiated the goitre. As previously stated relative iodine deficiency may account for some goitres, with goitrous individuals having a lower iodine uptake than non-goitrous controls (Alexander et al. 1962).

The following are common features of NTG which are not attributable to iodine deficiency (Studer and Ramelli 1982).

1. There is structural and functional heterogeneity between single follicles or between different regions of a goitre.
2. Goitres develop in spite of abundant supplies of iodine.
3. Development of single and multiple nodules.
4. Pathogenesis of hot and cold follicles.
5. Pathogenesis of hyperthyroidism in simple goitre.

1.4.2 Iodine Deficiency

Iodine deficiency although implicated has never received the attention that TSH has in sporadic goitre development even though it does so

possibly through an autoregulatory mechanism (Gartner and Pickardt 1990).

The main physiologic regulators of the thyroid gland are TSH and iodide. Doubts have been expressed regarding the role of TSH in goitre development (Garter and Pickardt 1990, Edmonds 1992). There is no doubt that functions such as iodine organification, thyroglobulin and thyroid hormone synthesis and secretion are controlled by TSH. It would be expected but there is no increase in TSH in sporadic goitre (Gutekunst et al. 1986).

The claim that the iodine deficient thyroid is more sensitive to TSH is true for function and hypertrophy (pathological enlargement of cells) and is easily demonstrated but has not been demonstrated for hyperplasia (increase in total cell number) argues Gartner (1994). The experiments carried out by Brady (1968) on hypophysectomysed rats have also been criticised. The rats used were either given a low iodine diet or fed goitrogens, these experiments were carried out over only 12 days during which time it would be impossible to demonstrate an increase in the total cell number, instead it is claimed that it is the enlargement of cells which is induced by short term TSH stimulation (Gartner 1994). In support of this is the fact that cell divisions are very rare in goitre only 3 % of cells and <1 % of normal thyroid cells are in mitosis (Dumont et al. 1991). In vitro intact isolated thyroid follicles with preserved polarity, thyroglobulin content, cell to cell contact and biological extracellular matrix have no spontaneous growth behaviour in the presence of low concentrations of foetal calf serum (FCS). They primarily organify

iodine and secrete thyroid hormones when stimulated with TSH and only proliferate when iodine content of the follicle decreases. This is because of the lack of iodine in the culture medium, however if adequate iodine is available there is no increase in cell proliferation even in the presence of high TSH concentrations (Gartner 1992).

In contrast thyroid epithelial cells when dispersed onto plates grow to confluence in the presence of FCS most are in mitosis and can be modulated by growth factors, TSH and CAMP, this cannot be demonstrated in vivo.

1.4.3 Thyroid Growth Immunoglobulins (TGI)

The involvement of a TGI, specific for thyroid cell growth, in the pathogenesis of NTG would certainly implicate autoimmunity and would provide an attractive explanation for the phenomenon of NTG (Drexhage et al. 1980). Unlike Graves' IgG this TGI does not act through adenylate cyclase nor specifically bind to the TSH receptor (Drexhage et al. 1983). The existence of such would account for (Studer and Ramelli 1982):-

1) the female prevalence

2) increase in familial incidence

3) occurrence of goitres in non-iodine deficient areas

4) explain continuous growth despite suppression with thyroxine therapy

5) explain the colloid rich structure.

TGI may account for different thyroid sizes in Graves' patients and explain atrophy of the thyroid in non-goitrous hypothyroidism. Patients

in whom autoimmune non-toxic goitre is thought most likely are those in whom other autoimmune phenomena are present in themselves or their families, e.g. pernicious anaemia, Addison's disease, diabetes mellitus, myasthenia gravis, or goitres returning after sub-total post thyroidectomy, although more evidence is required to support this finding (Valente et al. 1983a).

Evidence as to the immunological nature of the thyroid growth stimulator was shown by Van der Gaag et al. in 1985 who demonstrated that the growth response seen in a group with simple goitre could be abolished by pre-treating the immunoglobulin (Ig) with antihuman Ig, confirming the immunoglobulin nature of the growth stimulator.

TGI have been detected by indices of growth i.e. labelled thymidine incorporation, increase in DNA content and increase in cell number in cultured thyroid cell systems (Drexhage et al. 1980, Smyth et al. 1982, Wilders–Trushing et al. 1990).

Neither TSH nor TGI explains why long standing NTG becomes nodular. NTG is characterised by anatomical and functional heterogeneity and functional autonomy, presumed to be due to hyperstimulation by TSH or from repeated cycles of hyperstimulation and involution thus leading to areas of hyperplasia (autonomy and atrophy). After a time autonomous tissue is sufficient to suppress TSH secretion giving a subnormal response to TRH administration.

1.4.4 Growth Factors

There is evidence to indicate that other paracrine and autocrine growth factors, such as Insulin like growth factor 1 (IGF1) and Insulin like growth factor 2 (IGF2), may also be involved in modulating thyroid growth (Westermark et al. 1983, Tramontano et al. 1986, Shin-Ichiro et al. 1990). The local hormone transforming growth factor β (TGFβ) has been shown to inhibit thyroid growth (Grubech-Loebenstein et al. 1989, Bidey 1990). Subconfluent human thyroid cells isolated from non-toxic thyroid tissue obtained at surgery responded to human epidermal growth factor (EGF) in a dose dependant manner with an increase in incorporation of tritiated thymidine (^{3}H-thymidine), ranging from 25 - 60 %. There was also inhibition of T3 secretion of between 20 and 45 % (Ollis et al. 1985).

In more recent studies IGF1, EGF, TGF (α and β) and βFGF have been studied extensively (Dumont et al. 1991, Gartner 1992). It is claimed that release of these growth factors is under the control of iodine supplementation of thyroid cells along with TSH. Changes in the iodine content of the follicle decreases the paracrine release of TGFβ and βFGF responsible for matrix formation and endothelial cell proliferation that are mainly released under the influence of EGF the most potent (Gartner 1992). In contrast high iodine content of thyroid follicles decreases this paracrine release of TGFβ and βFGF and the growth response to EGF. When stimulated by TSH IGF1 is released but only when the iodine

content of the follicles are low. When the iodine content is normal TSH stimulation leads only to thyroid hormone secretion and not to the release of IGF1 or the expression of IGF1 mRNA. Blocking the IGF1 receptor with an antibody specific to it results in the abolishment of any growth effect to TSH on thyroid epithelial cells (Rafferzeder et al. 1993). This would imply that TSH is only indirectly involved in thyroid cell proliferation and strongly dependent on iodine, although TSH is able to modify iodine intake.

In rat thyroids it was first demonstrated that intense replication of stromal cells occurred simultaneously with the multiplication of follicular epithelia (Santler 1957). Since then it has been hypothesised that stromal cells play an actual role in thyroid growth. The stroma is a minor component of the thyroid gland in terms of volume with little importance attached to it. A thin sheath of fibroblasts and capillary network surround each thyroid follicle, it is therefore evident that epithelial and stromal growth must be closely co-ordinated by humoral communication (Dumont et al. 1991). Growth response to TSH, in vitro, of isolated normal thyroid follicular cells was shown to be IGF1 dependent (Smith 1988, Williams et al. 1988). As IGF1 is not normally an autocrine product of thyroid epithelium, the IGF1 must therefore be from another source. Fibroblasts have been shown to produce IGF1 (Gartner et al. 1987), suggesting that epithelial and stromal cells could be interdependent for thyroid growth control.

1.4.5 Autoimmune Associations

Multinodular goitres have been associated with autoimmune thyroiditis, but there is a poor association between simple goitre and thyroid autoantibodies. Even so several workers support the theory that most sporadic non-toxic goitres involve disorders of immune regulation and self-recognition, with disturbances of T suppressor cell function (Von Blomberg and Kokje 1984, Van der Gaag et al. 1985).

The following evidence indicates a link with autoimmune disease.

1. Autonomous thyroid growth; autonomy has been shown by the fact that thyroid growth continues in iodine deficient and non-deficient goitres and also with TSH suppression (De Smet 1960, Peter et al. 1985 and 1986, Studer et al. 1989).
2. Goitre regrowth post thyroidectomy; experiments on mice with inherited absence of TSH showed that the remaining lobe still hypertrophied after hemi-thyroidectomy (Lewinski et al. 1983). The very variable growth potential of individual thyroid cells together with local and systemic growth factors could itself be important in the evolution of sporadic goitres (Studer et al. 1989).
3. The purported existence of thyroid growth immunoglobulins discussed in 1.4.3.

It has been suggested that some varieties of NTG may be precursors to Graves' disease. This is due to the observation that after long term follow up some cases of non-toxic adolescent goitre have resulted in a high frequency of toxic goitre, even when suppressive doses of thyroid

hormone were being administered. In NTG iodine deficiency is obviously more important than genetic factors. A study on twins by Phillips (1994) demonstrated that heredity does play a role in the genesis of NTG. Confirmation of this has also come from studies on the increase in goitre frequency among children with two goitrous parents, compared to those with one such parent, with those neither of whose parents had a goitre showing the lowest frequency of all (Freidman and Fialkow 1983).

1.4.6 Clonal Heterogeneity

Studer proposed his theory of NTG development in 1982. This concept is of clonal heterogeneity and results from clonal differences among those cells that give rise to thyroid follicles some being more and some less responsive to external stimulation by TSH and some being autonomous at the outset. Individual responses to TSH might also vary from clone to clone with respect to iodine accumulation, exocytosis of thyroglobulin or resorption of colloid. This concept implies that the basis of anatomical and functional heterogeneity exists within the thyroid at the outset of the disease and is exaggerated by prolonged stimulation.

1.4.6.1 Goitre Development Microscopically

The basic process in every simple goitre is the increase in epithelial cell mass and the generation of new follicles (Gerber et al. 1981). Histologically the predominant cells are follicular epithelia, although no

single type of follicle characterises simple goitre. Diseased glands consist of endless varieties and shapes of follicles (Studer et al. 1978, Milone and Studer 1980).

In rat models three phases of thyroid growth have been shown due to sustained elevation of serum TSH induced by goitrogen administration. Firstly there is a phase of rapid growth lasting 1 - 2 months, followed by a plateau period of 3 - 6 months where there is little increase in weight and the third is the appearance of multiple follicular thyroid tumours (Purves and Greisbach 1946, Philp et al. 1969). This limitation of the initial growth response occurs despite undiminished TSH stimulation. This results in the maintenance of functional activity but loss of proliferative responses (Wynford-Thomas et al. 1982). This altered response is thought to be mediated by an intracellular change in the follicular cell either at the receptor or post-receptor level rather than by a chalone (Stringer et al. 1985). (A chalone is an internal secretion of an inhibitory nature opposing the action of hormones (Chambers Science and Technology Dictionary 1984).

Two factors are pre-requisite to the development of NTG:-

1) Low intensity goitrogenic stimulus. This was used to induce simple goitres in experimental animals (Olen 1969, Strum and Karnovsky 1971). Heavy goitrogenic stimulation leads to the diffuse hypertrophy seen in Graves' disease (Marine 1928, Taylor 1953).

2) The second factor is time. It has been proposed that the typical microscopic, macroscopic and functional heterogeneity seen in NTG only develops if the mild goitrogen acts intermittently (Gerber et al. 1981) and is present over a long period of time (De Smet 1960).

1.4.6.2 Goitre Development Macroscopically

Long-standing heterogeneous goitres can be identified by gross differences in structure and natural colour within and between different nodules, nodular growth of the parenchyma, strands of connective tissue, along with fresh haemorrhagic necroses and scarred calcified areas of necrosis (Taylor 1953, Correa 1980). The mechanism is probably similar to that causing tissue necrosis in tumours i.e. as newly growing tissue requires new capillary networks, failure to meet the ever increasing demand for blood supply, to the growing number of follicles, is likely to cause tissue death (Studer and Ramelli 1982).

NTG is initially diffuse and later multinodular. There is diffuse hypertrophy and hyperplasia of the epithelium lining follicles, the cells increase in height and number and may protrude into the follicular lumen with an accompanying decrease in colloid and an increase in vascularity (Larsen and Ingbar 1992a).

Multinodular goitre is long standing and results from repeated cycles of hyperplasia and involution leading to formation of nodules of involuted tissue surrounded by more hyperplastic tissue. A goitre progresses from being diffuse and symmetrical to a multinodular goitre characterised by

structural and functional heterogeneity with areas of functional autonomy (De Smet 1960).

1.4.6.3 Functional Characteristics of Simple Goitre

The increase in cell number (Wollman and Breitman 1970) is not exclusively due to the multiplication of epithelial cells but is also caused by the proliferation of capillary endothelia and other non-epithelial cells (Wollman et al. 1978, Wollman 1980, Denef et al. 1981).

In the normal human thyroid there is a highly significant correlation between the number of cells and the thyroglobulin content, (the mean thyroglobulin content is 4 %), but this relationship is lost in simple goitre (Rentsch et al. 1982). There is often also evidence of poor iodination, (Ermans 1969, Rapoport et al. 1972, Studer et al. 1974). Variability of structure and function among the follicles of goitrous glands leads to huge regional interfollicular heterogeneity (Studer et al. 1978, Milloni and Studer 1980) with bizarre variation in iodine turnover among single adjacent follicles (Studer and Ramelli 1982). Homogeneity can also be demonstrated (Miller et al. 1967, Miller and Block 1970). Another feature is variability of growth, where in one region replication may be rapid producing many daughter follicles and within close proximity, follicles may grow extremely large and become distended by excessive accumulation of colloid (Studer and Ramelli 1982).

The normal thyroid gland has a predictable response to TSH i.e. all

follicles function in a similar way, they contain identically iodinated thyroglobulin, there is enhancement of iodide transport, thyroglobulin endocytosis and neosynthesis, cell replication and other follicular functions are all orchestrated in a co-ordinated manner (Tong 1974). In nodular goitres this is not the case and it is possible to find cold follicles, which no longer participate in iodine turnover, hot follicles with excessive iodine turnover and other follicles with assorted degrees of iodine turnover (Miller et al. 1967, Miller and Block 1970, Miller 1978). Hot follicles are thought to either start out normal and later become hyperactive or are hyperactive from the beginning and remain so for their lifetime. The same theory applies to cold follicles but they are characteristically inactive. It has been suggested, as a result of experiments on ageing mice, that normal follicles become cold by losing their ability to organify iodine, (Studer et al. 1978 and 1980).

1.4.6.4 Structural Characteristics

Long standing simple goitres often become nodular (Studer and Gebel 1986, Studer 1986, Smeds et al. 1987).

This may occur in two ways:-

a) The abundance of interstitial tissue makes the smooth expansion of replicating follicles impossible and the goitre cannot become diffuse. Nearby strands of connective tissue provide the material for encapsulating the goitre tissue giving it a nodule like appearance and peculiarities of blood supply do not permit the survival of

follicular epithelia.

b) Where uniform follicles multiply rapidly any new follicles with a higher intrinsic growth rate expand at the expense of slower growing cells, the surrounding tissue becomes compressed, the follicles collapse and atrophy, the stroma survives and forms a capsule around the expanding cluster of follicles.

There are two types of nodules (Studer and Ramelli 1982).

i) The first is architecturally uniform, is clearly distinguishable from non-nodular tissue and delineated from surrounding follicles. Follicles are of identical structure and equal function and are considered to be true clonal adenomas or benign tumours of oligoclonal origin. These are by far the exception. True adenomas are the result of a single homogeneous family of cells in a mother follicle replicating at a fast rate (Failkow 1974).

ii) The large majority of nodules consists of follicles which are functionally and morphologically identical to those of non-nodular tissue without the homogeneity of the true adenoma. These are the result of the neogeneration of many daughter follicles by many different mother follicles.

In reality it is impossible to draw a clear line between thyroid adenomas and heterogeneous nodules (Taylor 1980).

1.5 Treatment of NTG

1.5.1 Thyroid Hormone

The use of thyroid extract in the treatment of myxoedema was first described by Murray in 1891. Once the initial enthusiasm had dwindled, it rapidly fell into disrepute and was not revived until almost 60 years later by Greer and Astwood in 1953. The logic in using T4 for the treatment of NTG was to suppress TSH with a resulting decline in thyroid growth and function (Greer and Astwood 1953).

In TSH suppression the action of T4 is independent of the origin of the goitre and it is important that the dose given should be sufficient to produce a maximal state of thyroid inactivity. Where positive results have been obtained it has been suggested that this method of treatment may be due to an immunostimulatory effect of T3 and T4. In a series of experiments a significant increase was seen in blastogenic response of various lymphoid tissues to plant mitogens following T4 and T3 administration. This was not true however, in the case of spleen cells where the response was depressed. Restoration of blastogenic response of the lymphocytes of thyroidectomised rats following T3 therapy was also demonstrated (Chaterjee and Chandel 1983).

In the past, studies to evaluate the efficacy of T4 in decreasing goitre size have been hindered by not always being randomised nor group controlled. There has been poor assessment of thyroid size, no tissue diagnosis, relatively insensitive methods for assaying serum TSH levels

and the inclusion of patients with autoimmune goitres, which usually do reduce in size when T4 is given (Hedgedus et al. 1991). Occasionally nodules which were due to thyroid cancer were included, these may also be TSH dependant (Hill et al. 1976).

Recently, in an attempt to re-evaluate T4 therapy, whilst eliminating the aforementioned deficiencies, it was found on average that T4 had no significant effect on the size of solitary thyroid nodules. While there were a few patients where nodule size did decrease, this effect was also observed in the untreated group (Reverter et al. 1991). These findings oppose an earlier study by Morita et al. (1989), but are in agreement with a response to treatment in almost 60 % of patients and a mean decrease in thyroid volume of 25 %, in a study by Berghout et al. (1990).

Thyroxine administration for diffuse NTG is useful in the prevention of nodules. Radioiodine uptake should be < 0.5 %. Suppressive thyroid therapy is unlikely to cause hyperthyroidism but caution must be exercised as there may be autonomous nodules, especially in the elderly (Edmonds 1992). On performing a TRH test a normal or near normal response would indicate that there is some suppression still in reserve and therapy may go ahead. ^{131}I treatment eradicates autonomous foci and can forestall future thyrotoxicosis (Larsen and Ingbar 1992c).

Several studies, have failed to find any effect of post-thyroidectomy treatment with T4 on recurrence of sporadic goitre and indicate that prophylactic use of T4 is unjustified (Geersden and Frolund 1984, Hegedus et al. 1987, Berghout et al. 1989). There is also a risk of osteoporosis after prolonged suppressive therapy with thyroid hormones

(Ross et al. 1987, Paul et al. 1988, Wartofsky 1991).

1.5.2 ^{131}Iodine

Ablation of the thyroid gland with ^{131}I in the treatment of NTG has been used to a lesser extent. Two large German studies have shown decreases in two thirds of their group however goitre size in both were clinically assessed (Keiderling et al. 1964, Klein et al. 1989). In some recent smaller studies decreases in thyroid volume ranged from 40 - 59 % (Hegedus et al. 1988, Nygaard et al. 1993, Wesche et al. 1995). There are some disadvantages to this treatment ie., substernal goitres sometimes swell causing intra-tracheal compression (Becker and Hurley 1971, Lorentzen and Blichert-Toft 1983). Patients can also have a thyroiditis type reaction:- tenderness, fever, hypersedimentation, and hyperthyroidism caused by the release of thyroid hormones after the radiation induced destruction of follicular cells, this presents in 3 % of patients (Nygaard et al. 1993). Also hyperthyroidism which developed 3 months post treatment with ^{131}I has been documented in 3 % of patients (Nygaard et al. 1993). There is likewise a small risk of hypothyroidism and also of cancer (Shore et al. 1985, Williams 1991, Cooper 1998). The risk of cancer appears to be dose related (Hall et al. 1992). In a large study Holm et al. (1988) evaluated 35,000 patients and was unable to demonstrate an increased frequency in any type of cancer. In rats it has been shown that ^{131}I is a dose dependent carcinogen (Williams 1991).

1.5.3 Surgery

Surgical intervention is rapid and effective in goitre removal but within 10 years there is recurrence in around 10 - 20 % (Geersden and Hee 1982b, Geersden and Frolund 1984, Berghout et al. 1989, Hegedus et al. 1991). Complications to surgery are seen in 7 - 10 % of cases (Lorentzen and Blichert-Toft 1983, Jensen et al. 1986, Reeve et al. 1987, Agerbaek et al. 1988, Pilegaard and Hessov 1988). There is also increased frequency of complications seen with increasing goitre size (Agerbaek et al. 1988). The tendency to hypothyroidism after surgery varies from 0 - 70 % (Lorentzen and Blichert-Toft 1983, Jensen et al. 1986) also there are anaesthetic complications to be considered and the risk of hypoparathyroidism.

1.6 Aims of this Study

There were several aims to this study:-

1. To look for the presence of thyroid growth immunoglobulins in the serum of a group of patients with non-toxic goitre and compare this to a normal control group and a disease control group.
2. To compare the three following in vitro methods of measuring cell proliferation
    a) A colourimetric method using the dye 3-(4,5-dimethythiazole-2-yl)-2,5-diphenytetrazolium (MTT).

b) The incorporation of ^{3}H-thymidine.

c) A commercially available kit which measures 5-bromo-2'-deoxyuridine (BrdU) uptake.

3. To address the following clinically relevant questions:-

i) Was there any evidence of stimulation or suppression of FRTL5 cells in vitro when treated with IgG from the non-toxic goitre group, disease control patients or normal controls?

ii) Did treatment with thyroxine have any effect on non-toxic goitre size or thyroid volume?

iii) Was there a change in serum TGI levels in non-toxic goitre patients after thyroxine therapy?

iv) Did serum TGI levels pre-treatment have any value in predicting which non-toxic goitre patients would show a reduction in thyroid volume after thyroxine therapy?

Chapter Two Subjects and Methods

2.1 Subjects

2.1.1 Non-Toxic Goitre Group

The population of this study group comprised of patients with NTG 27; female 24, male 3, prior to suppressive therapy with thyroxine, mean age, 46.6 ± 13.2 years. Within this group 20 of the patients completed a 6 month course of suppressive therapy with thyroxine, (female 18, male 2, mean age 48.4 ± 13.0).

The NTG group were recruited from patients attending the thyroid clinic at Glasgow Royal Infirmary (GRI), who presented with goitre. Their suitability for entering the study was based on a clinical appraisal. The patients in the study all had a clinical diagnosis of goitre based on inspection and palpation. Goitres were described as simple, small single nodule or multinodular. Goitre size was estimated by the clinician and was referred to as 1, 2, 3, 4 or 5 times normal. The volunteers recruited had given their consent and were all clinically and biochemically euthyroid. Patients had 30 mls of venous blood taken (10 mls for thyroid function tests).

Thyroid function was assessed by measurement of serum T4, using a Fluorescence Polarisation Immunoassay, which combines competitive protein binding and fluorescence polarisation. On occasions serum T3 was measured, also by a Fluorescence Polarisation Immunoassay. TSH

was measured by Microparticle Enzyme Immunoassay Technology. These three parameters for thyroid function were measured using the IMX system from Abbot Laboratories UK and were carried out at the Institute of Biochemistry GRI.

Anti-microsomal [now known as autoantibodies to thyroid peroxidase (TPO)] and anti-thyroglobulin antibodies were measured at the Department of Immunopathology, Western Infirmary Glasgow, using kits from Wellcome Laboratories. The remainder of blood, taken at this first visit, was stored as serum at -20°C for later estimation of thyroid growth immunoglobulins. Cytological examination of a fine needle aspirate from the thyroid gland was carried out only on those nodules where malignancy was suspected. Arrangements for a pre-treatment ultrasound scan and a review appointment at three months were made.

A suppressive dose of sodium thyroxine is in the order of 0.15 - 0.2 mg/day which corresponds to its endogenous 24 hour production (Nilsson et al. 1977). The goitre group were prescribed sodium thyroxine for 6 months, 0.1 mgs/day for one month which was increased to 0.2 mgs/day for the remaining 5 months. Side effects from T4 can include the following:- cardiac arrhythmia's, angina, tachycardia, muscle cramps, headache, restlessness, excitability, flushing, sweating, diarrhoea and excessive weight loss.

2.1.2 Disease Control Group

2.1.2 (a) Multiple Sclerosis

Patients with multiple sclerosis (MS) 10; (female 9, male 1, mean age, 39.7 ± 8.4 years). These subjects attended the neurology out patient clinic at the Southern General Hospital, Glasgow. Patients were selected according to a clinical diagnosis of probable multiple sclerosis, which was supported by a positive oligoclonal pattern in the cerebrospinal fluid (CSF).

Multiple sclerosis is a demyelinating disease of the central nervous system. Its cause and pathogenesis are unknown but it is well linked with environmental and genetic factors. It affects approximately 1 in 700 people in the United Kingdom, age range at onset is from 10 - 59 years and mean age at onset approximately 29 - 33 years (Acheson 1985). In general early onset MS is accompanied by the most severe symptoms. There is general agreement that it is more common and that age of onset is slightly earlier in females than in males. A female to male ratio of 1.4 : 1 was found by (Acheson 1985), although one of the larger and more reliable American studies quotes 1.8 : 1 (Kurtze 1979). The higher incidence in females is thought to reflect a hormonal factor affecting susceptibility. The rise and fall of the incidence of MS corresponds closely with puberty and with the menopause (Fischman 1982). Other autoimmune diseases such as Hashimoto's thyroiditis and systemic lupus erythematosus (SLE) are also more common in females than males.

Multiple sclerosis shows an organ specific immune response directed against a target antigen within and restricted to the myelin sheath. The pathological lesion is a plaque which is an area of white matter in which myelin and oligodendrocytes are absent. Axons denuded of their myelin sheath are poor conductors of nerve impulses, giving rise to a neurological deficit.

2.1.2 (b) Environmental Factors

It is common in temperate climates, with a well recognised North/South gradient in the USA and Europe (Limburg 1950). Studies on migrants have shown that they assume the local risk of contracting MS if they migrate before the age of 15, but if they go later they retain the risk associated with their country of origin (Kurtze 1970). On occasion there have been epidemics, which have been accompanied by the proposal of a viral infective agent.

2.1.2 (c) Genetic Factors

Genetic factors are important as can be seen from the occurrence of MS in Caucasians. Its association with HLA-DRW2 antigens in both Northern Europeans and Americans is well documented (Jersild et al. 1972). Where MS is diagnosed in a family it is not unusual to find it in other siblings. There is also familial aggregation, indicating that it may be in part due to exposure to a common environmental influence, with

relatives being prone to other organ specific diseases (Bobowick et al. 1978).

