

**IMMUNOLOGICAL MECHANISMS
IN THE PATHOGENESIS OF
AUTOIMMUNE THYROTOXICOSIS**

A dissertation submitted to the University of Edinburgh
for the degree of Doctor of Medicine

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DECLARATION

I declare that I have written this dissertation entitled "Immunological Mechanisms in the Pathogenesis of Autoimmune Thyrotoxicosis". The studies contained in it have been designed, executed and analysed by me. Where the cooperation of others was involved, their contribution is duly acknowledged.

R.L. KENNEDY

ABSTRACT OF THESIS

The development, in recent years, of reproducible assays for TSH receptor antibodies and of monoclonal antibodies for the study of lymphocyte subsets has greatly advanced our understanding of the pathogenesis of Graves' disease.

The aim of this thesis is to consider both humoral and cell-mediated immunity in thyrotoxic Graves' disease. Particular attention is paid to the correlation of the immunological changes with clinical features of the disease and to age-related changes in immune parameters. Evidence is sought for heterogeneity within the disease to account for the variable expression of immunological markers. Spontaneous hyperthyroidism in the domestic cat has recently emerged as a clinical problem in veterinary practice. This has not been previously studied immunologically and is considered here as a potential model for autoimmune thyrotoxicosis in Man.

Methods used include the measurement of autoantibodies by haemagglutination and by indirect immunofluorescence, of TSH receptor antibodies by a binding inhibitory assay and of immune complexes using a C1q solid phase assay. HLA-DR typing has been performed using a B lymphocyte microcytotoxicity test. A wide range of monoclonal antibodies was employed in quantifying lymphocyte subsets including activated T lymphocytes.

TSH receptor antibodies have been measured in over 300 Graves' patients. They correlate with thyroid microsomal antibodies and their expression may be age related. The assay is compared using

immunoglobulin concentrates and neat serum, the latter being more sensitive. There is a strong relationship between elevations in antibody levels after radioactive iodine and the development of hypothyroidism. TSH receptor antibodies may persist for years after destructive anti-thyroid therapy and this persistence correlates with the presence of other autoimmune diseases.

Atrophic Graves' disease is commoner than is widely realised. These patients are older than the average Graves' patient, they have less severe thyrotoxicosis and a lower incidence of autoantibodies. A group of patients with goitre but without grossly elevated radioactive iodine uptake had a remarkable incidence of non-thyroidal autoantibodies, particularly parietal cell antibodies. This heterogeneity is not clearly related to DR types. A possible association between the DR2 phenotype and thyroid stimulation is identified and a group of young female patients, positive for DR2 and resistant to anti-thyroid therapy is described.

Immune complexes have been studied, not only in Graves' and other autoimmune thyroid diseases, but also in a unique, large series of patients with Addison's disease. The latter condition, though rare, is frequently associated with multiple autoimmune disorders. A role for immune complexes in modulating autoantibody expression is proposed.

Decreased suppressor cells and an increase in helper to suppressor cell ratio is confirmed using two separate monoclonal antibodies to suppressor cells. Furthermore, a decline in suppressor cells with age in normal female subjects is reported. Early activated T cells as

defined by the monoclonal antibody 4F2 are present in increased numbers and they show a negative correlation with TSH receptor antibodies. The expression of class II antigens on activated T lymphocytes was studied using a panel of monoclonal antibodies. These antigens could also be demonstrated on Graves' thyroid tissue by indirect immunofluorescence. Raised levels of killer cells were detected in the circulation of patients with active disease using a new monoclonal antibody.

By studying multiple immune parameters in Graves' disease, potentially important interactions have been found and their implications for the diagnosis, treatment and natural history of the disease are discussed.

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antibodies used. His enthusiasm for the subject is a source of continuing inspiration. The work on hyperthyroid cats was suggested by Mr Keith Thoday of the school of Veterinary Medicine in the University of Edinburgh and he was responsible for the clinical and biochemical aspects of this investigation.

In producing the final document, I am grateful to Dr. David Brown of the Department of Clinical Immunology, Addenbrooke's Hospital, Cambridge for his stimulating discussion. The use of the word processing facilities in the Department of Medicine, University of Cambridge and the help of Mr. Paul Smith have been invaluable as has the expert secretarial advice of Mrs. Agnes Hunt and my wife, Florance.

Chapter One

INTRODUCTION

INTRODUCTION

Theoretical and technical advances in immunology have, in recent years, revealed the variety of immunopathogenetic mechanisms involved in the development of an autoimmune disease. Our concept has changed from the early days of autoimmunity when the emergence of a self-reactive, "forbidden" clone of antibody producing cells was thought to be sufficient to produce disease (Burnet, 1961). We now recognise a complex immunoregulatory system with subsets of lymphoid cells, antibodies and soluble factors (lymphokines) controlling a variety of effector mechanisms, both humoral and cellular. These mechanisms operate against a complicated genetic background which is still far from completely understood. We have learned much about immunoregulation and about the genetic factors from the study of animal models, yet it is not always easy to translate observations on highly inbred strains of laboratory animals into useful information about human disease states. The thyroid gland is of particular importance to the study of human autoimmunity: its diseases are common, the anatomical accessibility of the gland and the profound metabolic effects of its hormone products make for early and accurate diagnosis of diseases, while in different individuals, the nature of the immunological response varies, producing a variety of hypo- and hyper-functioning states. This dissertation is largely concerned with the mechanisms involved in the pathogenesis of 'Graves' disease, how these immunological abnormalities interrelate and how they affect the presentation of the disease.

CURRENT THEORIES ON AUTOIMMUNITY WITH SPECIAL REFERENCE TO THYROID DISEASE

There is, at present, no single explanation which will on its own explain why some individuals develop autoimmune disease while others do not. Early work concentrated on the humoral immune system and a variety of organ specific and non organ specific autoantibodies was identified. Not all individuals with antibodies, however, developed disease and in many cases such as Graves' disease a proportion of individuals with disease had no detectable autoantibody. As techniques became available, cell-mediated immunity was also shown to be disturbed, with changes in both effector and immunoregulatory functions. The characterisation of the Human Leukocyte Antigen (HLA) system in the 1960s and the subsequent studies on the association between alleles of this system and disease states allowed us to identify at risk individuals but did not explain why only a proportion of the individuals with a disease associated allele were affected. It may be that newer HLA antigens will be better disease markers but one still has to explain the influence they have on the immune system. In briefly reviewing some of the current theories on autoimmunity, with special reference to thyroid diseases, it is possible to delineate four areas of interest:-

- 1) Changes in the target tissue which render it more immunogenic.
- 2) Increased autoaggression in susceptible individuals.
- 3) Defects in immunoregulatory mechanisms.
- 4) The genetic background to the autoimmune diseases.

CHANGES IN THE TARGET TISSUE

One obvious explanation for the development of an autoimmune disease would be a change in the antigenic composition of the target tissue, perhaps by random mutation. The best characterised autoantigen is thyroglobulin. There is no difference in the thyroglobulin of obese strain (OS) chickens, which develop spontaneous thyroiditis, and that of normal chickens (de Carvalho et al, 1982) and the response to injected thyroglobulin is the same whether it is self or non-self (Roitt & de Carvalho, 1982).

It is much more likely that changes in the way that antigen is presented to the immune system are important. It is known, for example, that viruses such as the influenza virus can incorporate host antigens and that cells so infected have increased immunogenicity (Lindermann & Klein, 1967). There is evidence that viral infections may be important in some cases of diabetes mellitus (for review, see Janson, 1980) but the evidence that infection is involved in the pathogenesis of Graves' disease is very limited (Joassoo et al, 1975; Shenkman & Bottone, 1976; Bech et al, 1977). Viral antigens may be synergistic with endogenous antigen in stimulating autoantibody production in much the same way as the polyclonal B cell activator, pokeweed mitogen (PWM) can act in the production of plaque-forming (antibody producing) cells (see Fauci, 1980). A much more likely explanation for increased antigen presentation to the immune system in Graves' disease comes from the studies of Bottazzo's group (1983) who have shown that a high proportion of Graves' thyroids express class II histocompatibility antigens which are not present on most normal thyroid cells (Hanafusa et al, 1983). Such Class II antigen

expression is probably important in the presentation of thyroid antigen to helper T lymphocytes. These authors further suggest that the increased reversal of thyroid epithelial polarity which occurs in Graves' glands leads to greater availability of antigens which are normally concealed (Hanafusa et al, 1984). Such "aberrant" HLA-DR expression has also been found on the beta cells in the pancreas of a diabetic patient. (Bottazzo G. F., personal communication), in acute graft-versus-host disease (Manson et al, 1981), in the colonic epithelium of patients with inflammatory bowel disease (Selby et al, 1983) and on the keratinocytes in certain dermatoses (Lampert, 1984).

Work on the Obese Strain chicken suggests that intrinsic thyroid abnormalities may precede the development of thyroiditis. Sundick et al (1979) have demonstrated incomplete suppression of ^{131}I uptake when T_4 is administered and there is an increased uptake of ^{131}I by glands transplanted to the chorioallantoic membranes of normal chickens (Sundick & Wick, 1976). The high functional activity of the thyroid may increase the amount of antigen released and thus stimulate an immune reaction. In support of this hypothesis, Sanker et al (1983) have found high levels of thyroglobulin in the circulation of Obese Strain chickens prior to the development of thyroiditis and it may well be, therefore, that a stage of thyroid hyperactivity precedes the initiation of autoimmune thyroiditis.

INCREASED AUTOAGGRESSION

The presence of hypergammaglobulinaemia in autoimmune diseases including Graves' disease (Briones-Urbina et al, 1982) has been taken as evidence of increased activity of the humoral immune system. Increased numbers of B cells in the circulation of Graves' patients have been reported by some authors (Hsu et al, 1976; Mori et al, 1980) although others have found them to be normal (Mulaisho et al, 1975). Although lymphocytes binding the common autoantigens thyroglobulin and DNA may be found in the circulation of normal individuals, the number of such cells is greatly increased in thyroid autoimmune disease and systemic lupus respectively (Bankhurst & Williams, 1973; Bankhurst et al, 1975). Selective IgA deficiency is commoner in patients with autoimmune diseases than in the normal population and may be one factor in predisposing the individual to polyclonal B cell activation by infectious agents. Increased spontaneous activity of antibody forming cells has been shown both in mice prone to autoimmune disease (Izui et al, 1978) and in human patients with SLE (Budman et al, 1977). In the latter study, the degree of humoral immune hyperactivity was highest in the patients with lowest complement C3 levels, who presumably had the most active disease. Antibody secretion has also been shown to be increased in vitro in SLE (Morimoto et al, 1977; Kallenberg et al, 1983). The primary immune response in the patients studied by Kallenberg was depressed and it was suggested that the hyperfunctioning of the immune system in these patients applied only to recall antigens. If this were the case, then foetal exposure to maternal autoantigens released in the course of an autoimmune disease and subsequent recall of the immune response during a time in later life when the

individual is susceptible to autoimmune disease may explain the observation that many of these diseases tend to breed true. Slaughter et al (1978) reported no difference in the total antibody secretion of lymphocytes from patients with rheumatoid arthritis when stimulated in vitro with Epstein-Barr virus, although they did produce much higher levels of IgM-anti-IgG and that antibody was of higher affinity for IgG than antibody secreted by normal lymphocytes.

The increased autoaggression in autoimmune diseases is not confined to the humoral immune system. Calder et al (1973) showed increased levels of cells cytotoxic to thyroglobulin coated erythrocytes in autoimmune thyroid disease. The same authors (Calder et al, 1976) have reported increased antibody dependent cellular cytotoxicity (K cell activity) in thyroid disease, although such activity has been found to be reduced in some autoimmune diseases (Feldman et al, 1976; Goto et al, 1981) possibly because of immune complexes or antilymphocyte antibodies binding to the surface of effector cells. Much recent interest has been in the natural killer (NK) cells which have particular autoaggressive potential since their cytotoxic activity is not HLA restricted. Increased levels of these cells are found in untreated Graves' disease (Kennedy et al, 1985).

DEFECTIVE IMMUNOREGULATION

Over the past 15 years, the concept has evolved of a dynamic immunoregulatory network with subsets of T lymphocytes having either helper or suppressor functions. It has been suggested that escape

from normal suppressor mechanisms may lead to breakdown of immune tolerance and thus the emergence of an autoimmune disease (Allison et al, 1961). The ideal method for quantifying suppressors would be not only reproducible but would assay some aspect of suppressor cell function and relate to the specificity of the cells involved. Such a method does not exist (for review of methodology, see Goodwin & Williams, 1979) and the method in widest use currently relies on monoclonal antibodies to define cell surface markers. Using these antibodies, Buschard et al (1983) have found reduced suppressors in diabetes of recent onset, as have Morimoto et al (1980) in active SLE and Sridama et al (1982) in Graves' disease and Hashimoto's. Decreased suppressors in Graves' disease were confirmed by Ludgate et al (1984) who also showed them to return towards normal with treatment. In their study of two HLA identical twins, one of whom had Graves' disease, Balazs et al (1984) showed that both twins had autoreactive T cells to the thyroid but only the patient had reduced suppressor cell activity. A possible basis for the reduction in suppressor cells may be the presence of antilymphocytic antibodies directed at this cell subset. Such antibodies are well recognised in SLE (Morimoto et al, 1980) and have been described in myasthenia gravis (Mishak & Dau, 1981) and more recently have been found in patients with Graves' disease (Pacini et al, 1983). However, not all antilymphocyte antibodies in autoimmune disease react with suppressor/cytotoxic cells, Morimoto et al (1981) have described antibodies in the circulation of patients with juvenile rheumatoid arthritis which react predominantly with helper/inducer cells. Heterogeneity of these lymphocyte binding antibodies may occur in

different disease states and may in part underlie the variable results seen in studies of lymphocyte subsets with monoclonal antibodies (see chapter 7).