2.1.2 (d) Diagnostic Criteria (McAlpine 1972)

Clinically definite multiple sclerosis is demonstrated by a history of acute retrobulbar neuritis or an episode of paraesthesiae, motor weakness, double vision, unsteadiness in walking and other signs of multiple lesions in the central nervous system. These may be minimal for many years, symptoms may improve or clear up and may be followed by relapses during the course of years. Later there is gradual onset of paraplegia followed by relapses and signs indicative of disease in the brain stem, cerebrum and optic nerve.

Probable multiple sclerosis; during the original attack, there is evidence of multiple lesions, this can be followed by a good recovery. There may be complete absence of fresh symptoms for a year with the return of original symptoms and the appearance of an extensor plantar response, nystagmus, tremor or temporal pallor of an optic disc and a history of one or more attacks of retrobulbar neuritis.

Possible multiple sclerosis has a similar history to that described in probable MS but with unusual features, or insufficient follow-up information. A patient may have a history of progressive paraplegia usually in early middle age with no evidence of relapse or remission, or of a lesion outside the spinal cord. When doubt exists attention is paid to

the age of the patient, a positive family history and laboratory findings in the cerebrospinal fluid (CSF). Diagnosis is assisted by expert neurological assessment, imaging (especially magnetic resonance imaging) and evoked potentials, such as visual evoked potentials.

Laboratory support is confined to the presence of one or more specific IgG bands, in the gamma globulin region when concentrated CSF is applied to agar gel electrophoresis (Lowenthal 1964). The more recent introduction of polyacrylamide gel electrophoresis has improved this diagnostic aid along with the refinement of calculating the CSF-IgG/albumin ratio in relation to the simultaneous serum levels (normal range 4 - 22 %). This expression in relation to another protein i.e. albumin which is not synthesised in the brain indicates the integrity of the blood brain barrier thereby eliminating other neurological diseases (Waigt 1980). There is some evidence that breach of the blood brain barrier may be a temporary phenomenon. This was shown in experiments in which isotope was administered and the brain scanned, the presence of isotope in the brain parenchyma indicated escape through the barrier. After 4 weeks, on repetition of the experiment, no abnormality was found (Gize and Mishkin 1970, Cohan and Fermalich 1975). Approximately 94 % of MS patients have a positive oligoclonal pattern, or increased production of IgG in the CSF (Thomson et al. 1979). The presence of this oligoclonal IgG appears to be independent of the clinical activity or type of the disease and is not abolished by immunosupression. In multiple sclerosis the total protein concentration

in the CSF is usually normal or only slightly elevated (normal range 0 - 0.4 g/l). This test is not entirely specific for MS as it is also positive in neurosyphilis, subacute sclerosing panencephalitis (SSPE) and Guillain-Barre syndrome (Waigt and Zgorzalewicz 1982). The presence of red blood cells in the CSF invalidates the test.

2.1.2 (e) Autoimmune Associations

Multiple sclerosis is not universally accepted as an autoimmune disease, however evidence of the autoimmune nature of MS is supported by the following abnormal immunological findings:-

1. Abnormal immunoglobulins can be found in the CSF protein, within the central nervous system (CNS) compartment.
2. It is common to find high titres of viral antibodies, especially measles (Adams and Imagawa 1962, Norrby 1978).
3. There are T cell subset abnormalities especially in relapses of the condition.

Like non-toxic goitre, MS is influenced by genetic and environmental factors, environmental probably being the most important. In autoimmune disease the state of self tolerance is lost, i.e. the body's own 'self' cells and molecules stimulate an immune response whereby the body's own structures are attacked. Whilst both MS and NTG are thought to be autoimmune in nature they are both disorders of non-specific immunity and although there are aetiological similarities between

both diseases, the clinical manifestation of one is remote from that of the other. It was for these reasons MS was chosen as a suitable disease control group, besides providing readily available samples of the required age and sex. The MS samples for this study were provided by Dr Hugh Willison at the Institute of Neurological Sciences Southern General Hospital Glasgow. These were received as serum samples and were frozen on receipt.

2.1.3 Normal Control Group

The normal control group (NC) numbered 27; (female 24, male 3, mean age, 46.6 ± 12.4years).

These were a group of normal individuals recruited mainly from laboratory staff at the Department of Medicine GRI. They were not known to have any current or past history of thyroid disease, nor any autoimmune disease. The normal control group included 24 females, of these 12.5 % were taking the oral contraceptive pill, 12.5 % were receiving hormone replacement therapy and the remaining 75 % were hormone free. They were also comparable for age and sex with the NTG and MS groups. Fifty per cent of the normal control group, were laboratory staff, while the remainder were students associated with the department and clerical staff. This group had 20 mls of venous blood taken.

2.2 Thyroid Imaging (McDougall 1992)

Thyroid ultrasound scans were performed at the X-ray department GRI, using an ALT Ultramark 9 scanner, confirming the diagnosis of goitre and providing thyroid volumes and dimensions.

Ultrasonography is a non-invasive, rapid, simple and relatively inexpensive method for studying the thyroid gland. The patient lies recumbent with neck extended. A sound wave of very high frequency (5 - 10 MHz) is pulsed into the area of interest, the echoes are detected by the receiver and reproduced as an image. Solid tissue produces many echoes at tissue interfaces and gives excellent resolution, theoretically <1.0 mm in the axial direction and 1.0 - 2 mm in the transverse direction (Scheible et al. 1979).

This method has an error of approximately ± 16 % for nodules with a volume of between 8 and 70 mls (Nygaard et al. 1996), this can be improved to ~ ± 10 % if cross sections are scanned at 0.5 cm intervals and the results computer calculated (Rasmussen and Hjorth 1974), even with this error it is still more accurate and reproducible than palpation (Ashcraft and Van Herle 1981a, Brunn et al. 1981, Gutekunst et al. 1986, Hay et al. 1986). In a study of clinically assessed goitre size, inter and intra-observer variation ranged from 0 - 170 % with an average error of 39 % when compared to those ultrasonically determined, giving poor correlation (Jarlov et al. 1991).

The sound beam is a major limitation in ultrasonography as it will only penetrate tissue a distance of 4 cm at 10 MHz. Fortunately in most

patients the thyroid gland lies within 4 cm of the skin surface enabling it to be imaged completely. Other inaccuracies of this method are chiefly in underestimating the volume of large multinodular goitres, where the shape may not be ellipsoidal and coupling the transducer to the neck surface may not be possible. Unfortunately it does not show differences in tissue characteristics and patients with intrathoracic thyroid tissue can prove problematic. Advantages include safety for patients as no ionising radiation is used, there is no patient preparation required and finally patients are not required to stop any medication before being scanned.

Ultrasound is also helpful in obtaining accurate measurement of the size of a nodule, if suppressive therapy is being used. Measurements taken regularly can show whether or not a nodule is growing and allows accurate calculation of thyroid gland volume. Thickness, width and length of both lobes are measured and thyroid volume calculated using the formula for a spherical ellipsoid.

Total thyroid volume = AP (cm) x width (cm) x length (cm), where AP is the anteroposterior diameter (Brunn et al. 1981).

In this study, a response to treatment was defined as a decrease in thyroid size of 20 % or more at the end of the follow-up as this degree of change is outwith the range of error of the method.

2.3 Sample Preparation

Serum was obtained by centrifugation of clotted blood at 2500 g for 10 minutes. This was stored at -20°C until required.

2.4 Gamma Globulin Separation

2.4.1 Separation of 7S Gamma Globulin

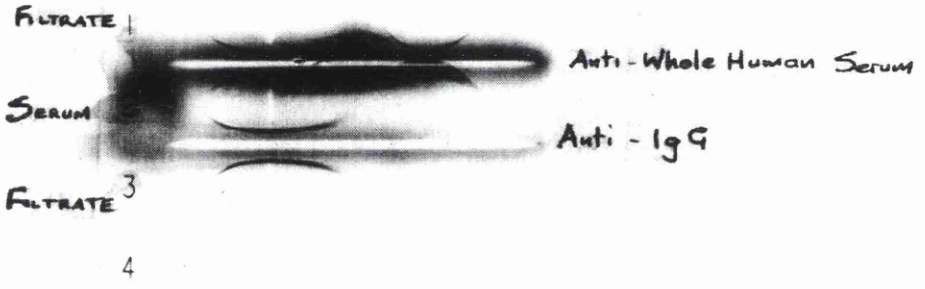
Gamma Globulin was separated from undialysed human serum using a two-stage batch procedure employing high resolution Sephacryl S-200 (Baumstark et al. 1964). In the procedure of gel filtration molecules are separated according to differences in their sizes. This method was chosen for its low cost, speed and ease of use. It does however produce a crude preparation of IgG. As the DEAE sephadex used in the Baumstark method is now obsolete, Pharmacia recommended replacing it with Sephacryl S-200.

Immuno-electrophoresis was carried out (figure 2.1), at the Institute of Biochemistry GRI, on the IgG sample prepared from the serum obtained from the Blood Transfusion Service (BTS) (section 2.4.4). This demonstrates that the sample is concentrated IgG, with a degree of contamination by other serum proteins.

Immuno-electrophoresis is a powerful analytical technique with great resolving power, as it combines prior separation of antigens by

electrophoresis with immuno-diffusion against antiserum. The antigen diffuses from a point source after the initial electrophoresis and interacts with the antiserum advancing on a plane front producing clearly defined arcs of precipitation at equivalence.

Figure 2.1 Immuno-electrophoresis of Normal IgG



Immuno-electrophoresis was carried out on the IgG prepared from the normal serum sample obtained from the BTS. This shows concentrated IgG, although there are other serum proteins also present.

2.4.2 Materials and Equipment

Sephacryl S-200 (Pharmacia)

Phosphate Buffer 0.01 M (pH 6.5); KH_2PO_4/K_2 HPO_4 molar ratio 2.45 (BDH)

HCL 0.5 N (BDH)

NaOH 1.0 N (BDH)

K_2HPO_4 1.0 M

pH meter (BDH)

Minisart filters 0.2 microns (Sartorius)

Watman number 1 filter papers (BDH)

Scintered glass funnel (BDH)

Conical side armed filter flask (BDH)

2.4.3 Gel Preparation

The Sephacryl gel, which comes pre-swollen and with fines removed, was washed with 1 litre of 0.5 N NaOH followed by distilled water until the effluent was free of alkali. The gel was then washed with 1 litre of 0.5 N HCL followed by distilled water until the effluent was free of acid. The preparation was suspended in 800 ml of distilled water and the pH adjusted to 6.5 with 1 N NaOH. The slurry was allowed to settle for 5 minutes, the supernatant liquid decanted and resuspended in 0.01 M phosphate buffer pH 6.5. The gel was allowed to stand with occasional

stirring for 15 minutes. It was then filtered and washed slowly on a Buchner funnel with 1L of cold 0.01 M phosphate buffer pH 6.5. This was stored at 4°C in 0.01 M phosphate buffer pH 6.5 to which some absolute alcohol had been added as preservative.

2.4.4 Method for 7S Gamma Globulin Separation

1.5 mls of serum was added to 3.0 grams of Sephacryl S-200. This was kept at 4°C with occasional stirring. After 1 hour the slurry was filtered using a scintered glass funnel and a double layer of Watman No 1 filter papers, then washed with 4 x 2.5 ml volumes of 0.01 M phosphate buffer, taking the sephacryl to dryness. A further 5 mls of 0.01 M phosphate buffer was added to the filtrate and mixed for 60 minutes at 4°C, after which the resin was again washed with 10 x 2.0 ml volumes of 0.01 M phosphate buffer and taken to dryness as before. The filtrate pH was immediately adjusted to 7.5 with 1.0 M K_2HPO_4 and filter sterilised using a 0.2 μ Minisart filter. These IgG samples were then aliquoted and stored frozen at -20°C for further use.

2.5 Protein Content of IgG Samples

At the outset it was decided to estimate the protein content based on its absorption of UV light at 280 nm, which it does in a concentration dependent manner (Hudson and Hay 1989a).

Total protein concentration (mg/ml) = 1.55 x A280 - 0.77 x A260

Although this is a simple and direct means of estimating the total protein content of a solution it is faulted by the fact that many detergents and some buffers absorb strongly at this wavelength. The bovine serum albumin (BSA) quality control (section 2.5.2) which had been diluted in order to give a final protein concentration of 600 μg/ml when measured, did not give this value. The method was repeated many times without success. The only explanation was perhaps that samples and the QC material were diluted in phosphate buffer and not the recommended 0.2 M NaCL. This method for estimating total protein was not pursued further and that of Lowry used in its place (section 2.5.3).

2.5.1 Estimation of Total Protein (Hudson and Hay 1989b)

The Lowry method is essentially a biuret reaction which uses Folin-Ciocalteau reagent to enhance colour development. It is used preferentially as it is 10 times more sensitive than the biuret reaction (Lowry et al. 1951).

Different concentrations of a known protein are treated with alkaline copper sulphate in the presence of tartrate. Following incubation, the Folin phosphotungstomolybdic acid reagent is added. This is reduced to a blue colour and is read at 650 nm. The unknown protein solutions are converted into concentration units by reference to the standard curve. Shortcomings of the method are that (i) the alkaline copper reagent is unstable and requires fresh preparation for each assay (ii) the assay is photosensitive and care should be taken to ensure that samples are subjected to the same amount of light (iii) interference is common from substances such as detergents, carbohydrates, glycerol, disulphide containing compounds, magnesium and calcium. In other ways the assay is rapid, technically simple and reliable.

This adaptation (Hudson and Hay 1989b) of the Lowry method differs from the original in that absorbances are read at 650 nm and not 750 nm. To demonstrate that this made no difference to the results 26 samples of BSA (600 μg/ml) were analysed and read at both wavelengths. Statistical comparison was carried out using a Mann Whitney U Test. Both sets of results are shown in table 2.1. No significant statistical difference could be demonstrated between the results at these two different wavelengths, $p = 0.41$ enabling either wavelength to be used, but because the method of Hudson and Hay stated 650 nm that was the wavelength used. At 650 nm mean = 667.2 ± 42.9 (1SD.), cv = 6.5 % and at 750 nm mean = 672 ± 37.9 (1SD.), cv = 5.6 %.

Table 2.1 Total Protein QC measured at 2 wavelengths

Total protein conc. (µg/ml)	
650 nm	750 nm
665	670
685	700
685	690
670	700
770	765
580	600
615	630
685	700
620	630
630	650
630	650
645	655
700	705
675	680
705	700
680	690
660	665
615	630
650	640
640	660
650	650
760	750
640	650
700	710
645	660
630	645

2.5.2 Materials and Equipment

Stock protein standard; human serum albumin (HSA); 1 mg/ml in 0.01 M PB (Sigma)

Unknown protein solutions

Sodium carbonate; 2 % w/v in 0.1 M NaOH.

Cupric sulphate; 1 % w/v in distilled water.

Folin and Ciocalteu's reagent (BDH)

Spectrophotometer

Phosphate buffer 0.01 M (PB)

BSA; 600 μg/ml in 0.01 M PB (Sigma)

Bovine serum albumin prepared at the above concentration was used as a quality control check in the protein method. It was aliquoted, stored frozen at -20°C and analysed with each protein assay.

2.5.3 Total Protein Method (Hudson and Hay 1989b)

A standard curve as detailed in table 2.2 was prepared and dilutions of the unknown protein solutions, usually 1/8 or 1/10, were made in PB. The total protein was measured as follows; equal volumes of copper sulphate and sodium potassium tartrate solutions were combined. One ml of this mixture was removed and mixed with 50 ml of sodium carbonate solution. One ml of this final mixture was added to 100 μl of each of the standards and unknown protein solutions to be determined.

Finally 100 µl of Folin Ciocalteu's reagent was added to each tube, mixed vigorously and incubated at room temperature for 15 minutes. Absorbances were read at 650 nm and by plotting values against concentrations of the standard solutions, the protein concentrations of the unknown solutions were determined.

2.5.4 Total Protein Standard Curve Construction

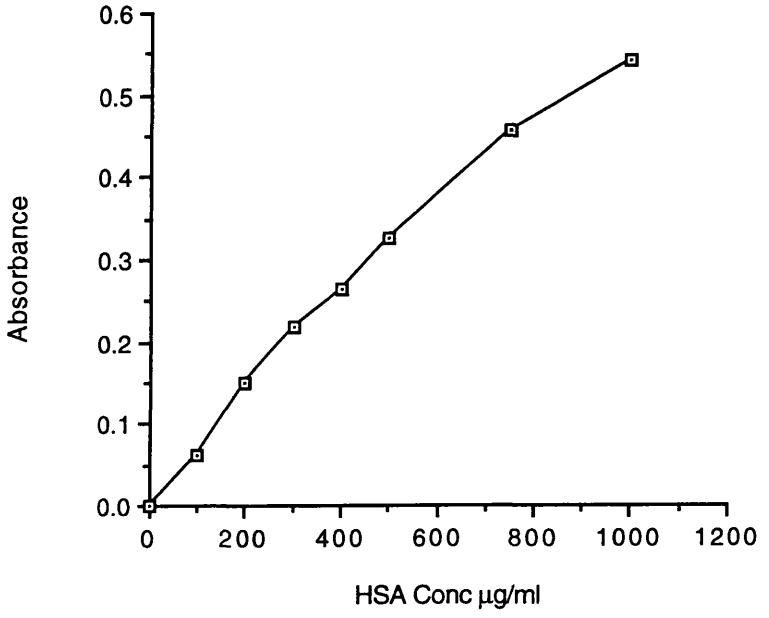
Stock HSA (1 mg/ml) was diluted in 0.01 M PB. to produce concentrations ranging from 100 - 1000 µg/ml.

Table 2.2 Standard Curve Construction

HSA conc. µg/ml	Volume (µl) of HSA	Volume (µl) of 0.01 M (PB)
0	0	100
100	10	90
200	20	80
300	30	70
400	40	60
500	50	50
750	75	25
1000	100	0

A typical standard curve is shown in figure 2.2 when the concentrations above are plotted versus absorbance. A new standard curve was constructed for each batch of proteins assayed.

Figure 2.2 Total Protein Standard Curve



Total protein standard curve, showing concentrations of HSA from 100 - 1000 µg/ml plotted vs absorbance of light at 650 nm.

In the preceding methods the gamma globulin prepared from each of the samples in each of the groups was analysed for protein content using the method of Lowry (1951) adapted by Hudson and Hay (1989b) (section 2.5.3). These IgG rich preparations from the NTG patients, disease control group and normal controls were then diluted in 'O'H + 0.1 % BSA media to give a protein concentration of 100 μg/ml (once the optimum IgG concentration had been established section 2.8.8) and stored at -20°C for use in each of the proliferation assays.

2.6 FRTL5 Cell Line

The FRTL5 cell line, is a non-transformed, differentiated strain of thyroid follicular cells derived from adult Fischer rats. They express important aspects of thyroid differentiation, i.e. the synthesis and secretion of thyroglobulin, trapping and concentrating iodide but do not organify it and as a consequence produce no thyroid hormones. This cloned cell line is totally dependent on TSH at physiological concentrations for cyclic AMP stimulation and growth and can be maintained in culture indefinitely, providing large quantities of a homogeneous functional thyroid cell population for study (Ambesi-Impiombato et al. 1980).

2.6.1 Problems Encountered with the FRTL5 Cell Line

2.6.1 (a) Contamination

On occasion cultures were contaminated with yeast but more frequently with fungus (mould). Contamination was not observed when cells were grown in flasks passaged weekly. However, assays carried out in micro-titre plates are more open and therefore more susceptible to airborne infection and maintaining them for 14 days with no contamination became increasingly difficult. The precaution of filter sterilising media which already contained antibiotics, penicillin/streptomycin (P/S), gentamycin and fungizone before use did not resolve the problem. As the amount of fungizone used was relatively small (0.25 μg/ml) it was decided to increase this 5 fold to 1.25 μg/ml. This had the desired effect with no apparent toxic effect to the cells. Fungizone can be used safely up to a concentration of 10 μg/ml (Hudson and Hay 1989c).

2.6.1 (b) Cell Passage

Collagenase and trypsin in chicken serum (CTC) was used to remove the adherent FRLT5 cells (section 2.6.2). This worked well but when it came to counting it was evident that the cells were in clumps and not single cell suspensions as expected. The clumping could not be tolerated if accurate cell counting and plating out at specific concentrations was to be achieved. Vigorous mixing with a pastette was not effective enough. A

cytospin was used in an attempt to evenly distribute the cells onto slides, this also was ineffective and only after checking the original paper of Ambesi-Impiombato et al. (1980), it was discovered that in transcribing the method the ethylenediaminetetraacetic acid (EDTA) had been omitted from the CTC solution.

2.6.2 Method of Cell Passage

Once the cells have grown to confluence, they are passaged weekly. This is done with a split ratio of 1/10, using a 5 ml mixture of CTC, containing collagenase, 20 units per ml, trypsin, 0.75 mgs/ml and 2 % heat inactivated chicken serum, all in calcium and magnesium free Hanks Balanced Salt Solution (HBSS). EDTA is added as a 0.2 M solution in 1.0 M NaOH/HBSS, 1 : 1. Cells were removed by the addition of one 5 ml aliquot of CTC to a 75 cm^2 flask containing the adherent FRTL5 cells and incubated at 37°C for 20 minutes. Cells were washed with 6H medium to remove the CTC and resuspended in 1 ml of 6H, in preparation for counting.

After passage the FRTL5 cell concentration was calculated as follows.

A ½ dilution of cells in Trypan Blue dye was counted in a haemocytometer (Weber Scientific Ltd, Middlesex England), the exclusion of the Trypan Blue indicating live cells. Cells should be at least 80 % viable.

Cell concentration /ml = number of cells counted x dilution factor x 10^4 (10^4 represents the area of the 4 x 4 matrix of the haemocytometer).

2.7 MTT Proliferation Assay (Mosmann 1983)

This cell proliferation method is used routinely in our department and was to prove useful in providing a convenient starting point from which to develop the MTT assay. In section 2.7.3.1 - 2.7.3.3 the method is optimised, (the results obtained influenced the decision to switch to that used by Claffey et al. 1993).

FRTL5 cells were cultured according to Ambesi-Impiombato et al. (1982). A colourimetric assay using 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was used to quantify cell proliferation (Mosmann 1983).

2.7.1 Materials and Equipment

6H media (section 2.8.2)

5H media (6H without bovine TSH)

Acidified Triton X (10 % Triton X in 0.04 N HCL)

MTT 5 mgs/ml in PBS (Sigma)

Equipment see section 2.8.2

2.7.2 Method

A single cell suspension of FRTL5 cells at a concentration of 1 x 10^6/ml in 6H media was plated out into 96 well flat bottomed plates, 100 µl / well.

After a growing period of 4 days, the cells were washed with 1 x HBSS and the media replaced with 100 µl of thyrotrophin free media (5H). The cultures were thus maintained, feeding every 72 hours.

After 7 days and in triplicate, 100 µl of the IgG samples were added and incubated for 72 hours at 37°C in a humidified atmosphere of 5 % CO_2. MTT, 10 µl per 100 µl medium was added to all wells and plates incubated at 37°C for 4 hours. The plates were inverted and drained onto absorbent paper, this was followed by the addition of 100 µl of acidified Triton X to each well. The plates were then mixed for 20 minutes to dissolve the dark blue crystals and absorbances read within 1 hour, at test wavelength 570 nm, reference wavelength 630 nm and calibration setting 1.99 (Mosmann 1983).

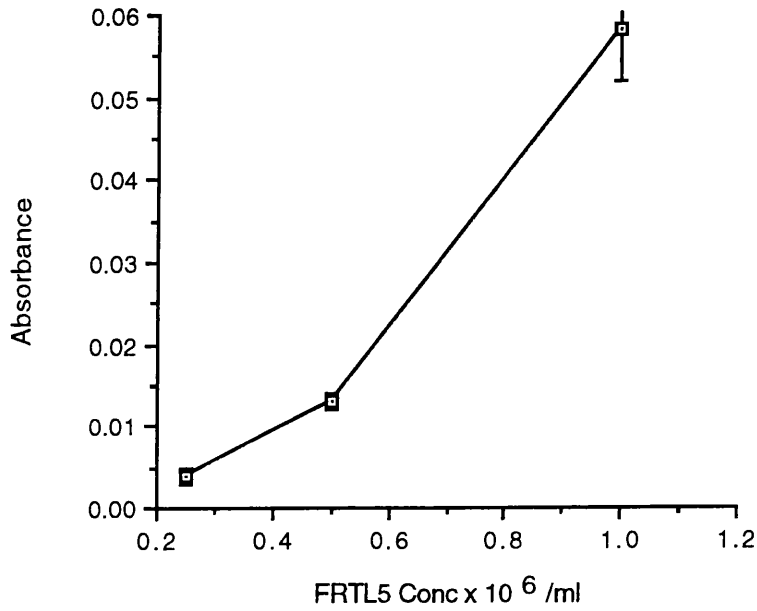
Caution must be exercised when handling MTT as it may cause heritable genetic damage.

2.7.3 Method Development

2.7.3.1 Establishing Optimum FRTL5 Cell Concentration

To establish the optimum FRTL5 cell concentration 3 different concentrations of FRTL5 cells were used, ranging from 0.25 to 1.0 x 10^6/ml. These were used in conjunction with the "low QC" material (section 3.1.2), which had been diluted to give an IgG concentration of 100 μg/ml. Results are shown in figure 2.3. As a result of this a cell concentration of 0.5 x 10^6/ml was chosen for further experiments, as at this concentration the graph shows that there was still potential growth.

Figure 2.3 Optimum FRTL5 Cell Concentration



To define optimum FRTL5 cell concentration the low QC material, IgG concentration of 100 μg/ml, was analysed in conjunction with FRTL5 cells at 3 different cell concentrations; 0.25, 0.5 and 1.0 x 10^6/ml (n = 3). 0.5 x 10^6/ml was chosen as the optimum cell concentration.

2.7.3.2 Optimum IgG Concentration

In order to establish the optimum IgG concentration four concentrations of, again, the low QC material were analysed ranging from 50 - 200 μg/ml and using an FRTL5 cell concentration 0.5 x 10^6/ml. Results of this can be seen in figure 2.4. Results indicated that 100 μg/ml would be the best IgG concentration to use. On reflection it would have been more advantageous to combine the optimum FRTL5 cell concentration with optimum IgG concentration in one assay, but apart from this the main concern was that absorbances were very low.