It was Jerne (1973) who first suggested that the immune system may be subject to regulation by immunoglobulins directed at determinants (idiotypes) on the variable regions of other antibody molecules. This idiotypic network may influence not only antibody expression but also the function of immunoregulatory cell subsets through immunoglobulin bound to their cell surface. One way in which this network could be involved in the pathogenesis of an autoimmune disease is shown in FIGURE 1.1. If an antibody to a virus or an antigen released during tissue damage shared an idiotypic with an autoantibody, then induction of the former by either infection or cell damage might lead to the latter being expressed.

Shechter et al (1982) have shown that mice immunized with insulin develop antibodies both to insulin and to the insulin receptor. The receptor antibodies displaced labelled insulin from its receptor, stimulated the oxidation of glucose and its incorporation into lipids as well as inhibiting lipolysis. These receptor antibodies could be blocked by or bound to the antibodies to insulin and were therefore thought to be anti-idiotypic. Similarly, immunization of rabbits with Bis Q, an agonist at the acetylcholine receptor, led to the production of antibodies capable of binding to the receptor (Wasserman et al, 1982). Immunization of rabbits with the purified anti-Bis Q led to the development of a myasthenic syndrome. Dwyer et al (1983) have recently demonstrated the existence of a naturally occurring anti-idiotypic antibody in patients with myasthenia gravis:

they found that 40% of over 50 patients had an antibody which bound to a monoclonal antibody directed at the acetylcholine receptor and that such antibodies were most likely to be found in patients with low anti-receptor antibody titres. That antibodies may develop to receptor agonists and then anti-idiotypic antibodies to these antibodies may in turn bind to the receptor (see FIGURE 1.2) may be a concept which is fundamental in the pathogenesis of the receptor antibody diseases. The recent detection of naturally occurring anti-TSH antibodies (Kajita et al, 1983; Akamizu et al, 1984; Copping et al, 1985) is therefore of great interest.

GENETIC

Although a genetic component clearly exists in autoimmune diseases such as Graves' disease - for example, about 50% of Graves' patients have a family history of thyroid disease (for review of the genetics of Graves' disease, see Friedman & Fialkow, 1978) - but the precise mode of inheritance has not been worked out. Graves' is not entirely genetically determined. Only 50% of sets of monozygotic twins are concordant for Graves'. The role of the environmental factor remains to be clarified but may account for the variable timing of onset of the different diseases in the polyendocrine deficiency syndromes (Solomon et al, 1975). Most of the work on the genetics of Graves' relates to the HLA system but the best marker, HLA-DR3, is only positive in approximately 65% of patients (Farid & Bear, 1981) and its precise relationship to the immunopathogenetic mechanisms is still largely a mystery. Adams (1978) has suggested that the genetic basis for autoimmune disease may lie in the genes coding for the

FIGURE 1.1 PROPOSED MECHANISM BY WHICH VIRAL INFECTION MAY TRIGGER AN AUTOIMMUNE REACTION

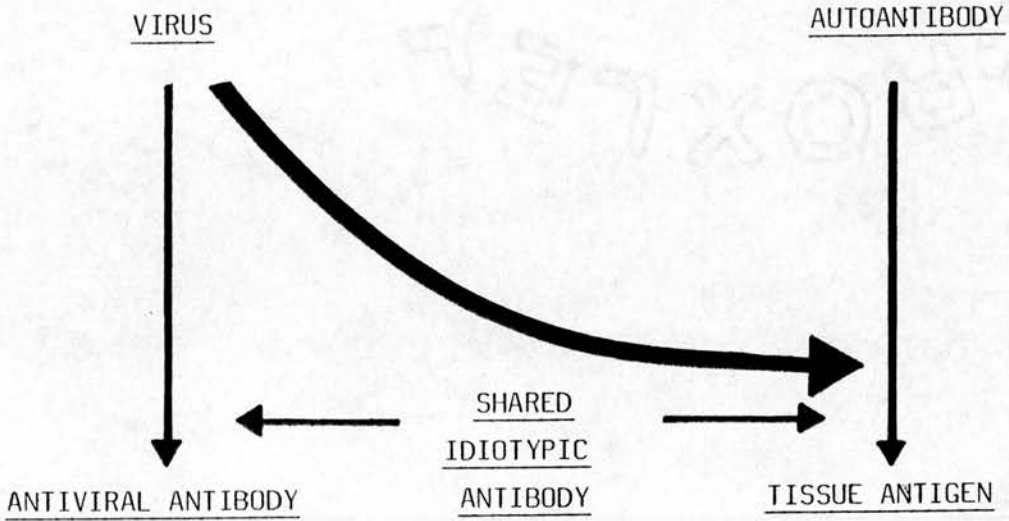
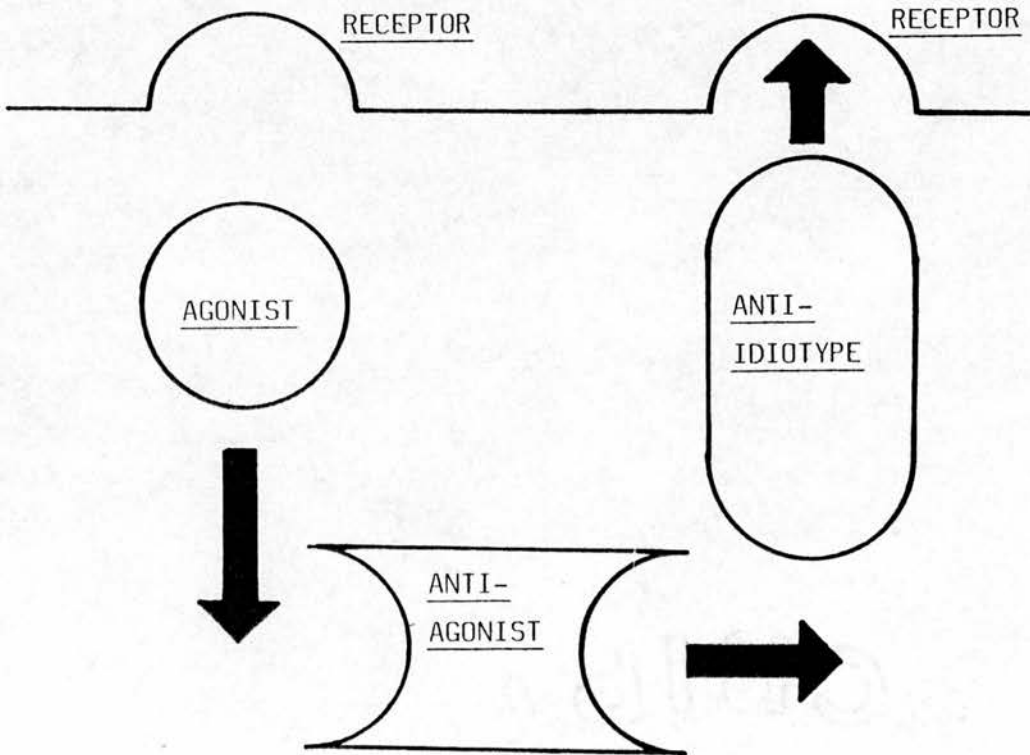


FIGURE 1.2 POSSIBLE ROLE OF ANTI-IDIOTYPIC ANTIBODIES IN THE PATHOGENESIS OF RECEPTOR ANTIBODY DISEASES



variable regions of immunoglobulin molecules (V genes). His hypothesis accounts for the recognised patterns of inheritance including the female predominance of many autoimmune diseases, the increased incidence of diseases with age, the association of the various autoimmune diseases and their link with the HLA system.

THE RECEPTOR ANTIBODY DISEASES

In this group of organ specific autoimmune diseases (see TABLE 1.1), the major factor in the pathogenesis is the production of antibodies to cell surface receptors.

TABLE 1.1 THE RECEPTOR ANTIBODY DISEASES

<u>DISEASE</u>	<u>RECEPTOR INVOLVED</u>
GRAVES' DISEASE	THYROID RECEPTOR FOR TSH
MYASTHENIA GRAVIS	ACETYLCHOLINE RECEPTOR ON POST SYNAPTIC NEUROMUSCULAR JUNCTION
INSULIN RESISTANT DIABETES	INSULIN RECEPTOR
ATOPIC ASTHMA	BETA ADRENERGIC RECEPTOR

Other immunological abnormalities are present in these diseases and the ability to measure an antibody which directly affects cell function allows us to study the effect of immunological control

mechanisms. Graves' is unique among the receptor antibody diseases in that the antibody actually stimulates the thyrocyte rather than blocking or destroying the receptor. Recently, a receptor mechanism has been proposed for SLE: Bennett et al (1986) have shown that the receptor on peripheral blood mononuclear cells which binds DNA and clears it from the circulation is defective in SLE and this defect is due to antibodies directed at the receptor.

1. GRAVES' DISEASE

The demonstration in the mid 1950s of circulating antithyroid antibodies in patients with thyroid disease (Doniach & Roitt, 1957) and, around the same time, the identification of an abnormal stimulator in the serum of thyrotoxic patients gave birth to the study of autoimmunity in thyroid disease. Most patients with the common thyroid diseases were found to have autoantibodies (Witebsky et al, 1957; Roitt & Doniach, 1960, Mori & Kriss, 1971). Thyrotoxicosis was shown to occur after hypophysectomy (McCullagh et al, 1960; McKenzie, 1962; Christensen & Binder, 1962), after pituitary stalk section (Furth et al, 1962) and after post-partum pituitary necrosis (Fajans, 1958). The thyroid of normal subjects would respond to the infusion of Graves' plasma (Arnaud et al, 1965). The Long-Acting Thyroid Stimulator (LATS) was identified as an immunoglobulin G (Dorrington et al, 1966) and its specific antigen, the TSH receptor, has now been characterised (Smith, B. R. et al, 1985). It is an integral membrane protein with a molecular weight of about 70,000 consisting of two subunits (45,000 and 25,000). Study of the receptor is hampered by the small amount present on cells (10

nanograms/gram of wet tissue, equivalent to about 1000 receptors per cell). Not all patients with Graves' disease have antibodies detectable by the methods currently in use. There are a variety of reasons for this but one may be that different populations of antibodies exist as has been shown for myasthenia gravis (Mittag et al, 1981).

2. MYASTHENIA GRAVIS

The target for autoimmune attack in myasthenia gravis is the nicotinic acetylcholine receptor (AChR) on the postsynaptic membrane of the neuromuscular junction. Patrick and Lindstrom (1973) described the syndrome in rabbits immunized with AChR, and it is partly because of such animal models and the fact that the receptor is so well characterised that myasthenia gravis is the best understood of the receptor antibody diseases at the cellular level. The receptor consists of five membrane glycoprotein molecules - two alpha subunits (MW = 38,000), one beta subunit (MW = 49,000), one gamma ((MW = 57,000) and one delta subunit (MW = 64,000). There are two acetylcholine binding sites, one on each alpha subunit and binding is followed by sodium influx and triggering of the action potential. Although the receptor is remarkably similar in different animal species, the antibodies appear to be highly species specific (Lindstrom et al, 1978).

Autoantibodies to AChR are rare in individuals who do not have myasthenia gravis, and, in the individual patient, the antibody level varies with disease activity (Newsom-Davis et al, 1978; Seybold & Lindstrom, 1981). When cases of myasthenia gravis are divided according to the pathology of the thymus, there is evidence of

clinical and immunological heterogeneity (Compston et al, 1980): In the 10% of patients with thymoma, the sex incidence is equal, onset is in middle age and the level of AchR antibodies is high. Anti-striated muscle antibodies are found in a large number of cases and the condition is not related to HLA-DR3 (although a weak association with HLA-DR7 has been suggested). Thymic hyperplasia is usually associated with disease onset before the age of 40 years, there is a female preponderance of 3:1, it is associated with intermediate antibody titres and HLA-DR3. A group of patients with thymic atrophy typically presents in middle to late life, is predominantly male, has low AchR antibody levels and a possible association with HLA-DR2.

There are three established mechanisms by which AchR antibodies bring about changes in the response to acetylcholine at the motor end plate: Firstly, the binding of divalent antibody cross links two receptor molecules and this is followed by an increased rate of internalisation and lysosomal breakdown (Heineman et al, 1977; Drachman et al, 1978; Fumagalli et al, 1981). Secondly, complement dependent lysis alters the morphology of the post synaptic folds thus reducing the surface area for insertion of AchR (Engel et al, 1976, 1979). Thirdly, there is reduced insertion of AchR into the post-synaptic membrane (Engel & Fumagalli, 1982). The precise mechanism for this is not clear but if it were not so, then presumably increased turnover of receptors might compensate for their loss from the post synaptic membrane. AchR antibody formation requires the cooperation of helper cells and can be suppressed by normal lymphocytes suspensions containing OKT8 positive cells (Shinomiya et al, 1984). Reduced suppressor cells as defined by

monoclonal antibodies have been described in the peripheral blood of patients with myasthenia gravis (Berrih et al, 1981; Skolnik et al, 1982) although this has not been a universal finding (Miller et al, 1982). Myasthenia gravis is associated with thyroid disease and such patients may have circulating TSH receptor antibodies (Kiessling et al, 1982).

3. INSULIN RESISTANT DIABETES MELLITUS

The insulin receptor consists of four glycoprotein subunits - two alpha (MW = 135,000) and two beta (MW = 90,000). There are two insulin binding sites per receptor and these are probably associated with the alpha subunit. Antibodies to the receptor raised in rabbits can simulate the effects of insulin in vitro e.g. enhancement of glucose transport and oxidation, stimulation of lipogenesis and ³H thymidine incorporation (for review, see Kahn et al, 1982). They have also been shown to down-regulate the receptor.