2.7.3.3 Dose Response to TSH

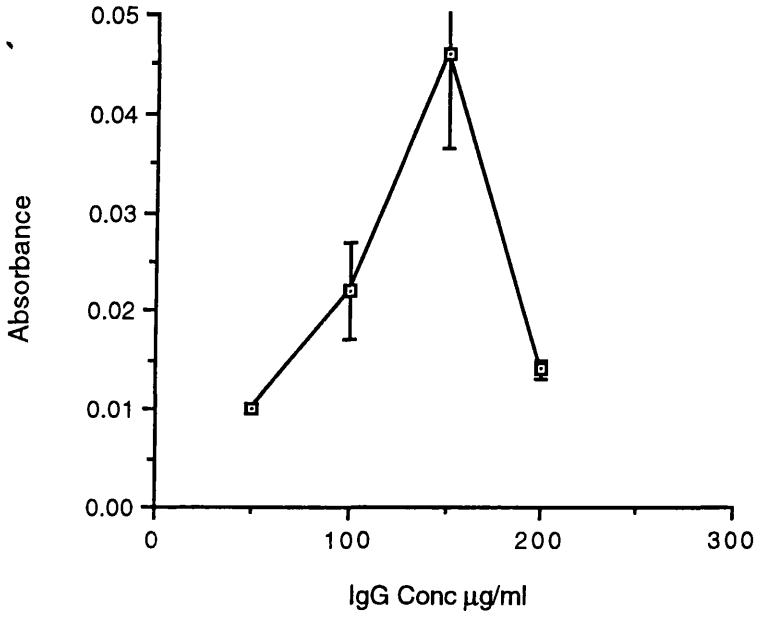
Bovine TSH (10 IU/ml) was diluted to a concentration of 100 mU/ml and a range of concentrations from 0.1 - 50 mU/ml made from this, table 2.3.

Figure 2.5 shows the proliferative response of FRTL5 cells at 0.5 x 10^6/ml to concentrations of bovine TSH which ranged from 0.1 - 50 mU/ml.

Table 2.3 TSH Concentrations used to Demonstrate Dose Response

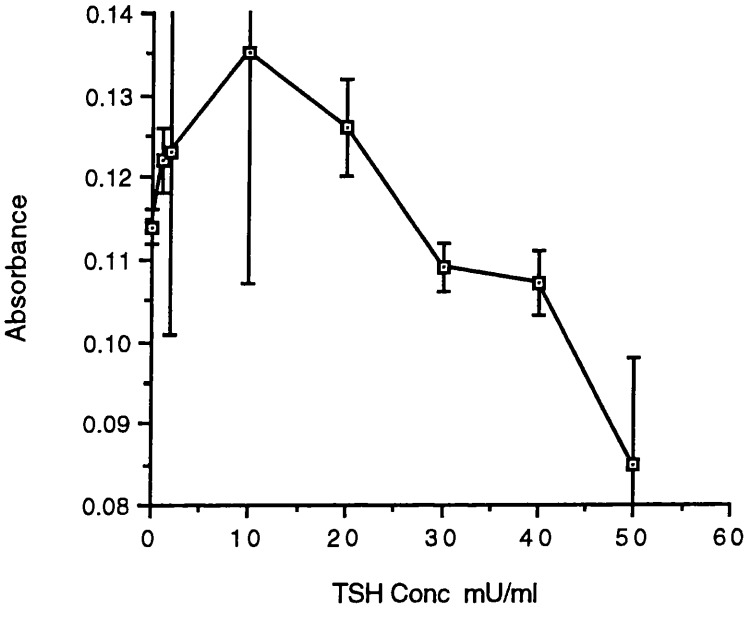
TSH conc. mU/ml	Volume (µl) of TSH (100 mU/ml)	Volume (µl) of 5H media
0.1	10	9990
1.0	10	990
2.0	20	980
10.0	100	900
20.0	100	400
30.0	150	350
40.0	200	300
50.0	250	250

Figure 2.4 Optimum IgG Concentration



Four different IgG concentrations of the low QC were analysed using an FRTL5 cell concentration of 0.5 x 10^6/ml (n = 3). From the results obtained 100 µg/ml was chosen as the optimum IgG concentration.

Figure 2.5 Dose Response to Bovine TSH



Dose response to bovine TSH was assessed by using concentrations of bovine TSH from 0.1 - 50 mU/ml (n = 3).

Two different samples were used to estimate the intra-assay variation; sample A from the goitre group, chosen at random and sample B the low QC. The results are shown in table 2.4. Although the sample size was small it was thought that this could only account in part for the poor cv.'s. Even after optimising for FRTL5 cell concentration and IgG concentration, there was no improvement in the low absorbances. It was decided at this stage to look for an alternative method. As previously mentioned this was achieved by changing to that of Claffey et al. (1993). Claffey uses OH + 5 % NCS and points out that by removing the 5 hormones from the culture media the sensitivity to these 5 hormones is diminished and at the same time the specificity of the bioassay is enhanced (Claffey et al. 1993).

Table 2.4 Intra-assay Variation

	Sample A	Sample B
n	8	8
mean	0.047	0.031
SD	0.018	0.008
% cv	38	27

2.8 Assessment of Cellular Growth

2.8.1 Assessment of Cellular Growth using MTT (Claffey et al. 1993)

This is a rapid, simple, colourimetric assay used for quantifying cellular growth and survival, showing a high degree of precision in which the mitochondrial enzyme succinate dehydrogenase of live cells reduces the yellow dye MTT to a water insoluble purple formazan product (Slater et al. 1963). The end product is solubilised and measured colourimetrically (Mosmann 1983). The amount of formazan generated is directly proportional to the cell number over a wide range, using a homogeneous cell population. The intracellular mechanism for formazan production is as yet unknown. One recent study has used 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium, inner salt (MTS) a contrasting tetrazolium salt and compared it with MTT. The aim of this was to elucidate the electron transfer pathway (Goodwin et al. 1996). The main advantages of this colourimetric assay are the speed with which samples can be processed and its non-use of radioisotopes.

2.8.2 Materials and Equipment

FRTL5 cells:- a gift from Dr N Marshall (University College and Middlesex School of Medicine London).

Fungizone 0.5 % Amphotericin B 250 μg/ml (Gibco BRL UK)

Hanks balanced salt solution (Gibco)

Heat inactivated chicken serum (Gibco)

BSA, Collagenase, Trypsin, (all Sigma)

Triton X, 10 % in 0.1 M HCL (Hopkin & Williams UK.)

EDTA and NaOH (BDH)

Dynatech MR 700 microtitre plate reader

75 cm^2 tissue culture flasks, 96 well microtitre plates (Nunc, Roskilde, Denmark)

Phosphate buffered saline (PBS) (Oxoid Ltd England)

Shaker (Luckham PLC UK)

37°C incubator with humidified atmosphere of 5 % CO_2/air

Laminar flow cabinet

MTT 5 mgs/ml in PBS.

Materials for 6H Media Preparation

This 6 hormone mixture plus non essential amino acids (Gibco) and 5 % heat inactivated new born calf serum forms the working solution of FRTL5 growth medium. Its constituents are as follows:-

Coon's modified Ham's F12 medium (Life Technologies Ltd Paisley Scotland), supplemented with the following hormones and at the following final concentrations; Somatostatin 10 ng/ml (Calbiochem), Bovine TSH 10 mU/ml (Armour, Pharmaceuticals, Illinois, USA),

Hydrocortisone 0.36 ng/ml, Transferrin 5 μg/ml, Glycyl-L-Hystidyl-L-Lysine Acetate 10 ng/ml, Insulin 10 μg/ml (Sigma). All of the aforementioned were stored in aliquots at -70°C. P/S, 5000 IU/ml/5000 μg/ml (Sigma), (5 mls/500mls Coons medium), Gentamycin 40 μg/ml (Roussel Ltd, Dublin, Republic of Ireland) (1 ml/500 mls Coons medium). Glutamine 200 mM (5 mls/500 mls Coons medium).

2.8.3 Cell Proliferation Method

FRTL5 cells, were seeded at a density of 5 x 10^4/ml in 6H medium into 96 well flat bottomed plates (150 μl/well).

After 4 days at 37°C the 6H medium was removed and replaced with '0'H medium which retained the 5 % NCS, 1 % glutamine, 1 % P/S and fungizone 0.5 % but lacked the hormones and supplements. The cultures were maintained thus in order to achieve quiescence, with media replacement every 72 hours.

At approximately 70 % confluence and after a period of 9 days the cultures were washed with '0'H medium supplemented with 0.1 % Bovine serum albumin, P/S, glutamine and fungizone as above. Triplicates of growth factors or test substances which had been diluted in '0'H + 0.1 % BSA to give protein concentrations of 100 μg/ml were incubated with the cells for 48 hours at 37°C using a final volume of 100 μl.

Prior to use the MTT was terminally filtered to sterilise it and to remove

any insoluble residue. After the 48 hour incubation with selected stimulators 10 µl MTT was added per well and incubated for a further 30 minutes at 37°C. The plates were inverted and blotted on absorbent paper. The formazan was then eluted from the cells by the addition of 50 µl of acidified Triton X-100 (10 % Triton X in 0.1 M HCL). The microtitre plates were mixed gently for 20 minutes at room temp. Absorbances of the wells were read on a Dynatech plate reader, within one hour of stopping the reaction. The test wavelength was 595 nm, reference wavelength 655 nm and the calibration setting 1.99 (Mosmann 1983).

The following amendments were made to the method during the course of this project.

1. The mixing period with acidified Triton X to dissolve the blue crystals was extended by 20 minutes to 40 as some crystals still appeared un-dissolved.
2. It was decided not to use the outer wells of the plates, as triplicates of results at the edges were not good. This was most likely due to evaporation in the outer wells. Sterile water was used to fill these wells in place of samples, the only drawback being a decrease in the number of samples processed per plate.

2.8.4 Optimising Conditions for MTT Cellular Growth

2.8.5 FRTL5 Growth Curve

In the log phase, cell growth is asynchronous and exponential and is the optimum time for cell use as the population is phenotypically at its most homogeneous and has its highest viability. By establishing the doubling time of the cells one is assured that the cells will be in the log phase (Hudson and Hay 1989c).

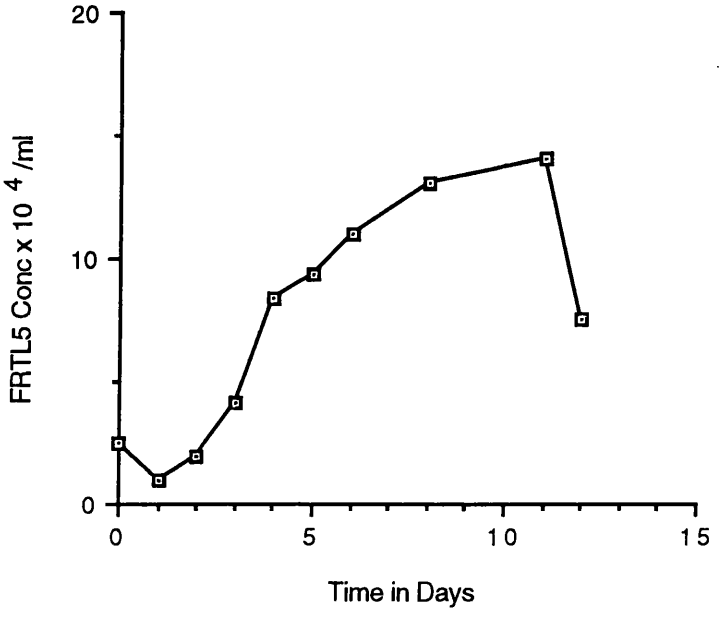
2.8.6 Growth Curve Method

1 ml of FRTL5 cells was added to each well of a 24 well plate at a cell concentration of 2.5 x 10^4/ml. At intervals of 24 hours 1 ml of media was removed from each of two wells and retained. Two hundred microlitres of CTC was added to each of the two wells and incubated for 5 minutes at 37°C, then 800 µl of the retained media was added to each well giving a final volume of 1 ml. Cells were then diluted x 2 in trypan blue to assess viability and counted as in section 2.6.2. This procedure was carried out over a period of 12 days. Table 2.5 shows the data obtained and figure 2.6 the FRTL5 cell line growth curve drawn from this data. The doubling time of the cells was approximately 30 hours.

Table 2.5 Growth Curve Data

Time in days	Mean No of cells $x10^4$/ml
0	2.5
1	1.0
2	2.0
3	4.1
4	8.4
5	9.3
6	11.0
8	13.0
11	14.0
12	7.5

Figure 2.6 FRTL5 Growth Curve

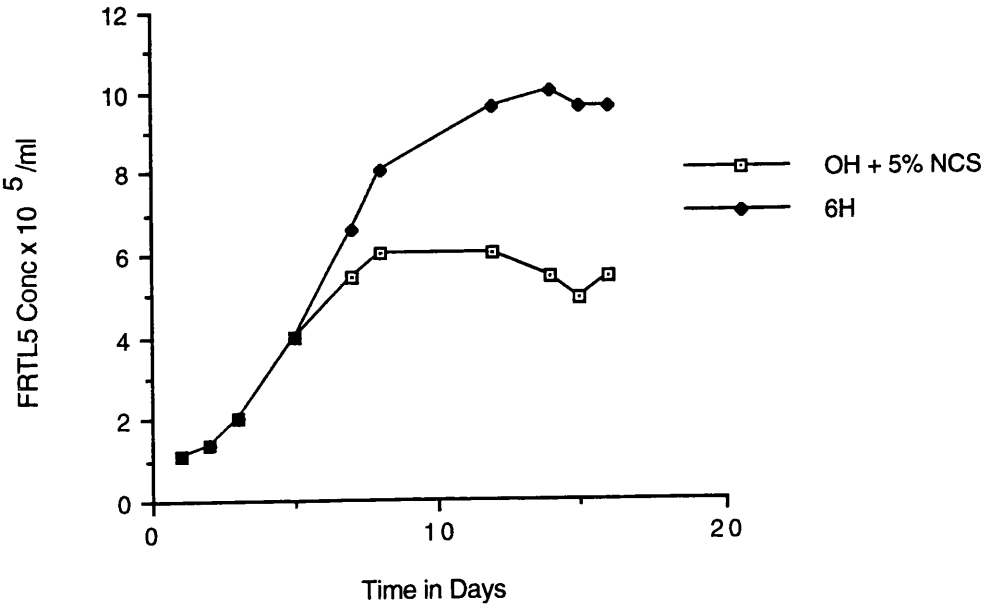


FRTL5 cells were seeded at a density of 2.5 x 10^4/ml. Cells were removed and counted daily, the curve demonstrates the growth phases of the cell line n = 3.

2.8.7 Demonstration of Quiescence

In order to demonstrate that FRTL5 cells quiesced upon removal of TSH from the growth media, a growth experiment as previously described in section 2.8.6 was carried out. On day 5 the FRTL5 cells in culture were divided into 2 groups, one fed with 6H media and the other with 'O'H + 5 % NCS media. The growth of each group was followed daily. Figure 2.7 shows that the cells which were switched to 'O'H + 5 % NCS reached a plateau at day 7 and those grown in 6H at day 14, demonstrating that the removal of TSH from the culture media resulted in the quiescence of the FRTL5 cells.

Figure 2.7 Growth Curve of FRTL5 Cells Showing Quiescence

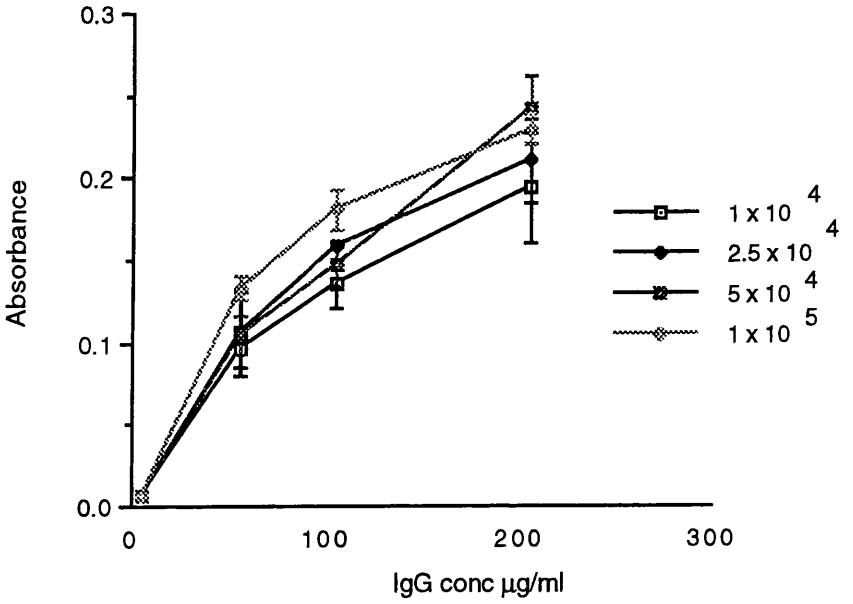


Growth experiment demonstrating the effect on FRTL5 cells after the removal of TSH from the growth medium (represented by the closed boxes). The growth plateau is reached at day 7 whereas those in 6H (with TSH) do not plateau until day 14.

2.8.8 Optimum IgG and FRTL5 Cell Concentration

Preliminary experiments were carried out in order to define optimum IgG concentration, optimum FRTL5 cell concentration and dose response to TSH. Figure 2.8 shows four FRTL5 cell concentrations ranging from 1 x 10^4 - 1 x 10^5. These were assayed in combination with 3 IgG concentrations made from the low QC material from 50 - 200 μg/ml. The concentration of IgG chosen for use was 100 μg/ml as this was halfway on the graph, thus allowing any growth or inhibitory effect to be observed. The FRTL5 concentration chosen was 5 x 10^4 as this appeared to give the maximum signal.

Figure 2.8 Optimum FRTL5 and IgG Concentrations

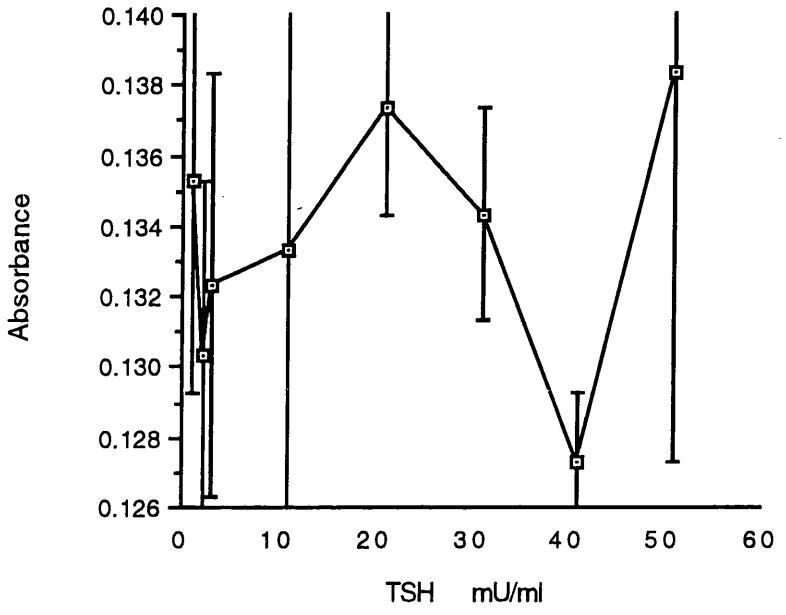


FRTL5 cell concentrations ranging from 1 x 10^4 - 1 x 10^5 in combination with IgG concentrations from 50 - 200 µg/ml were assayed colourimetrically using MTT (n = 3).

2.8.9 Dose Response to Bovine TSH

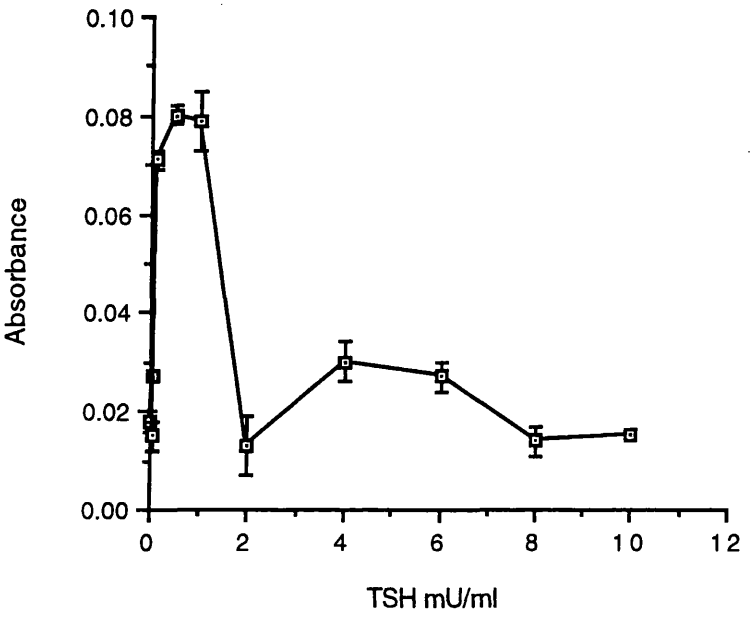
As described in table 2.3 bovine TSH (10 IU/ml) was diluted to 100 mU/ml and a range of concentrations from 0.1 - 50 mU/ml made from this. This dose response to TSH is shown in figure 2.9 (a). The response appeared to be tri-phasic, the experiment was repeated but using concentrations of TSH which covered a narrower range (0.025 - 10 mu/ml). This result is shown in figure 2.9 (b).

Figure 2.9 (a) MTT Dose Response to Bovine TSH (0.1 -50 mU/ml)



FRTL5 cells were incubated with dilutions of bovine TSH ranging from 0.1 - 50 mU/ml (n = 3). MTT was used to measure the effect of these different TSH concentrations on proliferation.

Figure 2.9 (b) MTT Dose Response to Bovine TSH (0.025 - 10 mU/ml)



FRTL5 cells were exposed to dilutions of bovine TSH ranging from 0.025 - 10.0 mU/ml (n = 3). Their response to this was measured using MTT.

2.9 Assessment of Cellular Growth using ^{3}H-thymidine

2.9.1 Labelled Thymidine

Tritiated thymidine is commonly used to detect changes in cell proliferation. It is a low energy β emitter which is less expensive and has a longer half life than other labelled equivalents. Tritiated thymidine is incorporated into replicating DNA, the amount of ^{3}H-thymidine taken up correlating well with the number of cells in the S-phase of the cell cycle (Sigal 1985). The use of ^{3}H-thymidine however does not always reflect true growth as will be discussed later.

2.9.2 Materials and Equipment

Packard/Direct beta counter (Packard Instrument company CT USA).

Gas 98.7 % Helium 1.3 % Butane pressure 15 psi

Self aligning glass fibre filter mats (Packard)

Microwave oven

'Cold' stock thymidine 5mM (Sigma)

Stock ^{3}H-thymidine solution (Amersham Int., Plc, UK)

'O'H Media + 0.1 % BSA

Laminar flow cabinet

Microtitre plates, flat bottomed (Nunc, Roskilde, Denmark)

Working ^{3}H-thymidine solution was made as follows:-

0.1 mls of cold thymidine, (final concentration 50 mM) and 0.5 mls stock radiolabelled thymidine solution (final concentration 50 μCi/ml), were added to 9.4 mls of 'O'H media containing 0.1 % BSA.

2.9.3 ^{3}H-thymidine Incorporation Method

FRTL5 cells were grown according to the method for assessment of cellular growth using MTT (section 2.8.3). After 24 hours incubation with selected stimulators, 20 μl of the working ^{3}H-thymidine solution was added (the equivalent of 1 μCi per well) and plates were incubated for a further 24 hours at 37°C Finally the adherent FRTL5 cells were removed from wells by the addition of 50 μl CTC mixture and incubated for 20 minutes at 37°C. Cells were then checked microscopically to ensure that they had lifted off. The plates were harvested and ^{3}H-thymidine uptake measured using the Packard/Direct beta counter (Packard/Canberra, Berks., England).

2.9.4 Cell Harvesting

Radiolabelled cells are suctioned from microtitre wells and are deposited in clearly defined discs onto a glass fibre filter mat. The unbound ^{3}H-thymidine passes through to the reservoir. Advantages of this method of harvesting cells are that multiples of 96 samples can be rapidly processed,

samples are recoverable and the hazards of using liquid scintillant are removed, although the hazards of using radiolabelled ^{3}H-thymidine itself remain.

2.9.5 Counting

The filter mats are counted using a Direct Beta Counter which uses a tritium sensitive avalanche gas ionisation detector (Packard/Canberra).

2.9.6 Method Development

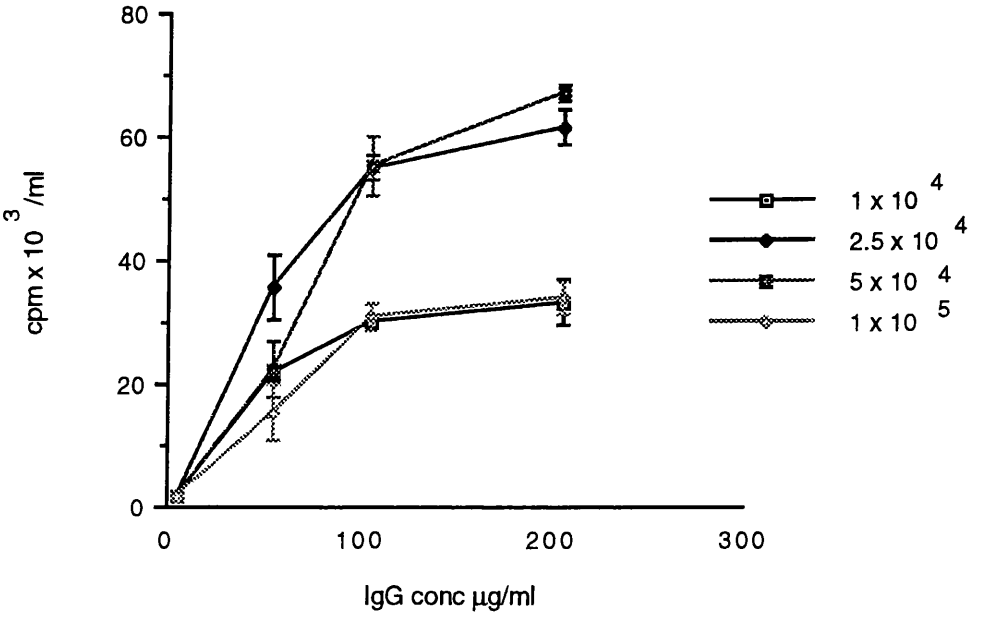
Preliminary experiments were carried out to establish optimum IgG, FRTL5 cell concentration and dose response to TSH, using ^{3}H-thymidine incorporation as a measure of cell proliferation.

An experiment which combined 4 FRTL5 concentrations ranging from 1 x 10^{4} - 1 x 10^{5} and 3 concentrations of IgG from 50 - 200 μg/ml was carried out. Results are shown in figure 2.10. The FRTL5 cell concentration of 5 x 10 ^{4}/ml was chosen as was 100 μg/ml IgG concentration as these gave the maximum signal. Using the same concentration of IgG as had been used in the MTT assay avoided different sample dilutions for different assays. Further experiments carried out would use these concentrations.