The rare syndrome of insulin resistance due to autoantibodies to the insulin receptor was first described in 1975 by Flier et al. It typically occurs in non-Caucasian, middle aged females and often in association with the skin lesion acanthosis nigricans. Patients often have features of other autoimmune diseases, although these are generally not organ specific. In vitro, the acute effect of these antibodies is initially a stimulatory one, and indeed patients with the syndrome may present with symptomatic hypoglycaemia, but with time the cells become resistant to the effects of insulin. Carpentier et al (1981) have shown that, following the binding of antibody, the

receptors may cap, internalise and become associated with the lysosomes. The phenomenon of post-receptor desensitization is also recognised to occur (Grunfeld et al, 1980).

4. ATOPIC ASTHMA

This condition is associated with changes in autonomic reactivity with impaired beta₂ adrenergic responsiveness and an increase in reactivity to alpha adrenergic and muscarinic agents. The beta receptor is a 59,000 molecular weight membrane protein which may form a dimer and which contains one catecholamine binding site per molecule. Autoantibodies were first described to this receptor in 1980 by Venter et al (for review, see Parker 1981). They are present in only a small proportion of asthmatics and their presence may relate to the severity of asthma (Blecher et al, 1981). They may also be found in a small proportion of normal subjects (Fraser et al, 1981) and their role, if any, in the pathogenesis of asthma is not known. Autoantibodies to the thyroid (Hall et al, 1966) and to smooth muscle (Turner-Warwick et al, 1970) are both found more commonly in asthmatics than in normal subjects and atopy has recently been associated with reduced suppressor cell function (Rose-Pleszynski & Blanchard, 1981).

THE SPECTRUM OF AUTOIMMUNE THYROID DISEASE

In addition to the classic autoimmune thyroid diseases - Graves' disease, primary atrophic hypothyroidism and Hashimoto's thyroiditis, a number of other entities are becoming recognised. The syndrome of painless thyroiditis with transient thyrotoxicosis (PTTT) may be associated with circulating thyroid antibodies and is linked to HLA-DR3 and DR5 (see Farid & Bear, 1981). The coexistence of Hashimoto's thyroiditis with Graves' disease is well recognised (Buchanan et al, 1961; Fatourechi et al, 1971) and may have important prognostic implications (see Chapter 4) while the common genetic background of the two conditions is demonstrated by the report of Volpe and colleagues (1974) of Hashimoto's occurring in the identical twin and Graves' in the other. Thyrotoxicosis with no goitre may account for over 20% of new cases and is associated with thyroid antibodies and ophthalmopathy (Greenwood et al, 1985) although its relationship pathogenetically to classic Graves' disease needs to be clarified. It may be that these patients simply lack a clone of cells normally present in Graves' which produce thyroid growth promoting antibodies. It has become necessary to identify the precise immunological abnormality causing each manifestation of an autoimmune disease. Thus, antibodies to melanocytes causing vitiligo (Hertz et al, 1977) and to orbital antigens in thyroid eye disease (Kodama et al, 1982) have recently been described.

Relatively little is known of the pathogenesis of multinodular goitre apart from its relationship to iodine deficiency in endemic areas. Evidence is however accumulating which suggests an overlap with autoimmune thyrotoxicosis. In addition to the presence of thyroid

microsomal and anti-thyroglobulin antibodies in multinodular goitre, changes in immunoglobulins with high IgG and IgA have been reported along with a positive response to thyroid antigen in the migration inhibition factor test (Mota et al, 1980; Kiy et al, 1981). In the study of Boukis et al (1983), performed in an endemic region of Greece, thyroid antibodies only developed after the patients had been treated with iodised oil. The release of antigen including thyroglobulin was thought to account not only for the autoantibody formation but also for the three cases of thyrotoxicosis which occurred among 58 patients. Unfortunately TSH receptor antibodies were not measured in this study. One of the major problems in interpreting epidemiological studies with nodular goitres is the geographical variation due to iodine status. A recent study in this country (Greenwood et al, 1985) reported thyroid antibodies in 58% of cases of nodular goitre and thyrotoxicosis, comparable to the incidence of antibodies in hyperthyroidism with diffuse glands. Even within this country however, considerable geographical variation exists with respect to the presence of thyroid autoantibodies including TSH receptor antibodies in populations of thyrotoxic patients (Phillips et al, 1985).

Using the radioreceptor assay for TSH receptor antibodies, most studies have not found an increased incidence of positive results in patients with nodular goitres (Bolk et al, 1972; Mukhtar et al, 1975; Strakosh et al, 1978) and this agrees with the results presented in Chapter 2. The one exception is the study from New England by Brown et al (1978) where a high proportion of patients with toxic nodular goitre were positive along with a smaller proportion of patients with euthyroid nodular goitre. This difference is hard to explain since

the patients selected and the assay method were similar to those used in the other studies cited. Smyth and colleagues (1983) have also found TSH receptor antibodies in a high proportion of patients with euthyroid goitre using a sensitive cytochemical section bioassay. Positivity in their assay correlated with the loss of TSH response to injected TRH indicating that the thyroid was either functioning autonomously or was being driven exogenously by immunological stimulators.

A detailed examination of the histology, including autoradiography to give some idea of the functioning of individual follicles, was carried out by Studer et al (1978) and revealed that thyroid autonomy was not necessarily related to or confined to the nodules in the gland. In autonomous goitre, scattered hyperfunctioning follicles or small clusters of such follicles might not be picked up clinically or scintigraphically. Of interest in this light is the study of Schleusener et al (1978) who identified a group of thyrotoxic patients with apparent diffuse goitres but neither ophthalmopathy or TSH receptor antibodies. This group did not show the expected association with HLA-DR3 but instead there was an association with HLA-DR5 and, in this group, possession of this antigen was a marker for thyrotoxic relapse following drug treatment. The association with an allele of the major histocompatibility complex makes an underlying immunological disturbance much more likely.

THE CLUSTERING OF AUTOIMMUNE DISEASES

Research has thus far failed to reveal entirely why the organ specific autoimmune diseases tend to be associated in certain individuals. Genetic factors must be important and are reflected in the association with HLA - for example, both Graves' and diabetes have an increased incidence of HLA-DR3. Defects in immunoregulation may also account for cases of multiple autoimmune disease although gross changes such as the reduction in overall percentage of suppressor cells seen in newly diagnosed Graves' disease tend to return to normal soon after diagnosis (Ludgate et al, 1984). Defective suppressor T cell function has been documented in patients with multiple endocrine deficiencies (Arulanantham et al, 1979). The possibility that target tissues share antigenic determinants has been raised by the recent finding of monoclonal antibodies which react with multiple organs (Haspel et al, 1983) while Onodera et al (1982) have shown that polyclonal B cell activation in SJL mice by virus infection leads to multiple organ specific antibodies being expressed.

AIMS OF THE THESIS

The aim of the work embodied in this dissertation is to consider the variety of immunopathogenic mechanisms, both humoral and cellular, which operate in autoimmune thyrotoxicosis and how these mechanisms interact with one another. An explanation is sought for the variable expression of immune markers in Graves' disease: whether different forms of the disease exist or whether variations may be caused by patients being studied at different stages in the natural history of their disease. The influence of the patient's age on immune parameters and on the clinical expression of the disease is considered. A major part of the work is devoted to how immunological tests may contribute to the diagnosis and management of autoimmune thyrotoxicosis.