With the optimum FRTL5 cell concentration now established it was possible to check dose response to TSH. Dilutions of bovine TSH which covered a range from 0 - 2.0 mU/ml were added to cultures at the stage of

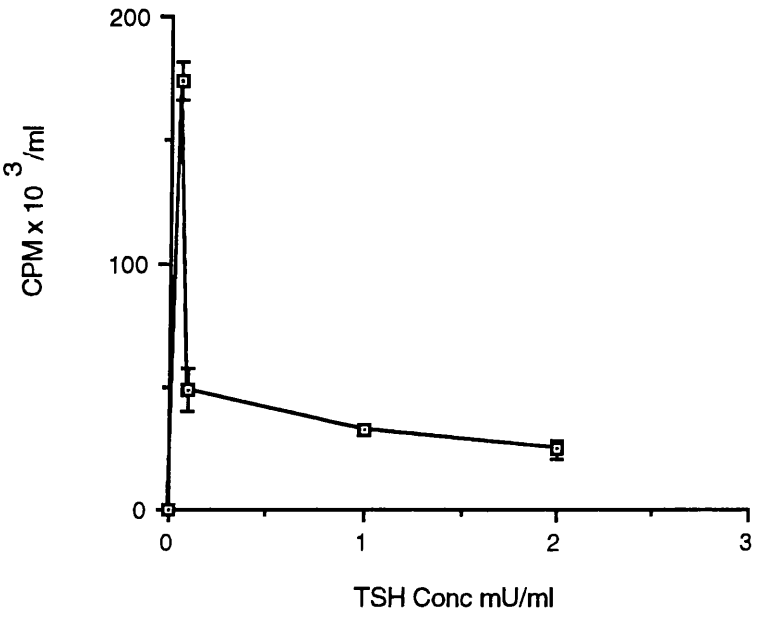
sample addition i.e., 24 hours prior to pulsing with ^{3}H-thymidine. This response is shown in figure 2.11. It was now possible to analyse the samples from the three groups now that optimum growing conditions had been established.

Figure 2.10 Optimum IgG and FRTL5 Cell Concentrations Using ^{3}H-thymidine



Determination of the combined optimum IgG and FRTL5 cell concentrations was carried out using four dilutions of FRTL5 cells which ranged from 1 x 10^4 - 1 x 10^5/ml and IgG ranging from 50 - 200 µg/ml (n = 3). Measured by the incorporation of ^{3}H-thymidine.

Figure 2.11 ^{3}H-thymidine Dose Response to Bovine TSH



The response to dilutions of bovine TSH ranging from 0 - 2.0 mU/ml (n = 3), using ^{3}H-thymidine to determine cell proliferation.

2.10 Assessment of Cellular Growth using Cell Proliferation Kit

This is an immunocytochemical system for monitoring cell proliferation using a monoclonal antibody to 5-bromo-2'-deoxyuridine (BrdU) to detect BrdU incorporated into cellular DNA. Replicating FRTL5 cells were exposed to selected thyroid stimulators from the non-toxic goitre group and proliferation detected by means of the kit. Statistical comparison was made with the normal and disease control groups.

Early work in the kinetics of cellular proliferation was based on the mitotic index and the use of agents such as colchicine to measure the flux of cells into mitosis (Leblond and Stevens 1948). The S phase was described as a discrete interval of DNA synthesis for chromosomal replication and was demonstrated by labelling replicating cells with a radioactive isotope of phosphorus (Howard and Pelc 1951). After the introduction of ^{3}H-thymidine as a marker for selective labelling in DNA synthesis (Taylor et al. 1957), it became easier to study cell kinetics. Tritium is a β emitter with a half-life of more than 12 years and therefore poses a hazard to users and the environment.

The development of a monoclonal antibody that identifies nuclei containing BrdU was an important breakthrough for cell kinetic studies (Gratzner 1982). BrdU is one of the halo pyrimidines and like thymidine is incorporated into the cellular nuclei at the time of DNA synthesis for mitosis (Dolbeare et al. 1983, Morstyn et al. 1983). Provided that the S phase cells are able to transport and incorporate sufficient labelled precursor to DNA on short term exposure to BrdU, then the BrdU

labelling index is a measure of the fraction of cells in the S phase (Hoshino et al. 1985). The kit provides a chemically defined antibody, a highly specific and consistent reagent which does not cross react with thymidine.

Five-bromo -2'-deoxyuridine is incorporated into replicating DNA and is localised by means of a specific monoclonal antibody. Cells are simultaneously exposed to 5-fluoro-2'-deoxyuridine which inhibits thymidilate synthetase increasing BrdU incorporation. Detection of bound antibody is achieved using peroxidase conjugated antibody to mouse immunoglobulin. Diaminobenzidine (DAB) in the presence of cobalt and nickel ions gives blue black staining at sites of BrdU incorporation. Care must be exercised with some of the reagents in the kit, i.e. inhalation and skin contact should be avoided. BrdU is teratogenic and mutagenic and may cause heritable genetic damage. DAB is possibly carcinogenic, cobalt chloride is also hazardous and all three are harmful if inhaled swallowed or absorbed through the skin.

2.10.1 Materials and Equipment

Cell proliferation kit (Amersham Plc)

Petri dishes 50 x 13 mm (Flow Laboratories UK)

Circular glass coverslips No 1 (Lamb UK)

DePeX mounting medium (Lamb UK)

Harris's Haematoxylin (Sigma)

Absolute alcohol (Hayman Ltd)

Hydrogen peroxide (H_2O_2) 2 % (BDH)

Glacial acetic acid (GCA) (BDH)

20, 40, 60, 80 and 100 % methanol (BDH)

6H medium (section 2.8.2)

'O'H medium + 5 % NCS + P/S glutamine and fungizone (section 2.8.3).

'O'H medium + 0.1 % BSA + P/S, glutamine and fungizone (section 2.8.3)

Normal sheep serum (NSS) from the Scottish Antibody Production Unit (SAPU)

PBS 0.01M (Oxoid Ltd England)

Phosphate buffer (pH 7.5)

37°C incubator with humidified atmosphere of 5 % CO_2/air

Humidified incubation chamber e.g. sandwich box lined with damp paper towel.

Jars for washing and staining.

Cotton wool swabs for drying around specimens after washes.

Microscope

Wax pen (Dako)

The FRTL5 cells were seeded at a density of 5 x 10^4 cells in 6H medium, into Petri dishes 3mls/dish and three sterile glass coverslips placed in each dish. After incubation at 37°C for 4 days the 6H medium was replaced with 'O'H medium and the cultures incubated for a further 9

days replacing the media every 72 hours. Samples were added for 48 hours prior to measuring cell proliferation by means of the kit.

2.10.2 Staining Procedure

1. Sites blocked with 10 % NSS in PBS for 20 minutes.

   Wash x 3 with PBS.

2. Apart from 1 control sample, label with BrdU (10 µl + 9.990 mls of 6H media) and incubate at 37°C for 60 mins.

   Wash x 3 with PBS.

3. Fix in acid/alcohol (95 mls methanol + 5 mls GCA) and incubate at room temperature room temp for 30 minutes.

   Wash x 3 with PBS.

4. Incubate at room temperature with 2 % H_2O_2 in methanol for 20 - 30 mins.

   Wash x 3 with PBS.

5. Incubate with BrdU/nuclease for 60 minutes at 37°C. Reconstitute nuclease with 4 mls of distilled H_2O and add 40 µl of BrdU. The outline of each coverslip is circled with the wax pen and covered with 100 µl of reagent.

6. Omitting a second control, incubate with peroxidase anti-mouse IgG2a for 30 mins at room temp.

   Wash x 3 with PBS.

7. Stain for 10 minutes with DAB pre-diluted 1/50 with phosphate buffer pH 7.5 to which 5 drops of colour intensifier has been added.

   Wash x 3 with H_2O.

8. Dehydrate in 20, 40, 60, 80 and 100 % methanol in ascending order.

9. Counterstain for 15 seconds in Harris's Haematoxylin blue in tap H_2O for 10 seconds, air dry, mount with DePeX. and coverslip.

2.10.3 Counting

Slides were looked over to check that staining was uniform and avoiding the periphery at least 1000 cells were counted, to determine the average S phase fraction. An ocular grid was used and X 40 microscopic magnification. Stained cells were counted from right to left, down and then left to right etc. The S phase fraction was expressed as the percentage of total BrdU labelled cells to the total number of cells counted e.g.

$$\text{S phase fraction} = \frac{\text{labelled cells} \times 100}{\text{Total cells counted}}$$

Of the 2 controls generated during the immunocytochemical procedure there should virtually be no positively stained cells.

2.10.4 Method Development

2.10.4 (a) Choice of Support Medium

There were a few developmental details which had to be worked out before commencing.

The FRTL5 cells were grown in 24 well plates, the cells grew well and staining with the kit was successful. It would be necessary to change from plastic to glass if permanent records of the staining were to be kept especially since cell counting is time consuming and tedious, this would enable slides to be stored for counting at a later date. Growing the cells onto a glass support medium such as a coverslip would then enable them to be dehydrated, counter-stained and mounted. It is not possible to use plastic with DePeX mounting media as it contains xylene.

The FRTL5 cells were seeded into the 24 well plates each of which contained a circular glass coverslip. The coverslips were washed in methanol to remove any dust and then autoclaved. Once the immunocytochemistry was complete the coverslips were removed, using watchmakers forceps, for counter-staining. They were then inverted and mounted onto a clean slide using DePex mountant. Great care is required as the circular coverslips are fragile. Eventually cells were grown in Petri dishes which contained the coverslips making their removal easier.

2.10.4 (b) Spurious Staining

Non-nuclear staining can result from non-specific binding of antibodies. This is preventable by blocking binding sites by pre-incubation with 10 % NSS in PBS for 20 minutes.

The following table 2.6 shows the results obtained when two different samples were stained and demonstrates the difference blocking with NSS made.

Table 2.6 Blocking with NSS

		NSS blocked sites	unblocked sites
Sample 1	Mean No of stained cells / 20 fields	38.5	41
Sample 2		86	118

n = 8

Mann Whitney analysis of the data from sample 1 above showed there was no significant statistical difference between the blocked and the unblocked $p = 0.19$, mean = 37.9 ± 3.94 (1SD.). Sample 2 did revealed a significant statistical difference, $p = 0.0009$, mean = 86.5 ± 4.69 (1 SD.) this result influenced the decision to block with NSS.

The second cause of false positives can be due to endogenous peroxidase activity. This is reduced post fixation by incubating for 20 - 30 minutes with 2 % hydrogen peroxide in methanol. If the control without label contains many positively stained cells, then this step should be included

in the procedure.

Using the kit (Amersham) and the conditions described in 2.10.2 analysis of the 3 different groups from this study were carried out, the results of which can be found in chapter 4.

Chapter Three Quality Control

3.1 Measuring Precision

Measuring the variability within and between both the MTT and ^{3}H-thymidine methods was carried out as follows. Quality control materials were made, stored frozen and used throughout this study, with a statistical evaluation of the final results. In the case of the cell proliferation kit this type of approach was not feasible owing to the increased costs which would be incurred.

The intra-assay variation of a method is an indication of the within batch precision of that method. This was measured by analysing the same sample several times, within the same assay. The results were expressed as the cv. In general the intra-assay variation should be approximately half that of the inter-assay variation.

The inter-assay variation reflects the between batch precision of a method. This was measured by analysing the same sample over several individual assays. Results were expressed as the cv. The 95 % confidence interval was calculated (mean ± 2 SD.), within which quality control results from further assays would be expected to fall. Any lying outwith these limits would result in the repetition of the assay.

3.1.1 Preliminary Quality Control

This preliminary quality control was carried out using the MTT method only.

In order to establish the intra-assay variation of the MTT method the following were analysed the specified number of times, within the same assay:-

1. 'O'H + 0.1 % BSA media.
2. 'O'H + 0.1 % BSA media spiked with bovine TSH giving a final TSH concentration of 0.1 mU/ml.
3. 'O'H + 0.1 % BSA media spiked with bovine TSH to give a final TSH concentration of 1.0 mU/ml.

The data are shown in tables 3.1 (a), (b) and (c). The cv. for 'O'H + 0.1 % BSA media was quite high at 21 % (n = 32) showing a probable lack of precision at this level. The cv's of the media containing 0.1 and 1.0 mU/ml TSH were 11.5 % and 12.9 % respectively. The data obtained from the measurement of the intra-assay variation at these three levels is summarised in figure 3.1.

From the results obtained it was decided that the mean absorbances of 2 and 3 were too similar to be useful, these were 0.078 and 0.089 respectively. It was also thought that it would be more appropriate for the QC materials to be serum based. This initial work on quality control materials led to the development of the materials finally used in 3.1.2.

Table 3.1 (a) Intra-assay Variation using 'O'H + 0.1 % BSA Media

Absorbance	
0.065	
0.041	
0.044	
0.040	
0.045	
0.043	
0.051	
0.057	
0.039	
0.041	
0.063	
0.067	
0.058	
0.058	
0.068	n = 32
0.051	mean = 0.04656
0.040	sd = 0.00982
0.044	cv = 21 %
0.049	
0.038	
0.038	
0.039	
0.040	
0.041	
0.041	
0.040	
0.058	
0.041	
0.035	
0.038	
0.039	
0.038	

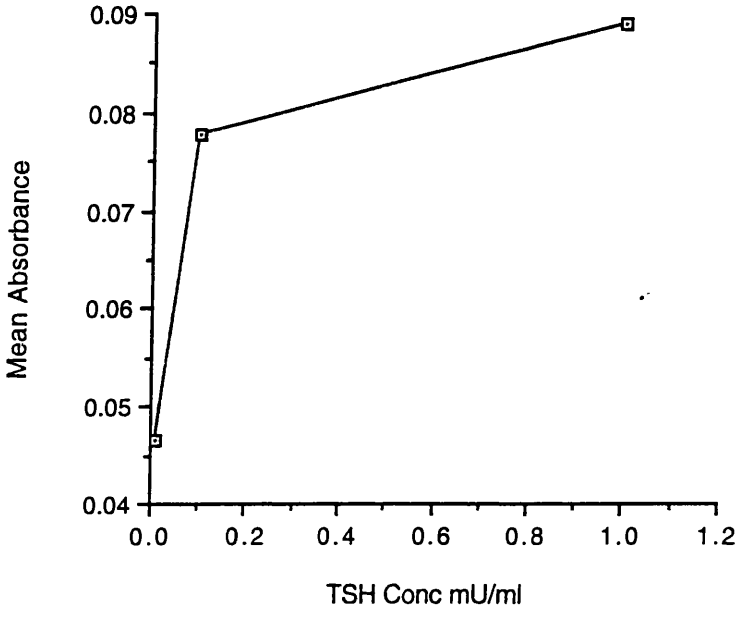
Table 3.1 (b) Intra-assay Variation using 0.1 % mU/ml Bovine TSH in 'O'H + 0.1% BSA Media

Absorbance	
0.074	
0.079	
0.094	
0.073	
0.080	
0.077	
0.097	
0.065	
0.070	
0.082	n = 23
0.084	mean = 0.07765
0.076	sd = 0.0089
0.076	cv = 11.5 %
0.071	
0.066	
0.064	
0.067	
0.078	
0.092	
0.080	
0.086	
0.075	
0.080	

Table 3.1 (c) Intra-assay Variation using 1.0 mU/ml Bovine TSH in'O'H + 0.1% BSA Media

Absorbance	
0.075	
0.075	
0.086	
0.082	
0.063	
0.072	
0.094	
0.087	
0.092	
0.083	
0.074	
0.104	
0.103	n = 28
0.099	mean = 0.08896
0.103	sd = 0.0011
0.102	cv = 12.9 %
0.098	
0.093	
0.094	
0.081	
0.080	
0.085	
0.105	
0.094	
0.081	
0.084	

Figure 3.1 MTT Quality Control Summary



Summary of the intra-assay variation for the MTT method at 3 different levels. Mean absorbance was plotted versus TSH concentration.

3.1.2 Final Quality Control Materials

It was necessary to find biological materials which would give values similar to those of the study samples.

1. Firstly whole plasma was obtained from the Blood Transfusion Service (BTS) at GRI. This was acquired from one donor on whom thyroid function tests were normal. Fabriquik, a thrombin preparation was added to the plasma (1ml of thrombin per litre of plasma and mixed using a magnetic stirrer. Once coagulation had occurred the clot was removed and the serum centrifuged to remove any particulate material. IgG was prepared from this by the method outlined in section 2.4.4 and the total protein content measured (section 2.5.3). This was diluted in 'O'H + 0.1 % BSA to give a final concentration of 100 μg/ml. Aliquots were stored frozen at -20°C and analysed in the MTT and ^{3}H-thymidine proliferation assays. This was designated the "low QC" material.

2. Serum was obtained from a patient with Graves' hyperthyroidism. This also had IgG prepared from it and the total protein measured, the results obtained from this sample were slightly higher than the low QC so it was used in further assays as the "medium QC" material.

3. The third quality control was made by spiking the low QC with bovine TSH to a concentration of 5 mU/ml, this was designated the "high QC".

The QC materials were aliquoted and stored at -20°C. All three QC's were analysed on each plate in each assay, along with a sample of the 'O'H + 0.1 % BSA media in which all samples had been diluted.

3.2 MTT Quality Control Results

3.2.1 MTT Intra-assay Variation

Measurement of the intra-assay variation at three different levels was carried out using the low, medium and high QC materials previously described in section 3.1.2.

The intra-assay variation of the MTT method was carried out by measuring 30 samples of the low QC within the same assay. This data is shown in table 3.2 (a). Results are given as means of triplicates, the cv. was 4.4 %.

Table 3.2 (b) contains the data obtained from the medium QC, n = 14. The cv. was 6.6 %.

The results in table 3.2 (c) show the intra-assay variation using the high QC material. The results are presented as means, the cv. was 8.6%.

Table 3.2 (a) MTT Intra-assay Variation (low QC)

Absorbance	
0.113	
0.109	
0.108	
0.110	
0.114	
0.116	
0.112	
0.117	
0.117	
0.116	
0.112	
0.116	
0.115	
0.116	n = 30
0.108	mean = 0.113
0.117	SD. = 0.005
0.119	cv. = 4.4 %
0.113	
0.120	
0.118	
0.115	
0.112	
0.114	
0.113	
0.112	
0.113	
0.107	
0.111	
0.111	
0.093	

Table 3.2 (b) MTT Intra-assay Variation (medium QC)

Absorbance	
0.149	
0.144	
0.162	
0.166	
0.142	
0.150	n = 14
0.134	mean = 0.154
0.165	SD. = 0.010
0.148	cv. = 6.6 %
0.154	
0.169	
0.159	
0.162	
0.158	

Table 3.2 (c) MTT Intra-assay Variation (high QC)

Absorbance	
0.210	
0.230	
0.240	
0.212	
0.192	
0.237	
0.237	n = 16
0.218	mean = 0.229
0.194	SD. = 0.020
0.228	cv. = 8.6 %
0.234	
0.261	
0.227	
0.244	
0.250	
0.223	

3.2.2 MTT Inter-assay Variation

The precision of the MTT assay was measured using the low, medium and high QC materials described in section 3.1.2.

Table 3.3 (a) shows the data obtained from the low QC material which was used to evaluate the between assay variation. These results are shown in the quality control graph in figure 3.2 (a). The error bars indicate the 95 % confidence interval (mean ± 2 SD.).

Similarly tables 3.3 (b and c) also show the inter-assay variation, using the medium and high QC materials respectively. These are shown in the corresponding quality control graphs in figures 3.2 (b and c) error bars also indicate the mean ± 2 SD.

The confidence interval is 95 %. Quality control results lying outwith this range resulted in the repetition of the assay.

Table 3.3 (a) MTT Inter-assay Variation (low QC)

Absorbance	
0.120	
0.124	n = 7
0.141	mean = 0.119
0.124	SD. = 0.014
0.114	cv. = 11.5 %
0.095	ci. = 0.092 - 0.147
0.118	

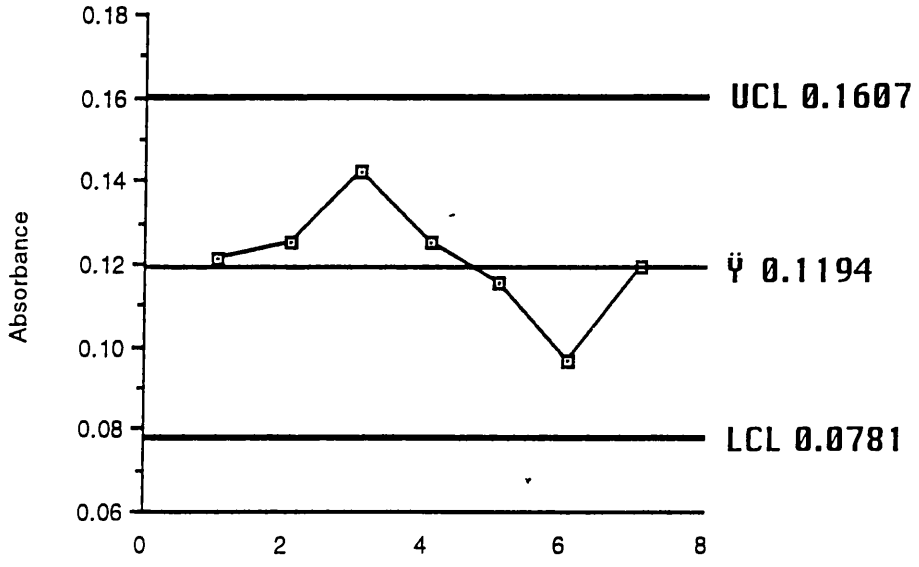
Table 3.3 (b) MTT Inter-assay Variation (medium QC)

Absorbance	
0.171	
0.163	n = 8
0.197	mean = 0.168
0.212	SD. = 0.025
0.146	cv. = 15.1 %
0.139	ci. = 0.117 - 0.219
0.166	
0.149	

Table 3.3 (c) MTT Inter-assay Variation (high QC)

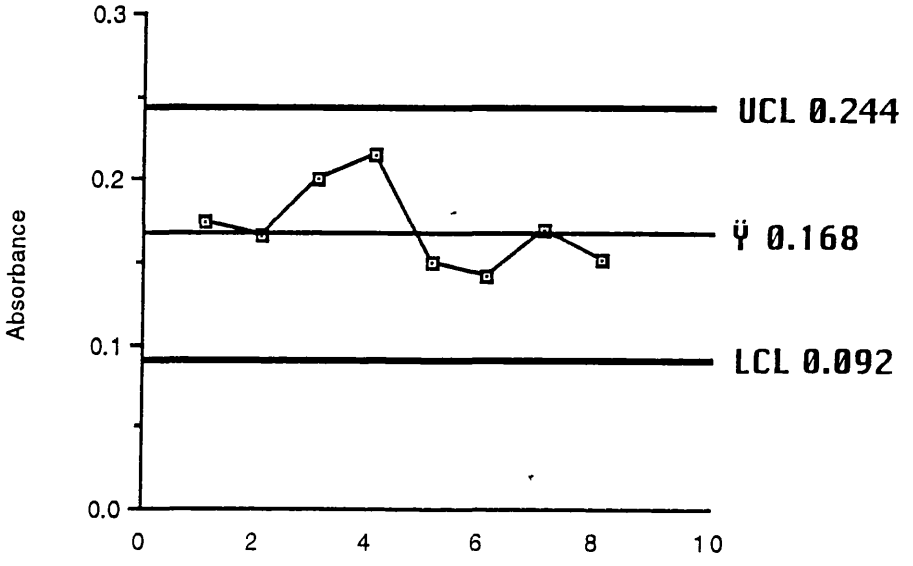
Absorbance	
0.273	
0.251	
0.275	n = 9
0.305	mean = 0.262
0.240	SD. = 0.027
0.232	cv. = 10.3 %
0.256	ci. = 0.208 - 0.316
0.297	
0.231	

Figure 3.2 (a) MTT Inter-assay Variation (low QC)



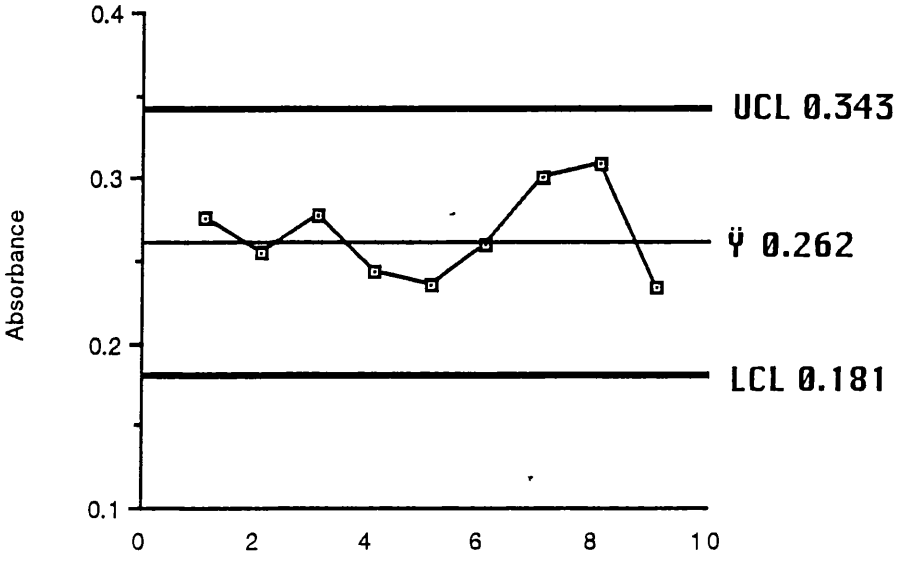
This graph shows the inter-assay variation using the low QC. The error bars indicate the mean ± 2 SD. Numbers 1 - 7 refer to the results obtained on each of the 7 different occasions on which the assay was performed.

Figure 3.2 (b) MTT Inter-assay Variation (medium QC)



Quality control graph demonstrating the inter-assay variation of the medium QC. The error bars show the mean ± 2 SD. Again, the numbers along x axis refer to the number of different occasions on which the assay was performed.

Figure 3.2 (c) MTT Inter-assay Variation (high QC)



Quality control graph showing the between assay variation using the high quality control material. The error bars indicate the mean ± 2 SD. Numbers 1 - 9 indicate the number of different occasions on which the assay was performed.

3.3 ^{3}H-thymidine Intra-assay Variation

Table 3.4 (a) shows the data, in counts per minute (CPM), used to calculate the within batch variation. The low QC was measured within the same assay the specified number of times (n = 14). The cv. at this level was 18 %.

The medium QC data is shown in table 3.4 (b). The sample number was 10 and the calculated cv. was 17 %.

Table 3.4 (c) also shows data used to calculate the intra-assay variation using the high QC. The error at this higher level is indicated by the cv. which was 21 %.

Table 3.4 (a) ^{3}H-thymidine Intra-assay Variation (low QC)

CPM	
3034	
2304	
2902	
3194	
2365	
2049	n = 14
2845	mean = 2789
1976	SD. = 509
3538	cv. = 18 %
3286	
3261	
3321	
2548	
2422	

Table 3.4 (b) ^{3}H-thymidine Intra-assay Variation (medium QC)

CPM	
35327	
23310	
35193	n = 10
31030	mean = 33573
29423	SD. = 5712
41311	cv. = 17 %
29351	
31459	
40784	
38545	

Table 3.4 (c) ^{3}H-thymidine Intra-assay Variation (high QC)

CPM	
66685	
98268	
98200	
85147	
124000	
85502	
72651	
98214	
96053	
84881	
83442	
93466	
117000	
85010	
79720	n = 31
95925	mean = 101558
141000	SD. = 21299
107000	cv. = 21 %
90677	
132000	
119000	
128000	
107000	
146000	
133000	
122000	
110000	
65382	
105000	
92295	
85791	

3.3.1 Inter-assay Variation

The data in table 3.5 (a) were obtained using the low QC (section 3.1.2) and demonstrates the between assay variation. These results are shown in the quality control graph figure 3.3 (a) The cv. was 50 %, error bars indicate the mean ± 2SD.

Tables 3.5 (b and c) show the data obtained using the medium and high QC. The inter-assay variation was poor for the medium QC cv. = 83 % and that for the high QC better at 22 %. The corresponding QC graphs are shown in figures 3.3 (b and c). The error bars indicate the mean ± 2SD.

Table 3.5 (a) ^{3}H-thymidine Inter-assay Variation (low QC)

CPM	
4371	
8359	
5808	
3749	n = 10
3271	mean = 5133
2152	SD. = 2559
9812	cv. = 50 %
7226	
3545	
3034	

Table 3.5 (b) ^{3}H-thymidine Inter-assay Variation (medium QC)

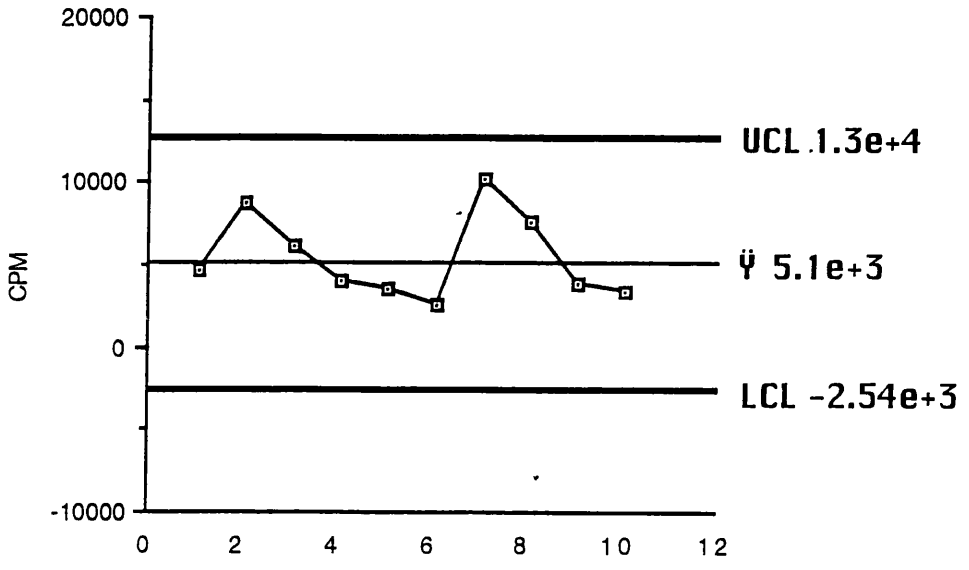
CPM	
41706	
14396	n = 6
15140	mean = 16446
2379	SD. = 13595
7450	cv. = 83 %
17605	

The cv. for the medium QC was unacceptably high , however this QC was assayed on the same plate alongside the low and the high QC which were within the 95 % confidence interval. In explanation, there may have been a problem with the sample or perhaps the precision was just bad at this level, the other factor could be the small sample size.

Table 3.5 (c) ^{3}H-thymidine Inter-assay Variation (high QC)

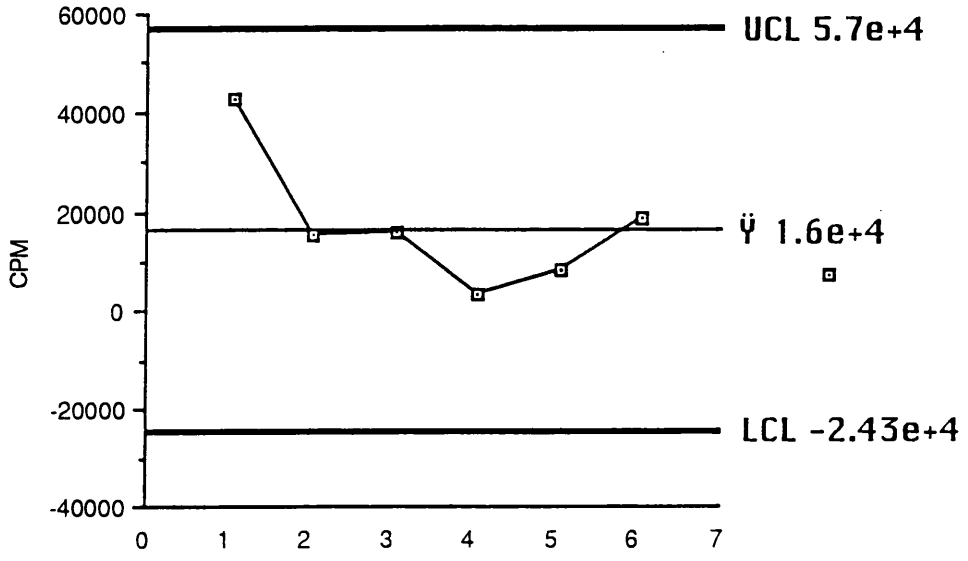
CPM	
110500	
89597	n = 9
70093	mean = 83883
79000	SD. = 18950
91677	cv. = 22 %
48220	
107110	
77814	
80937	

Figure 3.3 (a) ^{3}H-thymidine Inter-assay Variation (low QC)



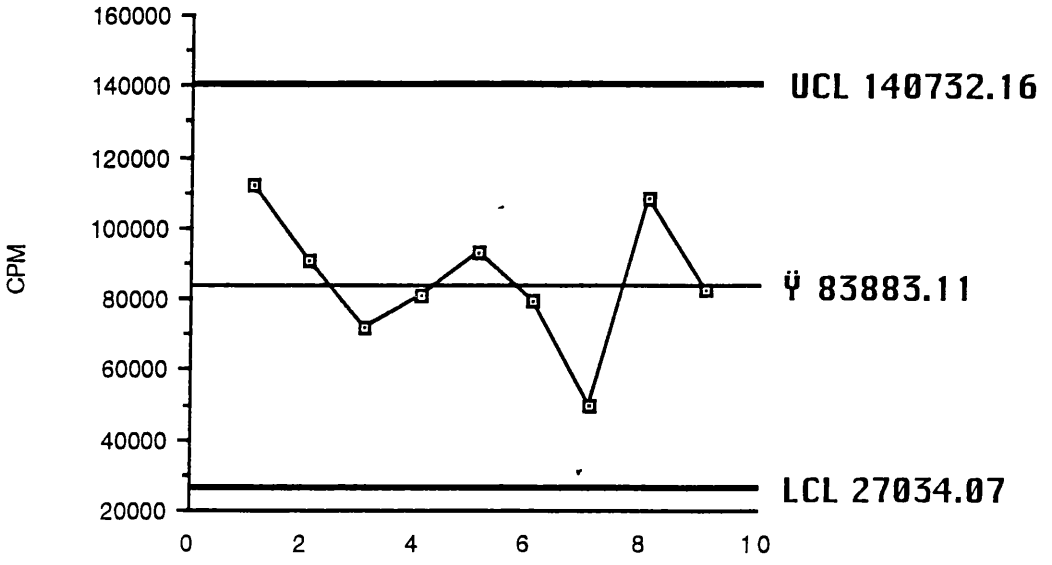
Quality control graph showing the between assay variation, of the low QC. Error bars indicate the mean ± 2 SD. Numbers 1 - 10 indicated the different occasions on which the assay was performed.

Figure 3.3 (b) ^{3}H-thymidine Inter-assay Variation (medium QC)



Quality control graph of the medium QC indicating the between assay variation. The error bars show the mean ± 2 SD. The numbering 1 - 6 refers to the number of different times the assay was performed.

Figure 3.3 (c) ^{3}H-thymidine Inter-assay Variation (high QC)



Quality control graph demonstrating the inter-assay variation of the high QC. The error bars show the mean ± 2SD. The numbering 1 - 9 indicates the different occasions on which the assay was performed.

3.4 Total Protein Quality Control

3.4.1 Total Protein Intra-assay Variation

Table 3.6 shows the data obtained when 26 samples of BSA concentration 600 μg/ml (section 2.5.2) were analysed consecutively. This data was used to calculate the within batch precision of the method, the cv. for which was 6.5 %.

Table 3.6 Total Protein Intra-assay Variation

Total protein concentration μg/ml	
665	
685	
685	
670	
770	
580	
615	
685	
620	
630	
630	
645	n = 26
700	mean = 662.7
675	SD. = 42.88
705	cv. = 6.5 %
680	
660	
615	
650	
640	
650	
760	
640	
700	
645	
630	

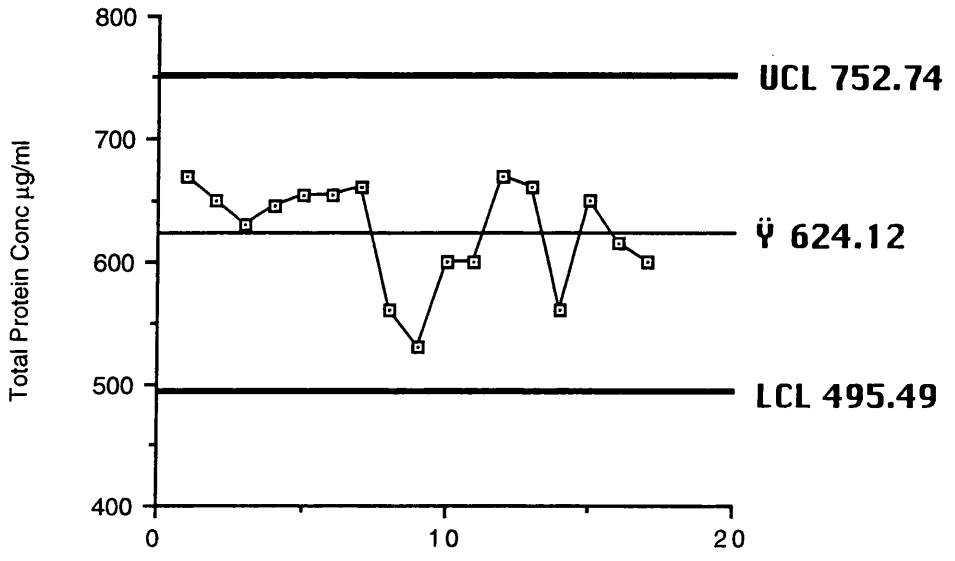
3.4.2 Total Protein Inter-assay Variation

Table 3.7 shows the data obtained when a sample of BSA (600 μg/ml) was analysed in 17 individual assays. Figure 3.4 is a quality control graph of this data, error bars denote the 95 % confidence interval. The cv. was 6.9 %.

Table 3.7 Total Protein Inter-assay variation

Total protein concentration (μg/ml)	
670	
650	
630	
645	
655	
655	
660	n = 17
560	mean = 624
530	SD. = 42.9
600	cv. = 6.9 %
600	
670	
660	
560	
650	
615	
600	

Figure 3.4 Total Protein Inter-assay Variation



BSA diluted to give a concentration of 600 µg/ml was analysed in 17 different assays. These results were used to calculated the inter-assay variation. The cv. was 6.9 %. Error bars indicate the 95 % confidence interval.

Chapter Four Results

4.1 Patient Data

The following groups of subjects were studied with the MTT, ^{3}H-thymidine and BrdU assays to detect FRTL5 cell proliferation.

1. Goitre group:-
    a) 27 with non-toxic goitre pre treatment; 24 female, 3 male.
    b) 20 of the same patients with non-toxic goitre after 6 months treatment with thyroxine; 18 female, 2 male.
2. Disease control group; 10 patients with multiple sclerosis; 9 female, 1 male.
3. 27 normal controls; 24 female, 3 male.

4.1.1 Demographic Characteristics of the Study Group

The study population is shown in table 4.1. Each group was matched for age and sex. Age data are presented as means ± 1SD. Goitre group pre Tx (1a) vs MS group (2), p = 0.073; goitre group post Tx (1b) vs MS group (2), p = 0.037, (comparison of this group now shows a significant age difference this was due to 2 of the original MS patients being removed from the study when their diagnosis of probable MS was not confirmed); goitre group pre Tx (1a)

vs normal control group (3), p = 0.99; goitre group post Tx (1b) vs normal control group (3), p = 0.63; MS group (2) vs normal control group (3), p = 0.067.

Table 4.1 Demographic Characteristics of Study Group

Group		n	Age (years) ± 1SD.	female (n)	male (n)
(1a)	NTG Pre Tx	27	46.6 ± 13.2	24	3
(1b)	NTG Post Tx	20	48.4 ± 13.0	18	2
(2)	MS	10	39.7 ± 8.4	9	1
(3)	Normal controls	27	46.6 ± 12.4	24	3

Age data were expressed as mean ± 1SD.

4.2 Statistical Analysis

All statistical analyses were carried out using Minitab Statistical Software. Means of triplicates were calculated and significant statistical differences between groups were tested for using the student's t test. The paired t test was used to show differences between the pre and post treatment goitre group. Statistical significance indicated where p = <0.05.

4.3 Goitre Grading

Table 4.2 shows the grade of goitre as determined by the clinician in those patients for whom data was available (n = 20). Goitres were described as simple, small single nodule, solitary large or multinodular which was sub-divided 1 - 5 according to the enlargement of the goitre defined in section 2.1.1.

Table 4.2 Goitre Grade

Simple goitre	small nodule	solitary large	Multinodular				
			1	2	3	4	5
1	1	2	1	7	5	2	1

n = 20

4.4 Thyroid Function Tests

Thyroid function test results pre and post treatment with thyroxine are shown in table 4.3. In the pre treatment group the means of all thyroid function tests were normal. In the post treatment group, as expected, serum T4 was elevated, comparison of the pre and post treatment serum T4 showed a significant difference, p = 0.0033. There was no significant difference between pre and post treatment serum T3 p = 0.28. Comparison of pre and

post treatment TSH results also showed a significant difference p = 0.017. Four of the 19 post treatment TSH results showed detectable TSH levels, suggesting incomplete suppression.

Table 4.3 Thyroid Function Tests

	T3 nmol/l (pre Tx)	T3 nmol/l (post Tx)	T4 nmol/l (pre Tx)	T4 nmol/l (post Tx)	TSH mU/ml (pre Tx)	TSH mU/ml (post Tx)
n	10	5	24	17	25	18
mean	2.21	3.00	106.25	151.9	1.22	0.21
SD	0.697	1.38	43.58	52.2	1.64	0.31

Normal ranges; T4 55 - 144 nmol/l, T3 0.9 - 2.8 nmol/l, TSH <5.0 mU/l

4.5 Thyroid Volumes

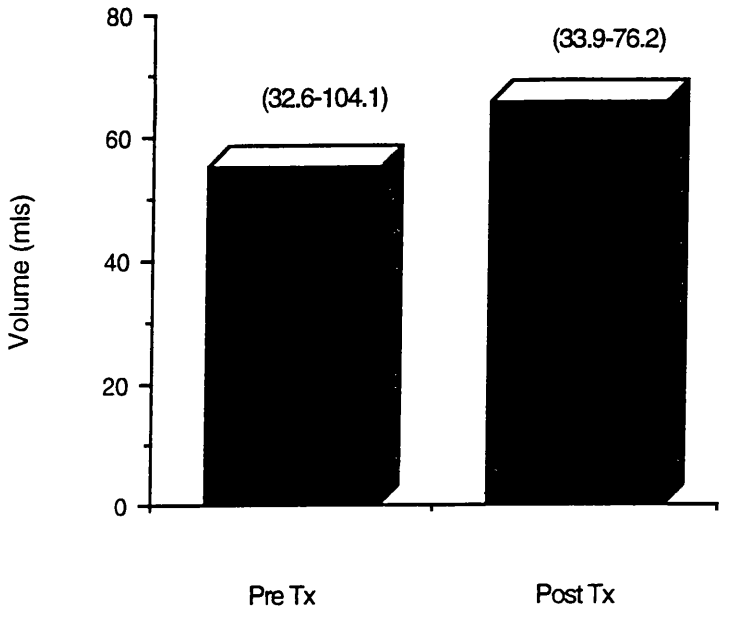
Table 4.4 shows median thyroid volumes, measured by ultrasound, of the goitre group pre and post treatment n = 11. These are also shown in figure 4.1 with inter-quartile ranges in parenthesis. A paired t test showed no statistically significant difference between thyroid volume pre and post Tx p = 0.70. Four (36 %) of the 11 on whom ultrasound measurements were available, had decreases in thyroid volume ranging from 21 - 51 %. In a further 4 an increase in thyroid volume was seen, these increases ranged from 38 - 44 %. The remaining 3 of the 11 showed no change in thyroid

volume.

Table 4.4 Thyroid Volumes of Goitre Group

	n	NTG pre Tx	NTG post Tx
median thyroid volume (mls)	11	55.1	65.6

Figure 4.1 Thyroid Volumes Pre and Post Tx (NTG Group)



Thyroid volumes of the NTG group were compared, pre and post treatment with thyroxine n = 11. Median thyroid volumes were plotted with ranges in parenthesis. Pre and post Tx volumes were compared using a paired students t test p = 0.70, showing no statistically significant difference.

4.6 Antithyroid Antibodies

Table 4.5 shows anti-microsomal and anti-thyroglobulin antibody results for the goitre group n = 13. Titres >1/32 for anti-microsomal and >1/100 for anti-thyroglobulin indicate the significant presence of these antibodies. There was no reference to Hashimoto's disease in the case notes of the patient with the high antibody titres, this patient previously had a right lobectomy.

Table 4.5 Antithyroid Antibodies

Patient	Anti-Microsomal	Anti-Thyroglobulin
t29	neg	neg
t7	neg	neg
t9	neg	neg
t12	neg	neg
t14	1/1600	1/2560
t20	1/100	neg
t27	1/6400	neg
t38	neg	neg
t39	1/6,553,600	1/1,638,400
t41	neg	neg
t36	neg	neg
t47	neg	neg
t17	neg	1/10

4.7 MTT Study Group Results

IgG from the 3 groups of subjects was used to stimulate growth in quiesced FRTL5 cells for 48 hours. Proliferation was demonstrated by an increase in absorption using the dye MTT. The data obtained are summarised in table 4.6.

Table 4.6 MTT Results Summary

	NC	Goitre pre Tx	Goitre post Tx	MS
n	27	27	20	10
mean	0.12	0.104	0.118	0.157
median	0.111	0.112	0.134	0.156
SD.	0.03	0.052	0.047	0.027

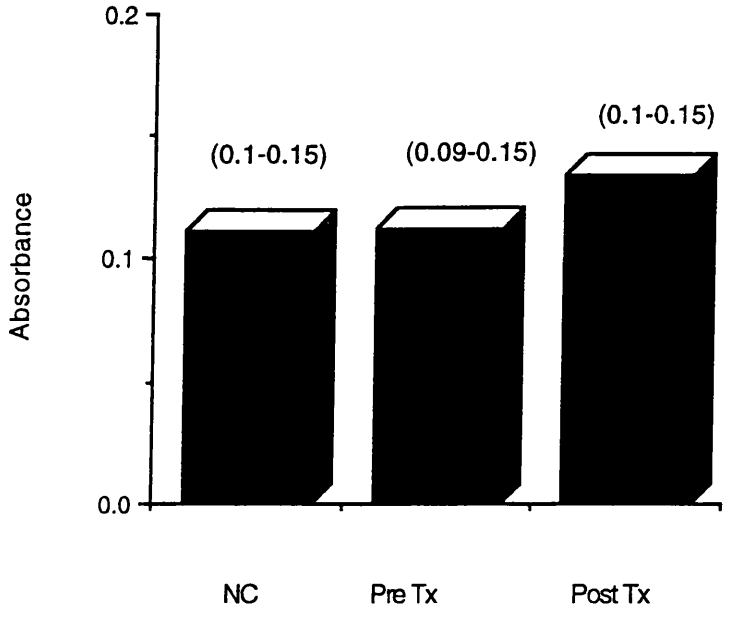
The following graphs were constructed using medians from table 4.6 with inter-quartile ranges in parenthesis.

Figure 4.2 (a) compares the results of the normal controls group (3) (n = 27) with goitre patients pre and post treatment groups (1a) (n = 27) and (1b) (n = 20), p = 0.17 and 0.91 respectively. These were compared using the student's t test for unpaired data and were found not to differ significantly.

A paired t test on the pre and post Tx groups (1a) (n = 20) and (1b) (n = 20) showed no significant difference p = 0.25.

Figure 4.2 (b) shows the comparison of the normal control group (3) (n = 27) with the MS group (2) (n = 10) p = 0.002 and was significantly different. Similarly when group(1a) (n = 27) and (1b) (n = 20) (NTG pre and post Tx) were compared with group (2) these were also found to differ significantly (1a) vs (2), p = 0.0003 and (1b) vs (2), p = 0.0085. Shown in figure 4.2 (c).

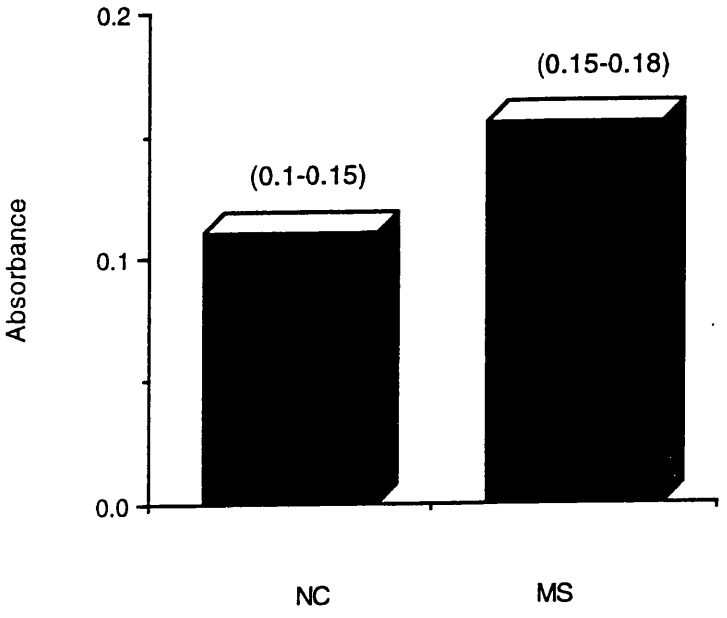
Figure 4.2 (a) FRTL5 Cell Proliferation using MTT in NC vs NTG Pre and Post Tx Groups



Exposure of FRTL5 cells to IgG from the normal control group n = 27, NTG pre Tx group n = 27 and the NTG post Tx group n = 20, for 48 hours. Subsequent proliferation was measured using MTT. Data were presented as medians with inter-quartile ranges in parenthesis, statistical significance indicated where p = <0.05. Normal control group vs NTG pre Tx group p = 0.17; normal control group vs NTG post Tx group p = 0.91. Comparison of the pre Tx with the post Tx group p = 0.25.

Values used were means of triplicates.

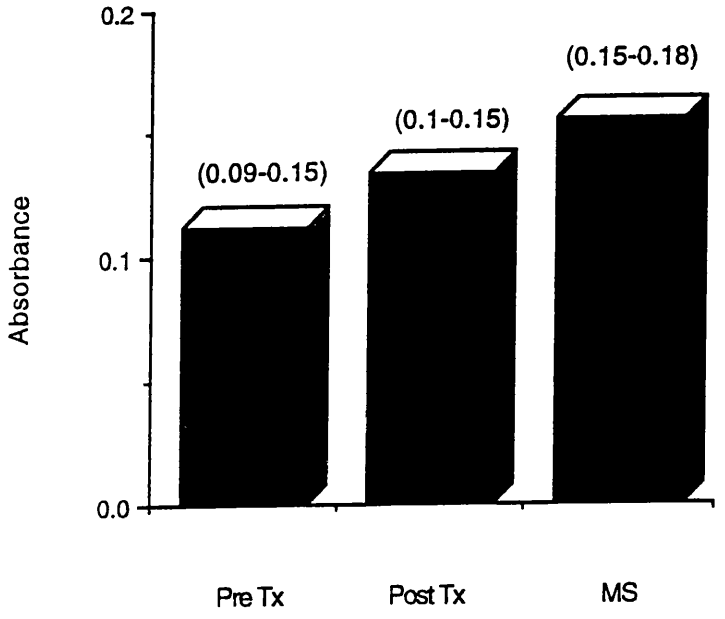
Figure 4.2 (b) FRTL5 Cell Proliferation using MTT in NC vs MS Groups



FRTL5 cells were stimulated with IgG from the normal control group n = 27 and the MS group n = 10, for 48 hours. Proliferation was measured using MTT. The data are presented as medians with inter-quartile ranges. Statistical significance expressed as a p value of <0.05. A significant difference was demonstrated between these 2 groups p = 0.002.

Values used were means of triplicates.

Figure 4.2 (c) FRTL5 Cell Proliferation using MTT in NTG Pre and Post Tx vs MS Group



IgG from the NTG pre and post Tx and the MS groups, was incubated with FRTL5 cells for 48 hours. Proliferation was measured using MTT. Data are presented as medians with inter-quartile ranges. Statistical significance indicated where p = <0.05. Statistically significant differences were shown between the goitre group pre and post Tx vs the MS group p = 0.0003 and 0.0085 respectively.

Values used were means of triplicates.

4.8 ^{3}H-thymidine Study Group

Data from the incorporation of ^{3}H-thymidine for each of the study groups are summarised in table 4.7.