Chapter Two

TSH Receptor Antibodies in Newly Diagnosed Graves' Disease

SUMMARY

TSH receptor antibodies (TBII) have been measured using a TSH binding inhibitory assay in two large series of patients with Graves' disease. In the first series of 138 patients, immunoglobulin concentrates prepared by polyethylene glycol precipitation of serum were used for the assay. 84 (61%) of the patients had detectable TSH receptor antibodies. In the second series of 150 patients, neat serum was used in the assay and TBII were found in 121 (81%) of the patients. When they were used simultaneously on the same sample, the correlation between the two assays was good ($r = 0.882$, $p < 0.001$). There was a very low incidence of TBII in patients with multinodular goitres, while patients with simple goitre and Hashimoto's thyroiditis were consistently negative.

There was a positive correlation in both studies between TBII and thyroid microsomal antibody titres although this was more marked for the assay using neat serum ($p < 0.001$). When immunoglobulin concentrates were used, a significant ($p < 0.01$) negative correlation between TBII and age at diagnosis was found. No such relationship was found when neat serum was used in the assay. In neither assay was any correlation found between TBII and biochemical measurements of thyroid function or four hour ^{131}I uptake.

Fourteen patients with recurrent Graves' thyrotoxicosis were compared to 28 age and sex matched patients with newly diagnosed Graves' (two new patients being matched to each recurrent case). There was no significant difference in thyroid hormone or ^{131}I uptake values but the median value for TBII (measured with neat serum) in the new

patients was 28 (normal: -10 to +9) compared to 16 in the patients with recurrent thyrotoxicosis ($p < 0.02$). By contrast, thyroid microsomal antibodies tended to be higher in the recurrent patients with 11 out of 13 (85%) having microsomal antibody titres of 40² or above compared to 9 out of 26 (35%) new patients ($p < 0.01$).

The TBII assay using neat serum is therefore more sensitive than the method using immunoglobulin concentrates in the diagnosis of Graves' disease without loss of specificity. TBII correlate with microsomal antibodies in newly diagnosed Graves' and the expression of TBII may be age related. Recurrent Graves' patients differ from new patients with lower levels of circulating TBII but higher microsomal antibody titres which may reflect the tendency to develop thyroiditis in patients with long standing Graves' disease.

INTRODUCTION

The presence, in Graves' disease, of circulating antibodies directed against the TSH receptor provides the major evidence for an autoimmune aetiology for the disease. In the past thirty years, a number of methods have been developed to measure these antibodies (see TABLE 2.1) While methods which assay some aspect of thyroid function provide more direct information about the pathogenesis of the disease, the radioreceptor assay enjoys the widest usage since it is both convenient and reproducible. The assay may detect either stimulatory antibodies or those which simply bind to the TSH receptor making it refractory to the action of circulating TSH. The

TABLE 2.1 METHODS DESCRIBED FOR TSH RECEPTOR ANTIBODY MEASUREMENT

a) Bioassays

1. Long-acting Thyroid Stimulator (LATS)
(¹³¹I release from thyroid of intact mice)
2. Long-acting Thyroid Stimulator Protector (LATS-P)
(Ability to inhibit neutralisation of LATS by human thyroid)

b) Changes in Thyroid Slices

1. Colloid droplet formation - human/mouse thyroid
2. Cytochemical section bioassay - guinea pig thyroid

c) TSH Binding Inhibitory Assays

- Inhibit binding of labelled TSH to human or porcine TSH receptors

d) Assays Based On Aspects Of Thyroid Function

1. Adenylate cyclase activity
 - thyroid slices
 - human thyroid membrane preparation
 - human cell culture
 - rat FRTL-5 cells
2. Iodide Uptake
 - rat FRTL-5 cells
3. Thyroid hormone release
 - porcine thyroid slices
4. NADPH generation
 - guinea pig thyroid slices

e) Growth Promoting Antibodies

- guinea pig thyroid
- rat FRTL5 cells

composition of the antibodies measured may thus vary from patient to patient and this, along with geographical variation, patient heterogeneity (discussed in Chapter 4) and methodological differences, accounts for the variable rate of positivity reported in the literature. In spite of its wide usage over the past 10 years, much remains unknown about the radioreceptor assay for TSH-receptor antibodies.

a) The Long Acting Thyroid Stimulator (LATS)

The bioassay for TSH, which relied on the discharge of radio-labelled thyroid hormone from a guinea pig thyroid when TSH was injected, was a well-established method in 1956 when Adams and Purves described an abnormal activity in the sera of thyrotoxic patients. TSH gave maximal stimulation between 90 minutes and three hours, while the maximal stimulation with thyrotoxic sera was between 16 and 24 hours. Furthermore, the magnitude of the maximal response was greater with the thyrotoxic sera (Adams, 1958). The assay was modified for use with mice (McKenzie, 1958) - partly for convenience and partly because human serum was often toxic to guinea pigs. In 1961 the term "Long Acting Thyroid Stimulator" (LATS) was coined (Adams, 1961) and the activity was intensively studied in the ensuing years.

LATS activity could be demonstrated when Graves' sera were administered to hypophysectomised mice and presumably did not therefore act by stimulating pituitary TSH release. Its half life in the circulation was considerably longer than TSH, and it was not

neutralised by antisera to TSH. It not only stimulated thyroxine production by the thyroid but also increased ^{131}I uptake and induced TSH-like changes in thyroid histology (for a review of these properties see Adams, 1965). Shishiba et al (1970) later showed that LATS stimulated glucose oxidation by the thyroid as well as intracellular colloid droplet formation, while Levey and Pastan (1970) demonstrated activation of the membrane bound adenylate cyclase.

It seemed to many, therefore, that the cause of Graves' disease had been found - but there were two major objections: firstly, all Graves' patients were not positive for LATS - typically only about 30%, although Carneiro et al (1966) found LATS in 55 of the 100 patients in their study. This was ascribed to assay insensitivity and the rate of positivity could be increased to 65% by using immunoglobulin concentrates (Adams, 1965). The second objection was the presence of LATS in patients who were not thyrotoxic - for example, Major and Munro (1962) found a small amount of activity in some normal sera and also in patients rendered hypothyroid by treatment for thyrotoxicosis. Kriss et al (1964) similarly found LATS in seven subjects with pretibial myxoedema but who were euthyroid after treatment, and Liddle et al (1965) detected LATS in five patients with severe exophthalmos but who were euthyroid. The thyroids of these latter five patients failed to respond to large doses of injected TSH. This finding was confirmed by Adams et al (1969) who found high titres of thyroid microsomal antibodies in three euthyroid patients with exophthalmos and a high LATS titre. Impaired thyroid reserve due to concurrent thyroiditis may thus be the reason why some LATS positive patients do not become thyrotoxic.

One further objection to LATS as the cause of thyrotoxicosis was that the degree of thyroid dysfunction did not correlate closely with the LATS titre. Some felt that LATS may simply be TSH bound abnormally to a serum protein, while others felt that it might be an epiphenomenon and that changes in cell-mediated immunity were more important (Volpe et al, 1972).