Table 4.7 ^{3}H-thymidine Incorporation Results Summary

	NC	Goitre pre Tx	Goitre post Tx	MS
n	27	27	20	10
mean	19371	13787	8286	19657
median	13281	4594	5887	11642
SD.	18571	18774	8214	17452

Figure 4.3 (a) shows the proliferation measured by ^{3}H-thymidine uptake into quiesced FRTL5 cells when exposed to IgG from the normal control group (3) (n = 27) and patients with NTG pre and post treatment groups (1a) (n = 27) and (1b) (n = 20). Data are presented as medians with inter-quartile ranges in parenthesis. The student's t test for unpaired data was used to test for differences between the groups. A p value of <0.05 was considered to be statistically significant. There was no significant difference between the normal control group (3) and the pre Tx group (1a), p = 0.28 however the post Tx group was significantly different from the NC group (p = 0.009).

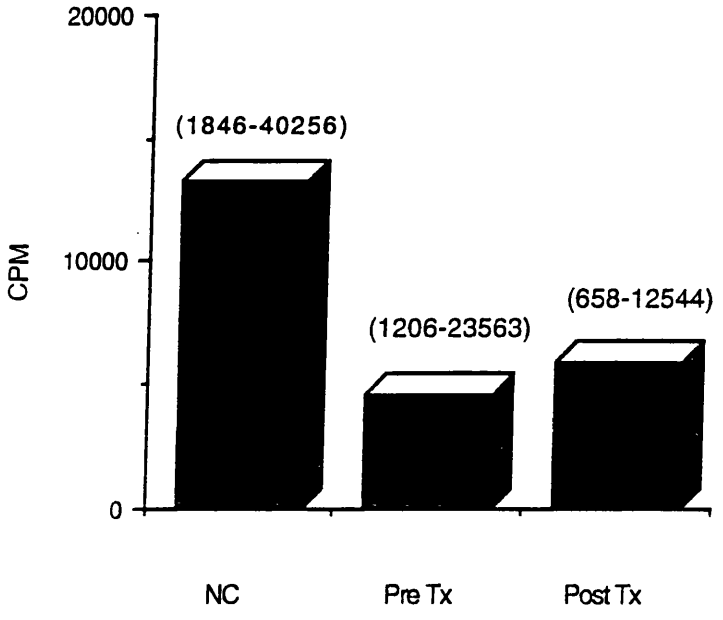
The students t test for paired data was used to compare the pre with the post Tx groups (1a) vs (1b) a p value of 0.30 showed that there was no

statistically significant difference between these groups.

Comparison of the normal control group (3) (n = 27) with the MS group (2) (n = 10) is shown in figure 4.3 (b). The student's t test for unpaired data showed no significant difference between these groups p = 0.97.

There was no significant difference observed also when the pre Tx NTG group (1a) (n = 27) was compared with the MS group (2) (n = 10) p = 0.39, likewise comparison of the post Tx NTG group (1b) (n = 20) with the MS group (2) showed no significant difference p = 0.076 [Figure 4.3 (c)].

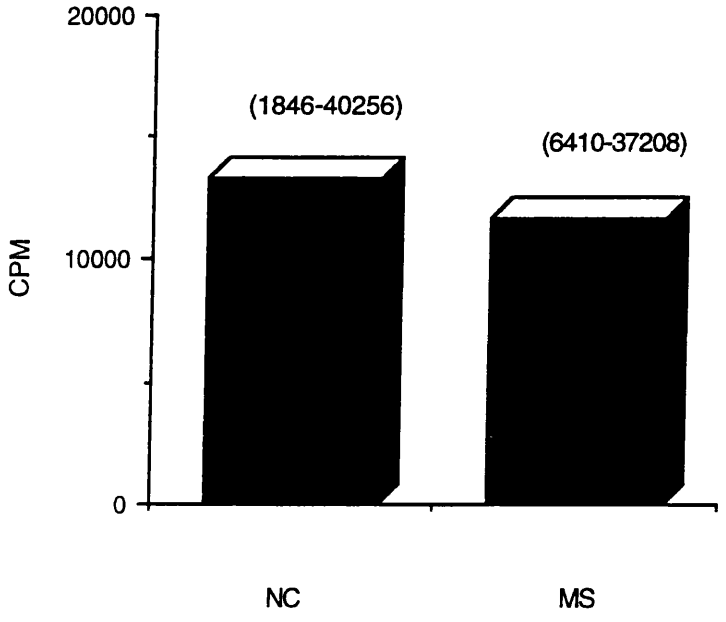
Figure 4.3 (a) ^{3}H-thymidine uptake by FRTL5 Cells in NC vs NTG Pre and Post Tx Groups



Exposure of FRTL5 cells for 48 hours to IgG from the normal control group and the NTG group pre and post Tx with thyroxine. Proliferation was measured by ^{3}H-thymidine uptake which was added 24 hours into the incubation. Data are presented as medians with ranges in parenthesis, statistical significance indicated where $p = <0.05$. Comparison of normal controls ($n = 27$) with NTG pre Tx ($n = 27$) $p = 0.28$; normal controls vs NTG post Tx ($n = 20$) $p = 0.009$; NTG pre Tx vs NTG post TX ($n = 20$) $p = 0.30$.

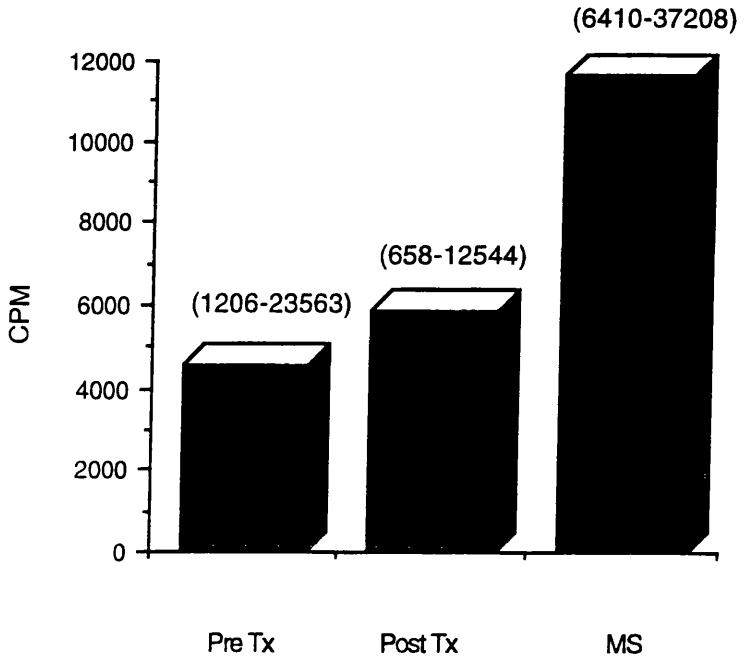
Values used were means of triplicates.

Figure 4.3 (b) ^{3}H-thymidine uptake by FRTL5 Cells in NC vs MS Group



FRTL5 cells were exposed to the IgG from the normal control and from the MS patient groups for 48 hours. FRTL5 cell proliferation was measured by ^{3}H-thymidine incorporation. Data are presented as medians with ranges in parenthesis and p = <0.5 indicating statistical significance. Comparison of the normal control group (n = 27) with the MS group (n = 10) p = 0.97. Values used were means of triplicates.

Figure 4.3 (c) ^{3}H-thymidine uptake by FRTL5 Cells in NTG Pre and Post Tx Groups vs MS Group



Following a 48 hour incubation with IgG from the NTG pre, post Tx and MS groups proliferation of FRTL5 cells was assessed following a 24 hour pulse with ^{3}H-thymidine. Data are presented as medians, ranges are shown in parenthesis. Comparison of the pre Tx group (n = 27) with the MS group (n = 10) p = 0.39 and post Tx (n = 20) vs MS (n = 10) p = 0.076.

Values used were means of triplicates.

4.9 BrdU Incorporation Results

Thyroid stimulators from groups (1a) NTG pre Tx, (1b) NTG post treatment and group (3) the normal controls were added to quiesced FRTL5 cell coated glass coverslips, after 48 hours the immunocytochemistry was proceeded with using the kits from Amersham. Owing to technical difficulties the numbers are slightly reduced. A summary of the data showing the S phase fractions is shown in table 4.8.

Table 4.8 BrdU Incorporation Results Summary

	NC	Goitre pre Tx	Goitre post Tx	MS
n	19	19	18	9
mean	8.37	8.13	9.57	3.49
median	6.1	7.5	9.1	3.9
SD	6.37	8.33	6.09	1.65

Figures 4.4 (a) - 4.4 (c) were constructed using medians from this data, with inter-quartile ranges in parenthesis.

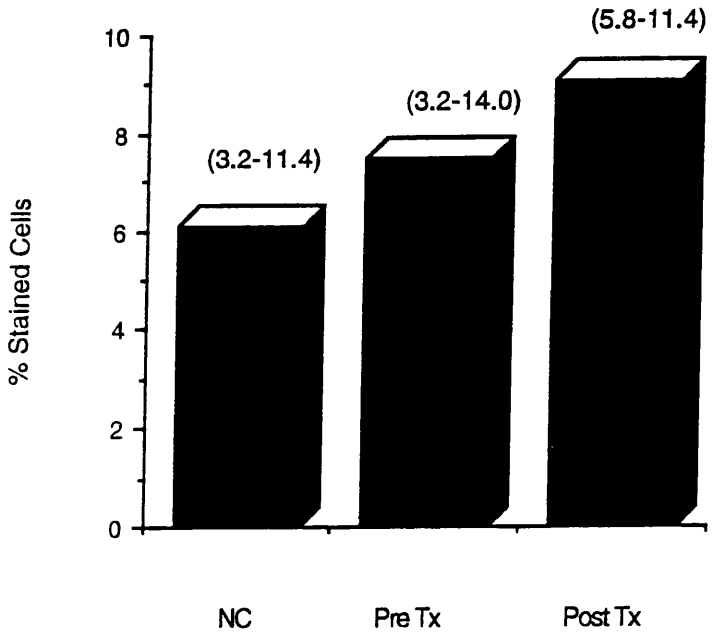
Unpaired data was compared using the students two sample t test. No significant statistical differences could be demonstrated, firstly between group (3) normal controls (n = 19) and the pre treatment group (1 a) (n = 19) p = 0.47, group (3) normal controls (n = 19) vs the post treatment group (1 b) (n = 19) p = 0.56 and finally using the students t test for paired data between

the pre and post goitre group (1 a and b) (n = 18) p = 0.61. Results are shown in figure 4.4 (a).

Figure 4.4 (b) shows the comparison between the NC group (3) (n = 19) and MS group (2) (n = 9). These groups were significantly different p = 0.0049

Figure 4.4 (c) compares the MS with the goitre group pre and post TX, p values were as follows p = 0.0032 and 0.007 respectively, also indicating a significant statistical difference, between these groups.

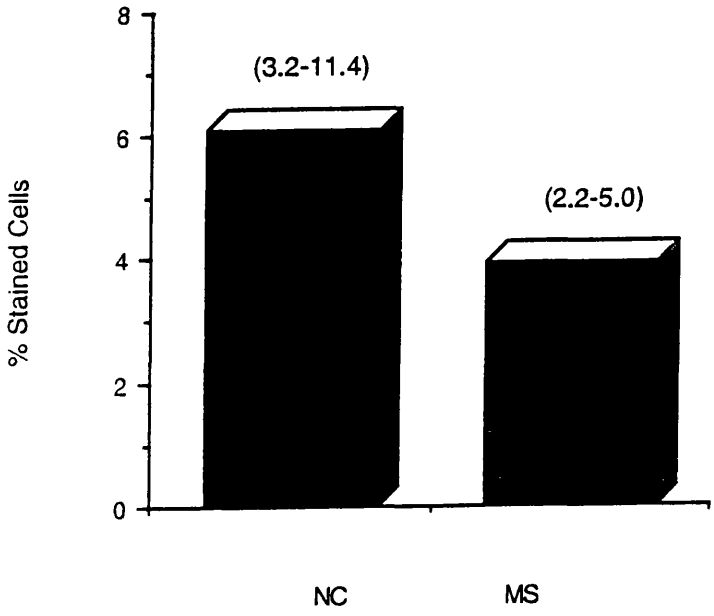
Figure 4.4 (a) BrdU Incorporation into FRTL5 Cells in NC vs NTG Pre and Post TX Groups



BrdU incorporation, into FRTL5 cells treated with IgG from the above groups, was measured using the kits from Amersham. Data are presented as the median number of cells in the S phase (% stained cells) with ranges in parenthesis. NC (n = 19) vs NTG pre Tx (n = 19) p = 0.47, NC (n = 19) vs NTG post Tx (n = 19) p = 0.56. The paired t test was used to test for statistical differences between the goitre group pre (n = 18) and post Tx (n = 18), p = 0.61.

Values used were means of triplicates.

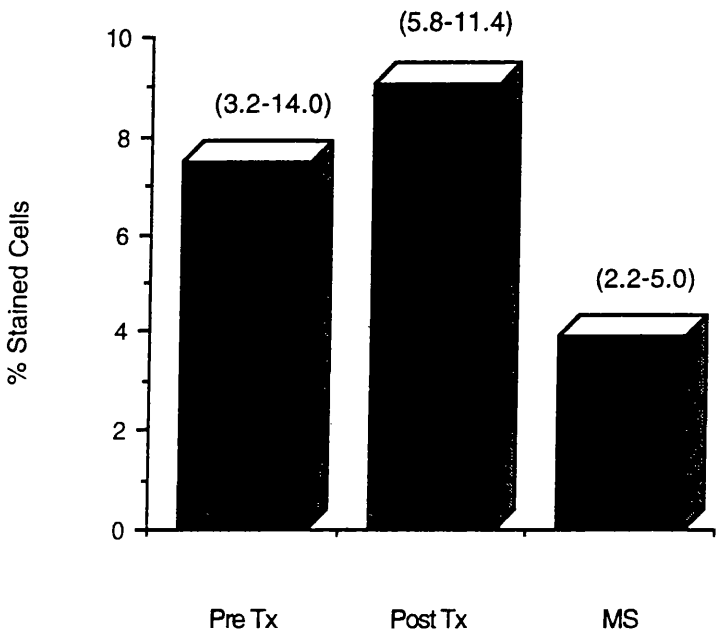
Figure 4.4 (b) BrdU Incorporation into FRTL5 Cells in NC vs MS Group



Possible stimulation of FRTL5 cells by IgG from the normal control and the MS groups was measured by the incorporation of BrdU, using an immunocytochemical kit. Data are presented as medians, ranges in parenthesis. A t test for unpaired data was used to test for a significant statistical difference between these two groups NC (n = 19) vs MS (n = 9) p = 0.0049, which was significant.

Values used were means of triplicates.

Figure 4.4 (c) BrdU Incorporation into FRTL5 Cells in NTG, Pre and Post Tx Groups vs MS Group



An immunocytochemical system was used to measure proliferation in FRTL5 cells previously treated with IgG from the MS and goitre groups pre and post Tx. Statistical analysis using the students t test for unpaired data showed a significant difference between these groups, NTG pre Tx (n = 19) vs MS (n = 9) p = 0.0032, and NTG post Tx (n = 18) vs MS (n = 9) p = 0.007. Values used were means of triplicates.

Chapter Five Final Discussion

5.1 T4 Therapy

Treatment of endemic and sporadic goitre with thyroxine is the standard textbook therapy (De Groot and Stanbury 1975). It is based upon the idea that TSH is the main growth stimulator for thyroid cells (Studer and Greer 1965, Greer et al. 1967).

We undertook to investigate this in a small study on the group of patients with non-toxic goitre (described earlier). Difficulties were experienced regarding compliance, for ultrasound scans, follow up appointments and to a lesser extent with medication. It was disappointing that only 11/20 attended for both thyroid ultrasound scans. Of these 11 patients there was a slight increase in mean thyroid volume in goitre group post treatment which did not differ significantly from the pre treatment group ($p = 0.70$). By clinical assessment 13/20 of the goitre group had no change in goitre size after 6 months treatment with thyroxine, the remaining 7 were unrecorded.

The lack of response to thyroxine therapy by both ultrasound and clinical assessment was disappointing. Reasons for this may be that a 6 month course of treatment was insufficient. Also the goitre population of this study was mixed, the majority were multinodular (~80 %), results from other studies indicate that a multinodular group would be the least likely to respond to treatment with thyroxine (Rojeski and Gharib 1985, Clark 1989,

Studer and Gerber 1991, Edmonds 1992, Ridgeway 1992).

Several studies on solitary benign nodules gave results ranging from no effect (Gharib et al. 1987, Cheung et al. 1989, Morita et al. 1989, Reverter et al. 1991, Papini et al. 1993) to >50 % Celani et al. 1990. No effect were in the majority but these studies did not include a precisely defined group. In 1990 Berghout et al. carried out a placebo controlled study of which 68 % had MNG, of these 63 % showed a mean decrease of 25 % and the group as a whole showed a mean decrease of 15 % with a return to pre treatment volume after a few months. In 1991 Reverter's was placebo controlled on solitary benign nodules but showed no appreciable change in thyroid volume, here the mean period of follow up was 8 - 12 months and all were fully suppressed.

A recent 5 year randomised trial using thyroxine for the treatment of benign cold nodules concludes that long term thyroxine therapy in the goitre group was effective in preventing new lesions and increases in nodule and thyroid volume. There was also a significant increase in thyroid size in the control group after 5 years (Papini et al. 1998). In conclusion, our finding that 6 months treatment with sodium thyroxine for NTG had no effect on goitre size, this compares with many other studies.

Disadvantages in treating with thyroxine include a risk of sub-clinical hyperthyroidism with prolonged therapy (Nygaard et al. 1996), there is also an increased loss of bone mass with its associated risks (Faber and Galloe 1994). Although even this claim is disputed suggesting that sodium

thyroxine administered at between 50 and 200 μg/day does not affect bone turnover and bone mineral density in pre and post menopausal women with NTG (De Rosa et al. 1995).

In the expectation that, in patients for whom thyroid volume had decreased after therapy, there would be a decrease in the presence of TGI and conversely for those whose thyroid volume increased, results were scrutinised, however there was no obvious pattern. It must therefore be concluded that it was not possible to predict those patients who would show a change in thyroid volume on the basis of TGI values.

5.2 Immunoglobulin Preparation

Criticisms have been made of many of the methods for IgG preparation. Ammonium sulphate precipitation yields a crude preparation of IgG and requires extensive dialysis of the end product, making it labour intensive.

Protein A-Sepharose; Protein A is a polypeptide isolated from staphylococcus aureas, yielding pure IgG but is more expensive and when many samples are to be purified it is a time consuming process.

The question of contamination has been addressed by Gartner et al. (1987) who proposed that contamination of immunoglobulin preparations with EGF was responsible for the increase in proliferation seen in sporadic goitre and even Graves' disease. IgG preparations made by ammonium sulphate precipitation were found to have the greatest contamination, with EGF

concentrations ~ twice that of the serum. When these samples were further purified on protein A-sepharose growth promoting activity could not be demonstrated. In the serum EGF was elevated in the sporadic goitre group in 13/23, in the group with Graves' disease 6/19 and in only 3/17 of the normal group. Although there is still the possibility that serum EGF is itself involved in goitre growth.

The method used in this study, Sephacryl 200-S, was chosen as it yields IgG more economically in expense and labour. In retrospect isolation of IgG using protein A-sepharose in terms of purity would have been the more logical choice, thus avoiding contamination by other serum proteins or growth factors which may have contributed to the growth stimulation of the FRTL5 cells.

5.3 TGI

Methods used to detect thyroid growth immunoglobulins have included ^{3}H-thymidine incorporation, the Feulgen histochemical assay and the cytochemical bioassay (CBA). The metaphase index assay (MIA) has also been developed as a screening assay for TGI (Ealey et al. 1988a). Reputedly the most sensitive of these methods is the CBA. The bioassay system ESTA (eluted stain assay) was derived from the CBA but is based on cultured cells rather than tissue segments (Ealey et al. 1988b) In the literature none have escaped criticism.

In choosing the methods for comparison in this study cost and reproducibility were considered. ^{3}H-thymidine and MTT consumable's were relatively inexpensive, equipment for both were available in our department. The BrdU kit was the most expensive costing £350 and providing 100 tests per kit. The MTT method was technically the least demanding followed by ^{3}H-thymidine, the BrdU kit however is very labour intensive. Although with the MTT assay it is important to monitor the cell culture environment as artefacts can be introduced into the system (Marshall et al. 1995). Design of the kit has to be criticised, as once the nuclease reagent (section 2.11.2) is reconstituted it cannot be stored, rendering it necessary to process 30 samples. Processing a batch of 15 samples was considered a more manageable number. However the high specificity provided by the kit gives it an advantage over the other 2 methods. Many labs have 'in house' methods using BrdU which would be less expensive and could be tailored better to ones requirements.

5.4 MTT Results

Table 5.1 summarises the results obtained for the subject groups using the MTT method.

Table 5.1 Statistical Summary of MTT Results

NC (27) vs. Goitre pre Tx (27)	p = 0.17
NC (27) vs Goitre post Tx (20)	p = 0.91
Goitre pre Tx (20) vs. Goitre post Tx (20)	p = 0.25
MS (10) vs. Goitre pre Tx (27)	p = 0.0003*
MS (10) vs Goitre post Tx (20)	p = 0.0085*
NC (27) vs. MS (10)	p = 0.0020*

asterisks denote statistical significance.

No significant differences were observed between groups with the exception of the MS group, which surprisingly showed evidence of thyroid growth immunoglobulins. With such a small sample number (10), confirmation of this finding could only come from a larger study. We are unaware of any published support for this finding, Strakosch et al. (1978) measured thyroid stimulating antibodies in a small group of patients with a variety of autoimmune disorders, which included rheumatoid arthritis, pernicious anaemia, myasthenia gravis, polymyositis and systemic lupus erythematosis (SLE). These were not found to differ significantly from the control group. It is well documented that the sera and spinal fluid of MS patients contains

increased numbers of a variety of antibodies for example anti-nuclear antibodies although they do fluctuate (Fukazawa et al. 1997). Also anti-myelin basic protein antibodies, measles antibodies (Adams and Imagawa 1962) and canine distemper virus-specific antibodies (Rohowsky-Kochan et al. 1995), there is also almost 100 % Epstein Barr virus seropositivity among patients with MS. More significant perhaps and linking MS with thyroid disease, was a Norwegian study where it was found that MS patients showed a significant increased frequency in coexistence with rheumatoid arthritis, psoriasis and goitre patients (Midgard et al. 1996). Patients receiving interferon treatment for MS have shown increases in anti-microsomal and thyroglobulin antibodies. Also one of the few studies carried out on organ specific antibodies in MS found a significant presence, (16.8 %) of anti-microsomal and/or anti-thyroglobulin antibodies compared with 5.9 % of the neurological controls the authors support a specific association between thyroid autoimmunity and MS (Ioppoli et al. 1990). Finally patients with organ-specific autoimmune disorders are likely to develop antibodies to other organs, although there is still no agreement as to whether MS is truly autoimmune and organ specific, but it is an accepted concept that the immune system contributes to the tissue destruction seen in MS (Lucchinetti and Rodriguez 1997).

5.5 ^{3}H-thymidine Results.

Results obtained with the ^{3}H-thymidine method are summarised in table 5.2 below, no significant statistical differences were seen with the exception of the normal control group when compared to the goitre group post Tx with thyroxine.

Table 5.2 Statistical Summary of ^{3}H-thymidine Results

	Raw Data	Stimulation Index
NC (27) vs. Goitre pre Tx (27)	p = 0.28	p = 0.27
NC (27) vs. Goitre post Tx (20)	p = 0.009*	p = 0.0071*
Goitre pre Tx (20) vs. Goitre post Tx (20)	p = 0.30	p = 0.30
MS (10) vs. Goitre pre Tx (27)	p = 0.39	p = 0.44
MS (10) vs. Goitre post Tx (20)	p = 0.076	p = 0.094
NC (27) vs. MS (10)	p = 0.97	p = 0.96

* = statistical significance.

In order to minimise the poor performance and variability within this method, samples were analysed in as few batches as possible and results only accepted when QC checks were within the limits set. Also it was thought that by expressing data as a stimulation index the wide variation in the method would be minimised. These results are also given in table 5.2. The stimulation index was calculated as follows:-

$$\text{S.I.} = \frac{\text{cpm stimulated cells - cpm unstimulated cells}}{\text{cpm unstimulated cells}}$$

Finally to investigate whether there was correlation between the ^{3}H-thymidine and the MTT methods, all the raw data from both methods were expressed as a stimulation index and compared directly. Correlation was poor (correlation coefficient r = 0.381). The significant difference demonstrated between the NC and goitre group post Tx is puzzling. There appears to be a decrease in the level of TGI present in the post treatment samples, this is what was hoped for after treatment with thyroxine with an accompanying decrease in thyroid volume. This finding is suspicious as you might have expected the pre Tx samples to have been increased initially followed by a reduction in TGI in the post Tx samples. The high variability in this method perhaps has a part to play.

5.5.1 Errors Associated with ^{3}H-thymidine Uptake (Maurer 1981)

The incorporation of ^{3}H-thymidine into DNA is widely used, as a measure of DNA synthesis and cell proliferation. Results based on ^{3}H-thymidine incorporation however require careful interpretation.

The assumption that the amount of incorporated precursor is proportional to the amount of newly synthesised DNA is incorrect as fluctuations in intracellular precursor pools entered by a radioactive precursor can reduce or stimulate incorporation rates without corresponding changes in the actual rate of DNA synthesis (Smets 1969). Radiation effects e.g. mutations, chromosome aberrations, DNA strand breaks, growth retardation and cell

death all effect DNA synthesis (Cleaver et al. 1972).

5.5.2 Impurities

^{3}H-thymidine contains radiochemical impurities which are the result of self decomposition. These products are incorporated rapidly into Tetrahymena cells and bind to proteins other than DNA and RNA (Wand et al. 1967). Using autoradiography extensive cytoplasmic labelling can be demonstrated, but not exclusive labelling of the nuclei. This finding was also observed by investigating the distribution of free and bound ^{3}H-thymidine in mouse intestinal crypt cells after injection of pure and impure ^{3}H-thymidine (Diab and Roth 1970).

5.5.3 Factors Relating to the Synthesis of DNA

In mammalian cells the rate of DNA synthesis is not constant and the DNA content does not increase linearly nor exponentially with time (Klevecz et al. 1974). Early replicating DNA with ^{3}H-thymidine incorporation may be subsequently catabolised and the nucleotides reutilised. The problem of reutilisation can be reduced by a shorter pulse e.g. 4 hours as opposed to 24 hours. In some epithelial cells ^{3}H-thymidine may be stored for use later which results in a delay between the appearance of ^{3}H-thymidine in the cells and its incorporation into DNA (Moffat and Pelc 1966, Potten 1971 and 1973).

5.5.4 DNA Repair

Mammalian cells are capable of repairing damaged DNA pieces using the known nucleotides including ^{3}H-thymidine (Howard-Flander 1973), although precise data are lacking as to the normal frequency of DNA repair in resting mammalian cells. Repair also occurs in non-proliferating cells therefore uptake of ^{3}H-thymidine into DNA repairing cells cannot necessarily be correlated with cell division (Harbers 1975). In rat bone marrow reutilisation of ^{3}H-thymidine following catabolism of the DNA of dead cells meant that 40 - 60 % of the ^{3}H-thymidine found in the blast cell was supplied by DNA of the dead bone marrow cells.

The disadvantages by far outweigh the advantages and true proliferation should be determined by a simple cell count and compared with ^{3}H-thymidine incorporation (Maurer 1981).

5.6 BrdU Incorporation

Of the three methods compared in this study that using BrdU has the highest specificity, but was the most technically demanding and expensive of the three. A summation of the statistical results is shown in table 5.3.

Table 5.3 Statistical Summary of BrdU Incorporation Results

NC (19) vs. Goitre pre Tx (19)	p = 0.47
NC (19) vs Goitre post Tx (19)	p = 0.56
Goitre pre Tx (18) vs. Goitre post Tx (18)	p = 0.61
MS (9) vs. Goitre pre Tx (19)	p = 0.0032*
MS (9) vs Goitre post Tx (19)	p = 0.007*
NC (19) vs. MS (9)	p = 0.0049*

asterisks indicate statistical significance.

As with the two preceding methods a statistical difference between the pre and post treatment groups was not observed and again it was surprising to find differences between the MS patients and the other groups. However in the case of BrdU incorporation results were the reverse of that found with the MTT method, in that sera from MS patients appeared to inhibit proliferation in FRTL5 cells. This may have been due to differences in assay technology. Confirmation of these results would be required, along with a larger sample size to confirm whether or not this is a true finding.

5.7 Future Developments

Since there is disagreement as to the existence of TGI in simple goitre it is unlikely that agreement will be reached unless methods used in their detection can be refined, standardised and positive samples compared

between laboratories. In relation to the detection of TGI, lack of uniformity in the measurement of TGI is very apparent and criticism has been levelled at most methods (Zakarija et al. 1990). Zakarija suggests a simple screening index of growth stimulation along with the DNA measurement, which would be technically undemanding and eliminate observer bias. There is also evidence supporting species differences, if the detection system is using a cell line, as growth factors for human cells may not recognise the receptors on cells from other species.

Future work would aim at increasing the study numbers and to recruit a precisely defined goitre group. In our opinion the MTT method had the most potential for further development, it was the most reliable, accurate, technically uncomplicated and the least expensive of the 3 methods compared.

In the event of an antigen being found in NTG it is unlikely that it could be characterised as characterisation of the antigen to thyroid stimulatory immunoglobulin (TSI) in patients with Graves' disease has so far been unsuccessful. Attempts have been made using the method that was used to identify and characterise the insulin receptor i.e. immunoprecipitation of the TSI antigen followed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-Page) and autoradiography (Rapoport et al. 1984). This produced a protein MW ~320,000 which was preferentially selected by TSI containing serum. Results were inconsistent with those other workers on the nature of the human thyroid TSH receptor (Kohn et al. 1983).

In a recent study patients with Graves' disease were given short-term steroid treatment in order to reduce goitre size. The authors were encouraged by achieving this in 3/5 patients. Perhaps there are possibilities for NTG (Mori et al. 1997).

Experiments have been conducted on a model of the athymic nude mouse and although the human is the ideal experimental model there is huge potential for this procedure. Both malignant and benign thyroid tissue have been successfully xenotransplanted for the study of thyroid stimulating antibodies (Gerber et al. 1996).

Finally a review on 10 years of published data on T4 suppressive therapy in patients with solitary and predominantly solid thyroid nodules concludes that T4 therapy fails to shrink most thyroid nodules with only between 10 - 20 % responding to this therapy, response to treatment was defined as a decrease of 50 % in nodule size or volume (Gharib and Mazzaferri 1998).

In summary

1. There was no evidence of stimulation or suppression of FRTL5 cells in vitro, when treated with IgG from the non-toxic goitre group. The disease control patients however showed evidence of FRTL5 cell stimulation with the MTT method and with the BrdU method suppression. The ^{3}H-thymidine method showed evidence of suppression in FRTL5 cell proliferation when the NC group was compared to the goitre group post treatment.

2. Treatment with thyroxine had no effect on thyroid volume or non-toxic goitre size.
3. There was no change in serum TGI levels in non-toxic goitre patients after thyroxine therapy.
4. Serum TGI levels pre-treatment had no value in predicting which non-toxic goitre patients would show a reduction in thyroid volume after thyroxine therapy.

References

Acheson E.D. (1985). The epidemiology of multiple sclerosis. In: Mathews W.B. (ed.). McAlpines Multiple Sclerosis. Churchill Livingstone, Edinburgh p 7-8.

Adams J.M. and Imagawa D.T. (1962). Elevated serum antibodies to measles in patients with MS. Proceedings of the Society for Experimental Biology and Medicine, **3**: 562-566.

Agerbaek H., Pilegaard H.K., Watt-Boolsen S., Spangsberg N., Madsen M.R., Laursen C.N. and Blitchert-Toft M. (1988). Complications of 2,028 operations for benign thyroid disease. Ugeskr. Laeger, Copenh., **150**: 533-536.

Alexander W.D., Koutras D.A., Crooks J. et al. (1962). Quantitative studies of iodine metabolism in thyroid disease. Quarterly J. of Medicine, **31**: 281-305.

Ambesi-Impiombato F.S., Parks L.A.M. and Coon H.G. (1980). Culture of hormone-dependant functional epithelial cells from rat thyroids. Proc. Natl. Acad. Sci., USA, **77; 6**: 3455-3459.

Ambesi-Impiombato F.S., Tramontani D. and Picone F. (1982). Influence of hormones and serum on growth and differentiation of the thyroid cell strain FRTL5. In: Sato G.H., Pardee A. and Sibasku D.A., (eds.). Growth of cells in hormonally defined media. Cold Spring Harbour Symposium on Cell Proliferation, New York, **9**: 483-492.

Ashcraft M.V. and Van Herle A.J. (1981a). Management of thyroid nodules. 1 History and physical examination, blood tests, X-ray tests and ultrasonography. Head and Neck Surgery, **3**: 216-230.

Ashcraft M.V. and Van Herle A.J. (1981b). Management of thyroid nodules. 11 Scanning techniques, thyroid suppressive therapy and fine needle aspiration. Head and Neck Surgery, **3**: 297-322.

Balsam A. and Ingbar S.H. (1979). Observations on the factors that control the generation of triiodthyronine from thyroxine in rat liver and the nature of the defect induced by fasting. J. Clin. Invest., **63**: 1145-1156.

Baumstark J.S., Laffin R.J. and Bardawil W.A. (1964). A preparative method for the separation of 7S gamma globulin from human serum. Arch. Biochem. Biophys., **108**: 514-520.

Becker D.V. and Hurley J.R. (1971). Complications of radioiodine treatment of hyperthyroidism. Semin. Nucl. Med., **1**: 442-460.

Berghout A., Wiersinga W.M., Drexhage H.A., Smits N.J. and Touber J.L. (1990). Comparison of placebo with L-thyroxine alone or with carbimazole for treatment of sporadic non-toxic goitre. The Lancet, **336**: 193-197.

Berghout A., Wiersinga W.M., Drexhage H.A., Van Trotsenburg P., Smits N.J., Van Der Gaag R.D. and Touber J.L. (1989). The long term outcome of thyroidectomy for sporadic non-toxic goitre. Clin. Endocrinol., **31**: 193-199.

Bidey S.P. (1990). Control of thyroid cell and follicle growth. Trends Endocrinol. Metab., **1**: 174-180.

Bobowick A.R., Kurtze J.F., Brody J.A., Hrubec Z. and Gillespie M. (1978). Twin study of MS: an epidemiological enquiry. Neurology, **28**: 978-987.

Brady G.A. (1968). Increased sensitivity of the thyroid in iodine depleted rats to the goitrogenic effects of thyrotropin. J. Clin. Invest., **47**: 1640-1647.

Braverman L.E. (1986). Iodine-induced thyroid disease. In: Ingbar S.H. and Braverman L.E. (eds.). Werner's: The Thyroid, 5th edn. J.B. Lippincott Company, Philadelphia, p 735-746.

Braverman L.E., Ingbar S.H. and Sterling K. (1970). Conversion of thyroxine (T4) to tri-iodothyronine (T3) in athyreotic human subjects. J. Clin. Invest., **49**: 855-864.

Brunn J., Block U., Ruf G., et al. (1981). Volumetrie der Schilddrusenlappen mittels real time sonographie. Dtsch. Med. Wochenschr., **41**: 1338-1340.

Burgus R., Dunn T.F., Desidiri D. et al. (1970). Characteristics of ovine hypothalamic hypophysiotropic TSH-releasing factor. Nature, **226**: 44-46.

Burrow G.N. (1989). Nodular goitre and thyroid cancer. In: Thyroid Function and Disease. W.B. Sauders, London, p 152-174.

Burrow G.N. (1989). Non-toxic goitre diffuse and nodular. In: Burrow G.N., Oppenheimer J.H. and Volpe R. (eds.). Thyroid Function and Disease. W.B. Sauders, London, p 141-151.

Burrow G.N., Mutjaba Q., LiVolsi V. and Cornog J. (1978). The incidence of carcinoma in solitary 'cold' thyroid nodules. Yale J. Biol. Med., **51**: 13-17.

Celani M.F., Mariani M. and Mariani G. (1990). On the usefulness of levothyroxine suppressive therapy in the medical treatment of benign solitary, solid or predominantly solid, thyroid nodules. Acta Endocrinol., **123**: 603-608.

Chambers Science and Technology Dictionary (1984). In: Collocott T.C. and Dobson A.B. (eds.). Ridland Clay (The Chaucer Press) Ltd, Bungay, Suffolk, p 207.

Chatergee and Chandel (1983). Immunomodulatory role of thyroid hormones: in vivo effect of thyroid hormones on the blastogenic response of lymphoid tissue. Acta Endocrinol., **103**: 95-100.

Chesney A.M., Clawson T.A. and Webster B. (1928). John Hopkins Hospital Bull. **43**: p 261.

Cheung P.S.Y., Lee J.M.H. and Boey J.H. (1989). Thyroxine suppressive therapy of benign solitary nodules: a prospective randomised study. World J. Surg., **13**: 818-821.

Christensen S.B., Ericson U.B. and Janzon L. (1984). The prevalence of thyroid disorders in a middle-aged female population, with special reference to the solitary thyroid nodule. Acta Chir. Scand., **150**: 13-19.

Claffey D.C., Yateman M.E., Lane P.A., Ealey P.A., Wass J.A., Marshall N.J. and Holly J.M.P. (1993). The development and characterisation of an eluted stain assay (ESTA) for Insulin-like growth factors. Growth Regul., **3**: 215-225.

Clark O.H. (1989). Invited commentary. World J. Surg., **13**: p 822.

Cleaver J.E., Thomas G.H. and Burki H.J. (1972). Biological damage from intranuclear tritium: DNA strand breaks and their repair. Science, **177**: p 996.

Cohan S.L. and Fermalich J. (1975). Abnormal brain scans in MS. J. Neurol. Neurosurg. Psychiatry, **38**: 120-122.

Cooper D.S. (1998). Radioiodine for hyperthyroidism, where do we stand after 50 years. Journal of the American Medical Association, **4**: 375-376.

Correa P. (1980). Pathology of endemic goitre. In: Stanbury J.B. and Hetzel B.S. (eds.). Endemic Goitre and Endemic Cretinism. Wiley and Sons, New York, p 303.

De Groot L.J. and Stanbury J.B. (1975). The thyroid and its diseases 4th ed. Wiley and Sons, New York, p 644.

De Groot L.J., Nakai A., Sakurai A. and Macchia E. (1989). The molecular basis of thyroid hormone action. J. Endocrinol. Invest., **12**: 843-861.

De Rosa G., Testa A., Maussier M.L., Call'a C., Astazi P. and Albanese C. (1995). A slightly suppressive dose of L-thyroxine does not affect bone turnover and bone mineral density in pre and post menopausal women with non-toxic goitre. Horm. Metab. Res., **27;11:** 503-507.

De Smet M.P. (1960). Pathological anatomy of endemic goitre. In: Endemic Goitre. WHO, Geneva, p 315.

Delange F. (1974). Endemic Goitre and Thyroid Function in Central Africa. Monographs in Paediatrics, Karger, New York, vol. 2.

Delange F. and Ahluwalia R. (eds.) (1983). Cassava toxicity and thyroid: Research and public health issues. International Development Research Centre, Ottawa, IDRC-207e.

Delange F., Hershman J.M. and Ermans A.M. (1971). Relationship between the serum thyrotropin level the prevalence of goitre and the pattern of iodine metabolism in Idjwi island. J. Clin. Endocrinol. Metab., **33**: 261-268.

Denef J.F., Haumont S., Cornette C. and Beckers C. (1981). Correlated functional and morphometric study of thyroid hyperplasia induced by iodine deficiency. Endocrinology, **108**: 2352-2358.

Diab I.M. and Roth L.J. (1970). Autoradiographic differentiation of free, bound, pure and impure thymidine-^{3}H. Stain Technol., **45**: 285-291.

Dolbeare F., Gratzner H., Pallavicini M.G. and Gray J.W. (1983). Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. Proc. Natl. Acad. Sci. USA, **80**: 5573-5577.

Drexhage H.A., Bottazzo G.F. and Doniach D. (1980). Evidence for thyroid growth stimulating immunoglobulins in some goitrous thyroid diseases. The Lancet, **2**: 287-292.

Drexhage H.A., Bottazzo G.F. and Doniach D. (1983). Thyroid growth stimulating and blocking antibodies. In: Chayen J. and Bitensky L. (eds.). Cytochemical Bioassay Techniques and Applications. Marcel Dekker, New York, p 153-156.

Dumont J.E. (1971). The action of thyrotropin on thyroid metabolism. Vitam. Horm., **29**: 287-412.

Dumont J.E., Maenhaut C., Pirson I., Baptist M. and Roger P.P. (1991). Growth factors controlling the thyroid gland. Bailliere's Clin. Endocrinol. Metab.: Growth factors in endocrinology. Bailliere Tindall, London, **5: 4** 727-754.

Dunn A.D. and Dunn J.T. (1982). Thyroglobulin degradation by thyroidal proteases: action of purified cathespin. Endocrinology, **111**: 290-298.

Ealey P.A., Mitchell S.D., Rowles P.M. and Marshall N.J. (1988a). An improved metaphase index assay for detecting thyroid growth stimulators using FRTL-5 cells cultured on a microtitre plate. Journal of Immunological Methods, **111**: 117-123.

Ealey P.A., Yateman M.E., Holt S.J. and Marshall N.J. (1988b). ESTA: a bioassay system for the determination of the potencies of hormones and antibodies which mimic their action. J. Mol. Endocr, **1**: R1-R4.

Edmonds C. (1992). Treatment of sporadic goitre with thyroxine. Commentary, Clin. Endocrinol., **36**: 21-23.

Eltom M., Hofvander Y., Torelem I. and Fellstrom B. (1984). Endemic goitre in the Darfur region (Sudan) epidemiology and aetiology. Acta Med. Scand., **215**: 467-475.

Ericsson U.B. and Lindgarde F. (1991). Effects of cigarette smoking on thyroid function and the prevalence of goitre, thyrotoxicosis and autoimmune thyroiditis. J. Intern. Med., **229**; **1**: 67-71.

Ermans A.M. (1969). Intra thyroid iodine metabolism in goitre. In: Stanbury J.B. (ed.). Endemic Goitre. Pan Amer. Health Org. Scientific Publications, Washington DC, **193**: p 1.

Ermans A.M., Mbulamoko N.B., Delange F. and Ahluwalia R. (eds.) (1980). Role of cassava in the aetiology of endemic goitre and cretinism. International Development Research Centre, Ottawa, IDRC-136e.

Faber J. and Galloe A.M. (1994). Changes in bone mass during prolonged subclinical hyperthyroidism due to L-thyroxine therapy: a meta-analysis. Eur. J. Endocrinol., **13**: 350-356.

Failkow P.J. (1974). The origin and development of human tumours studied with cell markers. N. Engl. J. Med., **291**: p 26.

Felig P., Baxter J.D. and Broadus A.E. (1987). Frohman L.A. (ed.). Endocrinology and Metabolism 2nd ed. McGraw-Hill Book Co., New York, p 389-409.

Fischman H.R. (1982). Multiple Sclerosis: a new perspective on epidemiological patterns. Neurology, **32**: 864-870.

Freidman J.M. and Fialkow P.J. (1983). Genetic factors in thyroid disease. In: Werner S.C. and Ingbar S.H. (eds.). The thyroid: a fundamental and clinical text. Harper and Row, New York, 634-650.

Fukazawa T., Kikuchi S., Sasaki H., Hamada K., Hamada T., Miyasaka K. and Tashiro K. (1997). Anti-nuclear antibodies and the optic-spinal form of multiple sclerosis. J. Neurol., **244;8**: 483-488.

Gaitan E. (1988). Goitrogens. In: Lazarus J.H. and Hall R. (eds.). Hypothyroidism and goitre: clinical endocrinology and metabolism. Bailliere Tindall-Saunders, Philadelphia, p 683-702.

Gaitan E. (ed.) (1989). Environmental goitrogens. Boca Raton, Fla, CRC Press.

Gartner R. (1992). Thyroid growth in vitro. Exp. Clin. Endocrinol., **100**: 32-35.

Gartner R. (1994). Thyroxine treatment of benign goitre. Acta Med. Austriaca, **21;2**: 44-47.

Gartner R. and Pickardt C.R. (1990). Pathogenese der Struma. In: Gerok W. et al. (eds.). Medizin der Gengenwart Bd 5: Teil B and Hesch R.D. (eds.). Urban and Schwarzenberg, Munche-Weinm, p 888.

Gartner R. Tsavella G., Bechtner G. and Greil W. (1987). Evidence that thyroid growth promotion activity of immunoglobulin preparations is due to contamination with EGF. Acta Endocrinol. (Copenh.), **281**: 256-259.

Geersden J.P. and Frolund L. (1984). Recurrence of non-toxic goitre with and without post-operative thyroxine medication. Clin. Endocrinol., **21**: 529-533.

Geersden J.P. and Hee P. (1982a). Non-toxic goitre. A study of the pituitary-thyroid axis in 14 recurrent cases. Acta Chir. Scand., **148**: 225-227.

Geersden J.P. and Hee P. (1982b). Non-toxic goitre. Surgical complications and long term prognosis. Acta Chir. Scand., **148**: 221-224.

Gemsenjager E., Staubb J.J., Girard J. and Heitz P.H. (1976). Pre-clinical hyperthyroidism in multinodular goitre. J. Clin. Endocrinol. Metab., **43**: 810-816.

Gerber H., Studer H., Conti A., Engler H., Kohler H. and Haeberli A. (1981). Reaccumulation of thyroglobulin and colloid in rat and mouse thyroid follicles during intense thyrotropin stimulation. A clue to the pathogenesis of colloid goitre? J. Clin. Invest., **68**: 1338-1347.

Gerber H., Wagner H.E., Burgi U. and Peter H.J. (1996). Model of the athymic nude mouse for the study of benign goitre disease. Exp. Clin. Endocrinol. Diabetes **104; 3**: 56-59.

Gharib H., James E.M., Charboneau J.W., Naessens J.M., Offord K.P. and Gorman C.A. (1987). Suppressive therapy with levothyroxine for solitary thyroid nodules . A double-blind controlled clinical study. N. Engl. J. Med., **317**: 70-75.

Gharib H. and Mazzaferri E.L. (1998). Thyroxine suppressive therapy in patients with nodular thyroid disease. Ann. Int. Med., **128; 5**: 386-394.

Gize R.W. and Mishkin F.S. (1970). Brain scans in MS. Radiology, **97**: 297-299.

Gmelin R. and Virtanen A.L. (1960). The enzymatic formation of thiocyanate (SCN) from a precursor in Brassica species. Acta Chem. Scand., **14**: p 507.

Goodwin C.J., Holt S.J., Riley P.A., Downes S and Marshall N.J. (1996). Growth hormone-responsiveness DT-diaphorase-mediated bioreduction of tetrazolium salts. Biochemical and Biophysical Research Communications, 226: 935-941.

Gratzner H.G. (1982). Monoclonal antibody to 5-bromo and 5-iododeoxyuridine: a new reagent for detection of DNA replication. Science, **218**: 474-475.

Greenspan S.L., Klibanski A., Schoenfeld D. and Chester Ridgway E. (1986). Pulsatile secretion of thyrotropin in man. J. Clin. Endocrinol. Metab., **63**: 661-668.

Greer M.A. and Astwood E.B. (1953). Treatment of simple goitre with thyroid. J. Clin. Endocrinol. Metab., **13**: 1312-1331.

Greer M.A., Studer H. and Kendall J.W. (1967). Studies on the pathogenesis of colloid goitre. Endocrinology, **81**: 623-632.

Grubeck-Loebenstein B., Buchan G., Sadeghi R. et al. (1989). Transforming growth factor beta regulates thyroid growth. J. Clin. Invest., **83**: 764-771.

Gutekunst R., Smolarek H., Hasenpusch U., Stubbe P., Freidrich H.J., Wood W.G. and Scriba P.C. (1986). Goitre epidemiology: thyroid volume, iodine excretion, thyroglobulin and thyrotropin in Germany and Sweden. Acta Endocrinol., **112**: 484-501.

Hall P., Berg G., Bjelkengren G., Boice J.D., Ericsson U.B., Hallquist A. et al. (1992). Cancer mortality after iodine-131 therapy for hyperthyroidism. Int. J. Cancer, **50**: 886-890.

Harbers E. (1975). Nucleinsauren. G. Thieme. Verlag, Stuttgart, p 153-160.

Hay I.D., Reading C.C., Weiland L.H. et al. (1986). Clinicopathological and high resolution ultrasonographic evaluation of clinically suspicious or malignant thyroid diseases. Frontiers of Thyroidology, **2**: 925-927.

Hegedus L., Hansen B.M., Hjalgrim H. and Gervil M. (1991). Is prophylactic thyroxine therapy after operation for non-toxic goitre justified? Thyroid, suppl. **1**: 1-2.

Hegedus L., Hansen B.M., Knudsen N. and Hansen J.M. (1988). Reduction of size of thyroid with radioactive iodine in multinodular non-toxic goitre. BMJ., **297**: 661-662.

Hegedus L., Hansen J.M., Veiergang D. and Karstrup S. (1987). Does prophylactic thyroxine treatment after operation for NTG influence thyroid size? BMJ., **294**: 801-803.

Hill C.S. Jr (1976). The syndrome of medullary carcinoma of the thyroid gland. In Charyulu K.K., Sudarsanam A. (eds.). Hormones and cancer. Stratton, New York, p 151-162.

Holm L.E., Wiklund K.E., Lundell G.E., Bergman N.A., Bjelkengren G., Cederquist E.S. et al. (1988). Thyroid cancer after diagnostic doses of iodine-131: a retrospective cohort study. J. Natl. Cancer Inst., **8**: 1132-1138.

Horlocker T.T., Hay I.D., James E.M., Readin C.C. and Charboneau J.W. (1986). Prevalence of incidental nodular thyroid disease detected by high-resolution parathyroid ultrasonography. In: Medeiros-Neto G. and Gaitan E. (eds.). Frontiers in Thyroidology. Plenum, New York, p 1309-1312.

Hoshino T., Kobayashi S., Townsend J.J. and Wilson C.B. (1985). A cell kinetic study on medulloblastomas. Cancer, **55**: 1711-1713.

Howard A. and Pelc S.R. (1951). Nuclear incorporation of ^{32}P as demonstrated by autoradiographs. Exp. Cell Res., **2**: p 178.

Howard-Flander P. (1973). DNA repair and recombination. Br. Med. Bull., **29**: 226-235.

Hudson L. and Hay F.C. (1989a) Total protein by absorption. Practical Immunology. London: Blackwell Scientific Publications, London, 1.4.

Hudson L. and Hay F.C. (1989b). Total protein by Lowry. Practical Immunology Blackwell Scientific Publications, London, 1.4.1.

Hudson L. and Hay F.C. (1989c). Tissue Culture Techniques. Practical Immunology Blackwell Scientific Publications, London, Appendix 111.

Ingbar S.H. (1972). Autoregulation of the thyroid: response to iodide excess and depletion. Mayo Clin. Proc., **47**: 814-823.

Ioppoli C., Meucci G., Mariotti S., Martino E., Lippi A., Gironelli L., Pinchera A. and Muratorio A. (1990). Circulating thyroid and gastric parietal cell autoantibodies in patients with multiple sclerosis. J. Neurol. Sci., (Ital.), **11; 1**: 31-36.

Jarlov A.E., Hegedus L., Gjorup T. and Hansen J.M. (1991). Accuracy of the clinical assessement of thyroid size. Dan. Med. Bull., **38**: 87-89.

Jensen M.D., Gharib H., Naessens J.M., van Heerded J.A. and Mayberry W.E. (1986). Treatment of toxic multinodular goitre (Plummers' disease): surgery or radioiodine? World J. Surg., **10**: 673-680.

Jersild C., Svejgaard A. and Fog T. (1972). HLA antigens and Multiple Sclerosis. The Lancet, **1**: 1240-1241.

Keiderling W., Emrich D., Hauswaldt C. and Hoffmann G. (1964). Ergebnisse der radio-jodverkleinrungs therapie euthyreoter strumen. Dtsch. Med. Wochenschr., **89**: 453-457.

Kilpatrick R., Milne J.S. Rushbrooke M. Wilson E.S.B and Wilson G.M. (1963). A survey of thyroid enlargement in 2 general practices in Great Britain. BMJ., **1**: 29-34.

Klein B., Klein E. and Horster F.A. (1989). Ergenbnisse de fractionierten radiojodtherapie bie 696 hyperthyreosen und 690 blanden strumen. Nucl. Med., **28**: 129-136.

Klevecz R.R., Kapp L.N. and Remington J.A. (1974). Intermittent amplification and catabolism of DNA and its correlation with gene expression. In: Clarkson B. and Baserga R. (eds.). Control of Proliferation in Animal Cells. Cold Spring Harbour Laboratory, p 817.