LATS was soon recognised to be confined to the gamma-globulin fraction of the serum (McKenzie, 1962a; Meek et al 1964), it had many of the properties of an antibody and it could be neutralised by antisera raised to normal IgG (Kriss et al, 1964). It was found to be an IgG molecule and the Fab fragment of the molecule was the active moiety (Dorrington et al, 1966). The antibody - like other IgG molecules - could cross the placenta causing transient neonatal thyrotoxicosis (Adams et al, 1964). LATS correlated with the presence of thyroid eye disease (Lipman et al, 1967) although not with its severity (Major & Munro, 1962). Much higher levels of LATS were found in patients with localised myxoedema and thyrotoxicosis than in those with thyrotoxicosis alone (Lipman et al, 1967; Carneiro et al 1968). This was recently confirmed by Hardisty et al (1984a) who also found a persistent LATS-P in this group after successful treatment of hyperthyroidism. Levels of these antibodies declined in some of their patients as the skin lesions resolved. In the 1960s, therefore, the identification of LATS as an immunoglobulin and its correlation with other features of the disease provided the major evidence for an immune aetiology for Graves' disease.

b) Long-Acting Thyroid Stimulator Protector (LATS-P)

The activity of LATS in the mouse bioassay can be reduced by preincubation with human thyroid tissue homogenate (Kriss et al, 1964). The antigen responsible is associated with the microsomal fraction of the cell (El Kabir et al, 1966) and is quite specific for the thyroid cell. A substance which blocked the neutralisation of LATS by thyroid extract, presumably by competing for microsomal antigen binding sites, was described in 1967 by Adams and Kennedy. It was named the LATS-Protector (LATS-P) and was found in the gamma-globulin fraction of the serum. It does not interact with the mouse thyroid, either in vitro or in vivo and is thought to be highly-specific for human thyroid (Adams & Kennedy, 1971).

LATS-P is present in most patients with Graves' disease - 14 of the 20 patients studied by Adams and Kennedy (1971) showed activity with neat serum, while the remaining six were all positive when immunoglobulin concentrates were used. In a series of 50 cases reported by Adams et al (1974) LATS-P was found in 45 (90%) while LATS was only present in 15 (30%). All of the LATS positive cases were also positive for LATS-P. Furthermore, LATS-P showed a good correlation with the rate of ^{131}I uptake. More recent studies by Hardisty et al (1981a; 1981b) confirm the high incidence of LATS-P - 90% of patients relapsing after antithyroid drugs and 81% of patients prior to thyroidectomy were positive.

Direct evidence that LATS-P stimulates the human thyroid has been obtained by Shishiba et al (1983), who showed a strong correlation between the presence of LATS-P and intracellular colloid droplet

formation when thyroid tissue was incubated with Graves' serum. When plasma containing LATS-P was infused into normal volunteers whose thyroids had been pre-loaded with ^{131}I , there was a discharge of radioactive iodine into the circulation (Adams et al, 1974b) - a modification of the McKenzie mouse bioassay!

c) Intracellular Colloid Droplet Formation and the Cytochemical Section Bioassay

Both morphological and cytochemical changes in thyroid slices have been used to assess the activity of thyroid stimulating antibodies. The formation of intracellular colloid droplets has been found to correlate with ^{131}I release in response to Graves' immunoglobulins using mouse thyroid slices (Shishiba et al, 1967). Unlike LATS-P, the antibody is not highly species specific - it will interact with both human and mouse, but not with chicken thyroid (Shishiba et al, 1972).

In the cytochemical section bioassay, thyroid stimulators induce a change in lysosomal membrane permeability to a chromogenic substrate for lysosomal naphthylamidase (Petersen et al, 1975). The assay has been adapted for use with cryostat sections of thyroid (Neylan & Smith, 1982) and stimulatory antibodies are found in a high proportion of Graves' patients. Antibodies detected by this method are thought to correlate with thyroid cell growth rather than thyroid hormone production (Smyth et al, 1985) and positivity in this assay correlates with lack of response to injected TRH in euthyroid women with a diffuse goitre (Smyth et al, 1983).

d) TSH Binding Inhibitory Immunoglobulins (TBII)

Radioimmunoassays have the benefits of reproducibility and a large sample capacity. It is not surprising, therefore, that the radioreceptor assay, more than any other assay for TSH receptor antibodies, has found its way into the routine clinical laboratory. The assay is based on the ability of immunoglobulins from Graves' patients to specifically inhibit the binding of labelled TSH to membrane preparations containing the TSH receptor. It was first described in 1974 by Rees-Smith and Hall who felt that TSH binding inhibitory immunoglobulins (TBII) were equivalent to LATS-P and that they might thus be specific for human thyroid membranes. TBII were found in 22 out of 25 patients with Graves' disease and produced linear Scatchard plots for binding to TSH receptor preparations. The slope of the line and intercepts varied from patient to patient. When the assay was further characterized (Rees-Smith & Hall, 1981), porcine thyroid was found to be as effective as human thyroid and guinea pig thyroid was also suitable. The major objection to this method has been its lack of sensitivity; usually 50% - 80% of Graves' patients are positive although this varies greatly from one published series to another (see TABLE 2.2). Shinozawa et al (1986a; 1986b) have recently developed an assay based on the binding of Graves' immunoglobulins to the TSH receptors on guinea pig fat cell membranes which have the advantage of not containing the thyroid microsomal antigen. The assay is sensitive and can be used either with immunoglobulin concentrates or with neat unfractionated serum. The presence of false positive results with the sera of patients with rheumatoid arthritis or systemic lupus could not be explained by these authors and positive results were also obtained in two cases of

Table 2.2. TB11 in untreated Graves' disease: percentage positivity in different published series

<u>Authors</u>	<u>IgG preparation</u>	<u>Thyroid</u>	<u>No.</u>	<u>Positive</u>
Fenzi et al (1978)	Ammonium Sulphate ppt.	Partic. Human	15	53%
Davies et al (1982)	Ammonium Sulphate ppt.	Partic. Human	43	53%
Bliddal et al (1982)	Ammonium Sulphate ppt.	Partic. Human	52	54%
Endo et al (1978)	Ammonium Sulphate ppt.	Partic. Human	27	60%
Kuzuya et al (1979)	Protein A - Sepharose	Partic. Human	16	63%
Bech et al (1982)	Ammonium Sulphate ppt.	Partic. Human	22	64%
Gossage et al (1983)	Ammonium Sulphate ppt.	Partic. Human	27	70%
Schleusener et al (1978)	Ammonium Sulphate ppt.	Partic. Human	49	71%
McGregor et al (1980)	Ammonium Sulphate ppt.	Partic. Human	42	76%
O'Donnell et al (1978)	Ammonium Sulphate ppt.	Partic. Human	42	76%
Iida et al (1982)	DEAE - Sephadex	Soluble Human	26	77%
Biro (1982)	Ammonium Sulphate ppt.	Soluble Human	104	78%
Teng et al (1980)	Ammonium Sulphate ppt.	Partic. Human	74	82%
Shewring & Rees-Smith (1982)	Polyethylene glycol ppt.	Soluble Porcine	11	90%
Southgate et al (1984)	Neat Serum	Soluble Porcine	10	100%
	Partic. = particulate	ppt. = precipitate		

insulin resistant diabetes associated with anti-insulin receptor antibodies. Enzyme-linked immunosorbent assays (ELISA) are increasingly replacing radioimmunoassays and Baker et al (1983) have described such an assay based on the TSH receptors in guinea pig fat cell membranes.