Klinck G.H., Oertel J.E. and Winship T. (1970). Ultra structure of normal human thyroid. Lab. Invest., **22**: 2-22.

Kohn L.D., Valente W.A., Laccetti P., Cojen J.L., Aloz S.M. and Grollman E.F. (1983). Multicomponent structure of the thyrotropin receptor: relationship to Graves' disease. Life Sci., **32**:15-30.

Koutras D.A. (1972). Iodine metabolism in endemic goitre. Ann. Clin. Res., **4**: p 55.

Kurtze J.F. (1970). A method of estimating the age at immigration of white immigrants to South Africa with an example of its importance. South African Medical Journal, **1**: 663-669.

Kurtze J.F. (1979). Epidemiology of MS in US veterans race, sex and geographical distribution. Neurology, **29**: 1228-1235.

Lamberg B.A. (1991). Endemic goitre - Iodine deficiency disorders. Ann. Med., **23; 4**: 367-372.

Larsen P.R. (1982). Thyroid-pituitary interaction. N. Engl. J. Med., **306**: 23-32.

Larsen P.R. and Ingbar S.H. (1992a). The Thyroid Gland. In: Wilson J.D. and Foster D.W. (eds.). Williams Textbook of Endocrinology 8th edn. W.B. Saunders Company, London, p 359.

Larsen P.R. and Ingbar S.H. (1992b). The Thyroid Gland. In: Wilson J.D. and Foster D.W. (eds.). Williams Textbook of Endocrinology 8th edn. W.B. Saunders Company London p 405.

Larsen P.R. and Ingbar S.H. (1992c). The Thyroid Gland; Simple or Non Toxic Goitre: Diffuse and Multinodular. In: Wilson J.D. and Foster D.W. (eds.). Williams Textbook of Endocrinology 8th edn. W.B. Saunders Company, London, p 465.

Leblond C.P. and Stevens C.E. (1948). The constant renewal of the intestinal epithelium in the albino rat. Anat. Rec., **100**: 357.

Leppuluoto J., Mannisto P.T. and Virkunen P. (1973). On the mechanism of goitre formation during lithium treatment in the rat. Acta Endocrinol., **74**: 296-306.

Lewinski A., Bartke A. and Smith N.K.R. (1983). Compensatory thyroid hyperplasia in hemithyroidectomised snell dwarf mice. Endocrinology, **113**: 2317-2319.

Limburg C.C. (1950). The geographic distribution of Multiple Sclerosis and its estimated prevalence in the United States. Journal Proceedings of the Association for Research into Nervous and Mental Diseases, **28**: 15-24.

Lorentzen M. and Blichert-Toft M. (1983). Surgical treatment of benign non-toxic goitre. Operative methods, operative morbidity and late sequelae. Ugeskr. Laeger, Copenh., **145**: 1771-1774.

Lowenthal (1964). Quantitative estimations of proteins by E.P. in agarose gel containing antibodies. Anal. Biochem., **15**: 45-55.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randal R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem., **193**: 265-275.

Lucchinetti C.F. and Rodriguez M. (1997). The controversy surrounding the pathogenesis of the multiple sclerosis lesion. Mayo Clin. Proc., **72;7**: 665-678.

Manari L. and Venturi S. (1986). Iodine and retarded immunity. Minerva Med., **77;19**: 805-809.

Marine D. (1928). Certain features of the morphologic pathology of endemic goitre. In: Comptes Rendues de la Conference Internationale du Goitre. Hans Huber, Berne, p 68.

Marshall N.J., Goodwin C.J. and Holt S.J. (1995). A critical assessment of the use of microculture tetrazolium assays to measure growth and function. Growth Regulation **5**: 69-84.

Maurer H.R. (1981). Potential pitfalls of ^{3}H-thymidine techniques to measure cell proliferation. Cell Tissue Kinet., **14**: 111-120.

McAlpine D., Lumsden C.E. and Acheson E.D. (1972). Multiple Sclerosis: a reappraisal. Churchill Livingstone, Edinburgh, p 202.

McDougall I.R. (1992a). Thyroid structure, development and developmental abnormalities. Thyroid Disease in Clinical Practice. Chapman and Hall Medical, London, **1**:4-5.

McDougall I.R. (1992b). Thyroid Disease in Clinical Practice. Thyroid physiology. Chapman and Hall Medical, London, **2**:13-19.

McMullan N.M. and Smyth P.P.A. (1984). In vitro generation of NADPH as an index of Thyroid Stimulating Immunoglobulins (TGI) in goitrous disease. Clin. Endocrinol., **20**: 269-280.

Midgard R., Gronning M., Riise T., Kvale G. and Nyland H. (1996). Multiple sclerosis and chronic inflammatory disease. A case control study. Acta Neurol. Scand., **94;5**: 322-328.

Miller J.M. (1978). Hyperthyroidism from the thyroid follicle with autonomous function. J. Clin. Endocrinol. Metab., **7**: 177-197.

Miller J.M. and Block M.A. (1970). Functional autonomy in MNG. JAMA., **214**: 535-539.

Miller J.M., Horn R.C. and Block M.A. (1967). The autonomous functioning thyroid nodule in the evolution of nodular goitre. J. Clin. Endocrinol. Metab., **27**: 1264-1274.

Miloni E. and Studer H. (1980). Functional and morphologic particularities of the autonomous follicles in human goitres. J. Mol. Med., **4**: p 7.

Moffat G.H. and Pelc S.R. (1966). Delay after plucking of hairs between the appearance of ^{3}H-thymidine in cells and its incorporation into DNA. Exp. Cell Res., **42**: 460-466.

Mori T., Sugawa H., Kosugi S., Ueda M., Inoue D. and Akamizu T. (1997). Effectiveness of a short-term steroid treatment on the reduction in goitre size in antithyroid drug-treated patients with Graves' disease. J. Endocrinol., **44; 4**: 575-580.

Morita T., Tamai H., Ohshima A. et al. (1989). Changes in serum thyroid hormone, thyrotropin and thyroglobulin concentrations during thyroxine therapy in patients with solitary thyroid nodules. J. Clin. Endocrinol. Metab., **69**: 227-230.

Morstyn G., Hsu S.M., Kinsella T., Gratzner H., Russo A. and Mitchell J.B. (1983). Bromodeoxyuridine in tumours and chromosomes detected with a monoclonal antibody. J. Clin. Invest., **72**: 1844-1850.

Mosmann T. (1983). Rapid colourimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods, **65**: 55-63.

Nilsson G., Petersson U., Levin K. and Hughes R. (1977). Studies on replacement and suppressive dosage of L-thyroxine. Acta Med. Scand., **202**: 257-260.

Norrby E. (1978). Viral antibodies in MS. Progress in Medicine and Virology, **24**: 1-39.

Nygaard B., Faber J., Hegedus L. and Hansen J.M. (1996). ^{131}I treatment of nodular non toxic goitre. Eur. J. Endocrinol., **134**: 15-20.

Nygaard B., Gideon P., Dige-Petersen H., Jespersen N., Solling K. and Veje A. (1993). Thyroid volume morphology and urinary iodine excretion in a Danish municipality. Acta Endocrinol., **129**: 505-510.

Nygaard B., Hegedus L., Gervil M., Hjalgrim H., Soe-Jensen P. and Hansen J.M. (1993). Radioiodine treatment of multinodular non toxic goitre. BMJ., **307**: 828-832.

Olen E. (1969). The fine structure of experimentally induced hyperplastic and colloid goitre in the hamster. Lab. Invest., **21**: 336-345.

Ollis C.A., Davies R., Munro D.S. and Tomlinson S. (1985). Relationship between growth and function of human thyroid cells in culture. J. Endocrinol., **108**: 393-398.

Oppenheimer J.H., Bernstein G. and Barks M.J. (1968). Increased thyroxine turnover and thyroidal function after stimulation of hepatocellular binding of thyroxine by phenobarbital. J. Clin. Invest., **47**: 1399-1406.

Papini E., Petrucci L., Guglielmi R., Pununzini C., Rinaldi R., Bacci V., Crescenzi A., Nardi F., Fabbrini R. and Pacella C.M. (1998). Long term changes in nodular goitre: a 5 year prospective randomised trial of levothyroxine suppressive therapy for benign cold thyroid nodules. J. Clin. Endocrinol. Metab., **83; 3**: 780-783.

Papini E., Bacci V., Panuzi C., Pacelle C.M., Fabbrinr F., Bizzarri G., et al. (1993). A prospective randomised trial of levothyroxine suppressive therapy of solitary thyroid nodules. Clin. Endocrinol., **38**: 507-513.

Patel Y.C., Alford F.P. and Burger H.G. (1972). The 24-hour plasma thyrotropin profile. Clin. Sci., **43**: 71-77.

Paul T.L., Kerrigan J., Kelly A.M., Braverman L.E. and Bara D.T. (1988). Long-term L-thyroxine therapy is associated with decreased hip bone density in premenopausal woman. JAMA., **259**: 3137-3141.

Perez C., Scrimshaw N.S. and Munoz A.J. (1960). Technique of endemic goitre. In: Pitt-Rivers R. and Trotter W.R. (eds.). The Thyroid Gland. WHO, Geneva, p 369.

Peter H.J., Gerber H., Studer H. and Smeds S. (1985). Pathogenesis of heterogeneity in human MNG. J. Clin. Invest., **76**: 1992-2002.

Peter H.J., Gerber H., Studer H., Schurch E. and Teuscher J. (1986). Pathogenesis of autonomous growth in human MNG. In: Madeiross-Neto G. and Gaitan E. (eds.). Frontiers in Endocrinology. Plenum Medical Book Company, New York, **2**: 951-956.

Pezzino V., Vigneri R. and Squatrito S., Filetti S., Camus M. and Polosa P. (1978). Increased serum thyroglobulin levels in patients with non-toxic goitre. J. Clin. Endocrinol. Metab., **46**: 653-657.

Philips D.I.W. (1994). Genetic factors in benign thyroid disease. In: Wheeler and Lazarus (eds.). Diseases of the Thyroid. Chapman and Hall, London, p 69.

Philp J.R., Crooks J., MacGregor A.G. and McIntosh J.A.R. (1969). The growth curve of the rat thyroid under a goitrogenic stimulus. Br. J. Cancer, **23**: 515-523.

Pierce J.G. (1971). The sub-units of Pituitary Thyrotropin. Their relationship to other glycoprotein hormones. Endocrinology, **89**: 1331-1344.

Pierce J.G. and Parsons T.F. (1981). Glycoprotein hormones: structure and function. Annu. Rev. Biochem., **50**: 465-495.

Pilegaard H. and Hessov I. (1988). Complications of surgical treatment of benign thyroid conditions in a teaching department. Ugeskr. Laeger, Copenh., **150**: 530-532.

Podoba J., Michajlovskij M. and Stukovsky R. (1973). Iodine deficiency, environmental goitrogens and genetic factors in the aetiology of endemic goitre. Acta Endocrinol., (suppl.), **179**: 36-39.

Potten C.S. (1971). Early and late incorporation of a tritiated thymidine into skin cells and the presence of a long-lived G_0-specific precursor pool. J. Cell Biol., **51**: 855-861.

Potten C.S. (1973). Further observations on the late labelling associated with stimulus-responsive cells in skin. Cell Tissue Kinet., **6**: 553-566.

Purves H.D. and Greisbach W.E. (1946). Studies on experimental goitre Vlll. Thyroid carcinomata in rats treated with thiourea. Br. J. Exp. Pathol., **27**: 294-297.

Querido A.F., Delange F., Dunn J.T., Fierro-Benitez R., Ibbertson H.K., Koutras D.A. and Perinetti H. (1974). Definition of endemic goitre and cretinism, classification of goitre size and severity of endemias and survey techniques. Pan Amer. Health Org. Scientific Publications, Washington DC, **292**: 267-272.

Rafferzeder M., Hofbauer L., Janssen O., Bechtner G. and Gartner R. (1993). Thyrotropin stimulated IGF 1-mRNA expression in isolated porcine thyroid follicles is under negative control of iodine. J. Endo. Invest. (suppl.), **16; 110**: 2-6.

Rapoport B., Niepomniszcze H., Bigazzi M., Hati R. and De Groot L.J. (1972). Studies on the pathogenesis of poor thyroglobulin iodination in non toxic multinodular goitre. J. Clin. Endocrinol. Metab., **34**: 822-830.

Rapoport B., Seto P. and Magnusson R.P. (1984). Immunoprecipitation of radiolabelled human thyroid cells and proteins by serum from patients with autoimmune thyroid disease. Endocrinology, **115**: 2137-2144.

Rasmussen S.N. and Hjorth L. (1974). Determination of thyroid volume by ultrasonic scanning. J. Clin. Ultrasound, **2**: 143-147.

Reeve T.S., Delbridge L., Cohen A. and Crummer P. (1987). Total thyroidectomy. The preferred option for multinodular goitre. Ann. Surg., **206**: 782-786.

Rentsch H.P., Studer H. and Bruggisser (1982). Pathogenesis of thyroid nodules in multinodular goitre. Am. J. Pathol., **109**: 215-223.

Reverter J.L., Lucas A., Salinas I., Audi L., Foz M. and Sanmarti A. (1991). Suppressive therapy with levothyroxine for solitary thyroid nodules. Clin. Endocrinol., **36**: 25-28.

Ridgeway E.C. (1992). Clinician's evaluation of a solitary thyroid nodule. J. Clin. Endocrinol. Metab., **74**: 231-235.

Robbins J., Cheng S.Y., Gershengorn M.C. et al. (1978). Thyroxine transport proteins of plasma: molecular properties and biosynthesis. Recent Prog. Horm. Res., **34**: 477-519.

Rohowsky-Kochan C., Dowling P.C. and Cook S.D. (1995). Canine distemper virus-specific antibodies in multiple sclerosis. Neurology, **45; 8**: 1554-1560.

Rojeski M.T. and Gharib H. (1985). Nodular thyroid disease evaluation and management. N. Engl. J. Med., **313**: 428-436.

Ross D.S., Neer R.M., Ridgeway E.C. and Daniels G.H. (1987). Subclinical hyperthyroidism and reduced bone density as a possible result of prolonged suppression of the pituitary-thyroid axis with L-thyroxine. Am. J. Med., **82**: 1167-1170.

Santler J.E. (1957). Growth in the cell populations of the thyroid gland of rats treated with thiouracil. J. Endocrinol, **15**: 151-161.

Scheible W., Leopold G.R., Woo V.L. and Gosnik B.B. (1979). High resolution real time ultrasonography of thyroid nodules. Radiology, **133**: 413-417.

Schou M., Amidisen A. and Jensen S.E. (1968). Occurrence of goitre during lithium treatment. BMJ., **3**: 710-713.

Schwartz H.L., Surks M.I. and Oppenheimer J.H. (1971). Quantitation of extrathyroidal conversion of L-thyroxine to 3,5,3′-triiodo-L-thyronine in the rat. J. Clin. Invest., **50**: 1124-1130.

Shin Ichiro T., Conti M. and Van Wyk J.J. (1990). Thyrotropin potentiation of insulin-like growth factor 1 dependent deoxyribonucleic acid synthesis in FRTL-5 cells: mediation by an autocrine amplification factor(s). Endocrinology, **126**: 736-745.

Shore R.E., Woodward E., Hildreth N., Dvoretsky P., Hemplemann L. and Pasternack B. (1985). Thyroid tumours following thymus irradiation. J. Nucl. Cancer Inv., **74**: 1177-1184.

Sigal N.H. (1985). Investigation of Cell-Mediated Immunity. In: Yoshida (ed.). Practical Methods in Clinical Immunology. Churchill Livingstone, Edinburgh, **13**: p 135.

Slater T.F., Sawyer B. and Strauli U. (1963). Studies on succinate-tetrazolium reductase systems lll. Points of coupling of 4 different tetrazolium salts. Acta Biochem. Biophys., **77**: 383-393.

Smeds S., Peter H.J., Jortso E., Gerber H. and Studer H. (1987). Naturally occurring clusters of rapidly replicating follicular cells in mouse thyroid glands. Cancer Res., **47**: 1646-1651.

Smets L.A. (1969). Discrepancies between precursor uptake and DNA synthesis in mammalian cells. J. Cell Physiol., **74**: 63-66.

Smith B.R., McLachlan S.M. and Furmaniak J. (1988). Autoantibodies to the thyrotropin receptor. Endocr. Rev., **9**: 106-121.

Smith P. (1988). In vitro analysis of a growth limiting mechanism in the rat thyroid epithelium. PhD thesis, University of Wales.

Smyth P.P.A., Neylan D. and O'Donovan D.K. (1982). The prevalence of thyroid stimulating antibodies in goitrous disease assessed by cytochemical section bioassay. J. Clin. Endocrinol. Metab., **54**: 357-361.

Spencer R.P. and Banever C. (1970). Human thyroid growth: a scan study. Invest. Radiol., **5**: 111-116.

Sterling K. and Lazarus J.H. (1977). The thyroid and its control. Annu. Rev. Physiol., **39**: 349-371.

Sterling K., Brenner M.A. and Newman E.S. (1970). Conversion of thyroxine to triiodothyronine in normal human subjects. Science, **169**: 1099-1100.

Strakosch C.R., Joyner D. and Wall J.R. (1978). Thyroid stimulating antibodies in patients with autoimmune disorders. J. Clin. Endocrinol. Metab., **47**: 361-365.

Stringer B.M.J., Wynford-Thomas D. and Williams E.D. (1985). In vitro evidence for an intracellular mechanism limiting the thyroid follicular cell growth response to thyrotropin. Endocrinology, **116; 2**: 611-615.

Strum J.M. and Karnovsky M.J. (1971). Aminotriazole goitre. Fine structure and localisation of thyroid peroxidase activity. Lab. Invest., **24**:1-12.

Studer H. (1982). A fresh look at an old thyroid disease: euthyroid and hyperthyroid nodular goitre. J. Endocrinol. Invest., **5**: 57-67.

Studer H. (1986). Growth control and follicular cell neoplasia. In: Medeiros G.A. and Gaitan E. (eds.). Frontiers in Thyroidology. Plenum Press, New York, p 131.

Studer H. and Gebel F. (1986). Simple and nodular sporadic goitre. In: Ingbar S.H. and Braverman L.E. (eds). The Thyroid, 5th edn. J.B. Lippencott, Philadelphia, **33**: 696-704.

Studer H. and Gerber H. (1991). Intrathyroidal iodine: heterogeneity of iodocompounds and kinetic compartmentalisation. Trends Endocrinol. Metab., **2**: 29-35.

Studer H. and Greer M.A. (1965). A study of the mechanisms involved in the production of iodine deficiency goitre. Acta Endocrinol., **49**:610-619.

Studer H. and Rameli F. (1982). Simple goitre and its variants: euthyroid hyperthyroid MNG's. Endocr. Rev., **3; 1**: 40-58.

Studer H., Forster R., Conti A., Kohler H., Haeberli A. and Engler H. (1978). Transformation of normal follicles to thyrotropin refractory 'cold' follicles in the ageing mouse thyroid gland. Endocrinology, **102**: 1576-158.

Studer H., Haeberli A. and Forster R. (1980). Cold follicles in the thyroids of old mice: loss of organic binding and of endo and exocytosis but preservation of inorganic iodide transport. In: Stockigt J.R. Nagataki S. (eds.). Thyroid Research Vlll. Proceedings of the 8th International Congress, Sydney, Australia, Academy of Science, Canberra, p 113.

Studer H., Kohler H. and Burghi H. (1974). Iodine Deficiency. In: Greer M.A. and Soloman D.H. (eds.). Handbook of Physiology, American Physiological Society, Washington, section 7, **3**: p 303.

Studer H., Peter H.J. and Gerber H. (1989). Natural heterogeneity of thyroid cells: the basis for understanding thyroid function and nodular goitre growth. Endocr. Rev., **10**: 125-135.

Taylor J.H., Woods P.S. and Hughes W.L. (1957). The organisation and duplication of chromosomes as revealed by autoradiographic studies using tritium labelled thymidine. Proc. Natl. Acad. Sci., USA, **43**: p 122.

Taylor S. (1953). The evolution of nodular goitre. J. Clin. Endocrinol. Metab., **13**: 1232-1247.

Taylor S. (1980). Thyroid tumours. In: De Visscher (ed.). The Thyroid Gland. Comprehensive Endocrinology, Raven Press, New York, p 257.

Thomson E.J., Kaufmann P., Shortman R.C., Rudge P. and McDonald W.I. (1979). Oligoclonal immunoglobulins and plasma cells in spinal fluid of patients with multiple sclerosis. BMJ, **1**: 16-17.

Toft A.D., Irvine W.J. and Hunter W.M. (1976). A comparison of plasma thyrotropin levels in patients with diffuse and nodular non-toxic goitre. J. Clin. Endocrinol. Metab., **42**: 973-976.

Tong W. (1974). Action of thyroid stimulating hormone. In: Greer M.A. and Solomon (eds.). Handbook of Physiology, Amer. Physiological Society, Washington DC, **7; 3**: p 255.

Tramontano D., Cushing G.W., Moses A.C. and Ingbar S.H. (1986). Insulin-like growth factor 1 stimulates the growth of rat thyroid cells in culture and synergises the stimulation of DNA synthesis induced by TSH and Graves' IgG. Endocrinology, **119**: 940-942.

Tunbridge W.M.G., Evered D.C., Hall R. et al. (1977). The spectrum of disease in a community. The Whickham Study. Clin. Endocrinol., **7**: 481-493.

Valente W.A., Vitti P., Kohn L.D., Brandi M.L., Rottele C.M., Toccafondi R., Tramontano D., Alij S.M. and Ambesi-Impiombato F.S. (1983b). The relationship of growth and adenylate cyclase activity in cultured thyroid cells: separate bio-effects of thyrotropin. Endocrinology, **112**: 71-80.

Valente W.A., Vitti P., Rottele C.M. Vaughan M.M., Alij S.M., Grollman E.F., Ambesi-Impiombato F.S. and Kohn L.D. (1983a). Antibodies that promote thyroid growth: a distinct population of thyroid stimulating autoantibodies. N. Engl. J. Med., **309**: 1028-1034.

Van der Gaag R.D., Drexhage H.A., Wiersinga W.M., Brown R.S., Docter R., Bozatto G.F. and Doniach D. (1985). Further studies on thyroid growth-stimulating immunoglobulin in euthyroid non-endemic goitre. J. Clin. Endocrinol. Metab., **60**: 972-979.

Van Herle A.J., Rich P., Ljung B-M.E., Ashcraft M.W., Solomon D.H. and Keeler E.B. (1982). The thyroid nodule. Ann. Intern. Med., **96**: 221-232.

Van Wyk J.J., Arnold M.B., Wynn J. and Pepper F. (1959). The effects of a soya bean product on thyroid function in humans. Paediatrics, **24**: 752-760.

Vitari L., De Dominicis G., Vito M. et al. (1985) Teratogenic and goitrogenic activity of Probineb and propylenethiourea in the rat. Boll. Soc. Ital. Biol. Sper., **61; 2:** 271-278.

Von Blomberg-van Flier B.M.E. and Kokje-Kleingeld M. (1984). Defects in the T-suppressor cells system in simple goitre. Ann. Endocrinol., Paris, **45**: 86-88.

Vought R.L., Brown F.A. and Wolff J. (1972). Erythrosine: an adventitious source of iodine. J. Clin. Endocrinol. Metab., **34**: 747-752.

Waigt A. (1980). Evaluation of the functional state of the blood brain barrier by means of the analysis of total protein, albumin level and albumin index of the cerebrospinal fluid in multiple sclerosis. Neurol. Neurochir. Pol., **14; 5**: 465-471.

Waigt A. and Zgorzalewicz B. (1982). Immunoglobulins of the cerebrospinal fluid in subacute sclerosing panencephalitis. Pol. Tyg. Lek., **37; 37**: 1089-1093.

Wand M., Zeuthen E. and Evans E.A. (1967). Tritiated thymidine: effect of decomposition by self-radiolysis on specificity as a tracer for DNA synthesis. Science, **157**: 436-438.

Wartofsky L. (1991). Use of sensitive TSH assay to determine optimal thyroid hormone therapy and avoid osteoporosis. Annu. Rev. Med., **42**: 341-346.

Weeke J. and Gundersen H.J.G. (1978). Circadian and 30 minute variations in serum TSH and thyroid hormones in normal subjects. Acta Endocrinol. Copenh., **89**: p 659.

Wesche M.F., Tiel-v-Buul M.M., Smits N.J. and Weirsinga W.M. (1995). Reduction in goitre size by ^{131} I therapy in patients with non-toxic multinodular goitre. Eur. J. Endocrinol., **136**: 86-87.

Westermark K., Karlsson F.A. and Westermark B. (1983). Epidermal growth factor modulates thyroid growth and function in culture. Endocrinology, **112**: 1680-1686.

Wilders-Trusching M.M., Drexhage H.A., Leb G. et al. (1990). Chromatographically purified immunoglobulin G of endemic and sporadic goitre patients stimulates FRTL5 growth in a mitotic arrest assay. J. Clin. Endocrinol. Metab., **70**: 444-452.

Williams D.W., Williams E.D. and Wynford-Thomas D. (1988). Loss of dependence on IGF-1 for proliferation of human thyroid adenoma cells. Br. J. Cancer, **57**: 535-539.

Williams E.D. (1991). Biological effects of radiation on the thyroid. In: Braverman L.E., Utiger R.D. (eds.). Werner and Ingbar's. The Thyroid: a fundamental and clinical text. 6th edn. J.B. Lippencott, New York, p 421-436.

Wolff J. (1964). Transport of iodide and other anions by the thyroid gland. Physiol. Rev., **44**: 45-90.

Wollman S.H, Herveg J.P., Zeligss J.D. and Ericson L.E. (1978). Blood capillary enlargement during the development of thyroid hyperplasia in the rat. Endocrinology, **103**: 2306-2314.

Wollman S.H. (1980). Structure of the thyroid gland. In: De Visscher M. (ed.). The Thyroid Gland: Comprehensive Endocrinology. Raven Press, New York, p 1.

Wollman S.H. and Breitman T.R. (1970). Changes in DNA and weight of thyroid glands during hyperplasia and involution. Endocrinology, **86**: 322-327.

Wynford-Thomas D., Stringer B.M.J. and Williams E.D. (1982). Dissociation of growth and function in the rat thyroid during prolonged goitrogen administration. Acta Endocrinol., **101**: 210-216.

Zakarija M. and McKenzie J.M. (1990). Do thyroid growth-promoting immunoglobulins exist? J. Clin. Endo. Metab., **70; 2**: 308-310.