In a ten year follow up study of Graves' patients, Hensen et al (1984) have shown that the percentage positivity in the TBII assay was increased with repeated determinations, particularly in patients with recurring hyperthyroidism. Two recent modifications of the binding assay have also shown increased sensitivity: Borges et al (1982) have preincubated receptor preparations with the IgG being tested and then washed the membranes prior to adding labelled TSH. TBII were found in 94% of the 50 patients studied, the increased sensitivity having been achieved by reducing binding by immunoglobulins from normal controls. Furthermore, the degree of binding inhibition correlated significantly with an assay based on cyclic AMP (cAMP) accumulation. De Bruin et al (1984) describe an immunoprecipitation assay in which solubilised receptors equilibrated with a small amount of ^{125}I -TSH are incubated with serum and then precipitated using a rabbit anti-human IgG. The assay was positive in 26 out of 32 patients (81%) although antibody levels correlated poorly with those in a standard TBII assay.

e) Antibodies Affecting Adenylate Cyclase Activity

The recognition by McKenzie & Zakarija (1976) that immunoglobulins from Graves' patients stimulated the adenylate cyclase in human thyroid slices, and that this reflected the activity of the disease during drug therapy (Zakarija et al, 1980) lent further support to a role for these antibodies in the pathogenesis of the disease. An assay based on a membrane fraction from a tissue homogenate (Orgiazzi et al, 1976) proved to be more convenient and was positive in over 80% of untreated Graves' (Bech & Madsen, 1978; Karlsson & Dahlberg, 1981). A sensitive assay based on human thyroid cells in tissue culture (Etienne-Decert & Winand, 1981) used infractionated serum and was positive in 55 out of 69 (80%) patients. Cryopreserved human thyroid cells have also been used (Davies et al, 1983) and a system based on the FRTL-5 rat thyroid cell line was recently reported (Vitti et al, 1983). Adenylate cyclase stimulating antibodies are not species specific and can be detected using thyroid from calf, dog and guinea pig (Zakarija & McKenzie, 1978a; 1978b). Antibodies which block the cAMP accumulation in response to Graves' immunoglobulins are now well recognised in patients with primary hypothyroidism (Konishi et al, 1983).

f) Other Metabolic Activities and Thyroid Hormone Production

The uptake of iodide by the rat FRTL-5 cell line has been used to complement the adenylate cyclase stimulation (Marcocci et al, 1983). When these two measurements were combined with ³H-thymidine uptake as an index of cell growth, thyroid stimulating antibodies were found in

100% of newly-diagnosed Graves' patients. A quantitative cytochemical assay, based on measuring the amount of NADPH generated when the cells are stimulated in guinea pig thyroid slices has been described (McMullan & Smyth, 1984). Assays based on the production of thyroid hormones from porcine thyroid slices have been well described although not widely-used (Laurberg & Weeke, 1975; Atkinson & Kendall-Taylor, 1981).

g) Antibodies which affect Thyroid Cell Growth

Garry and Hall (1979) found that LATS increased the mitotic rate of a rat thyroid cell culture. Growth promoting antibodies are now recognised in other goitrous thyroid diseases (diffuse euthyroid goitre and Hashimoto's) and can be measured by nucleic acid cytophotometry or ^3H -thymidine incorporation (Drexhage et al, 1980). Valente et al (1983) have recently confirmed the presence of growth-promoting antibodies in Graves' disease using rat FRTL-5 cells.

CORRELATION BETWEEN DIFFERENT METHODS FOR MEASURING TSH RECEPTOR ANTIBODIES

With the advent of different methods for measuring TSH receptor antibodies, it has become apparent that considerable heterogeneity exists. There is some confusion about how the different activities interrelate. A correlation between LATS and adenylate cyclase stimulating activity has been reported (Macchia et al, 1982),

although this is in conflict with the findings of Orgiazzi et al (1976). LATS has not generally been found to correlate with TBII (Clague et al, 1976; Endo et al, 1978), although this has been disputed by Ozawa et al (1978). LATS-P is present in nearly all LATS positive sera (Adams et al, 1974; Hardisty et al, 1981), and correlates with colloid droplet stimulating antibodies (Shishiba et al, 1973). Endo et al (1978) found LATS-P and TBII to correlate in LATS negative sera but Ozawa et al (1978) found a poor correlation in this group. In examining the two widely used methods for measuring TSH receptor antibodies, Ginsberg et al (1983) have distinguished three patterns: some patients exhibit high binding inhibitory and adenylate cyclase stimulating activities, while in others there is a predominance of one or other activity. In general when they have been measured simultaneously, the two activities have been found to correlate poorly (Kuzuya et al, 1979; Sugenoza et al, 1979; Macchia et al, 1981; Shishiba et al, 1982; Hardisty et al, 1983). Some authors have however found a positive correlation between the two methods (Hall et al, 1978; Bliddal et al, 1982; Hensen et al, 1984). In the modified binding assay of Borges et al (1982) levels of TBII also correlated with cAMP accumulation in thyroid slices.

There is no doubt that the various methods are measuring different immunoglobulin populations. When different assays are combined, circulating TSH receptor antibodies are found in nearly all cases (Biro, 1982; Marcocci et al, 1983; Hardisty et al, 1983). The study by Biro combined TBII, adenylate cyclase stimulation and colloid droplet formation. All 104 patients were positive in at least one assay but only 29 were positive in all three.

In routine laboratories, it is impractical to use several different methods simultaneously and, of the established methods, the TSH binding inhibitory assay is by far the most convenient and reproducible.

The aim of the work presented in this chapter is to assess the radioreceptor assay in the context of a routine clinical laboratory comparing the use of immunoglobulin concentrates as described by Shewring and Smith (1982) with the modified assay using neat serum (Southgate et al, 1984). Correlation has been sought between the clinical features of the disease and the expression of TSH binding inhibitory immunoglobulins. Recurrent Graves' thyrotoxicosis is compared to that occurring in newly-diagnosed Graves' disease.

RESULTS

A: Measurement of TSH Receptor Antibodies Using Immunoglobulin Concentrates.

PATIENTS

This study included 138 patients (122 female, 16 male; mean age 45.1 years, SD 13.6) with thyrotoxicosis plus a diffuse goitre and/or scintiscan. Thyrotoxicosis was diagnosed by raised triiodothyronine (T_3 , normal up to 2.8 nmol/l) and/or raised thyroxine (T_4 , normal up to 150 nmol/l) along with a flat TRH test. Thyroid hormone and TSH measurements were performed in the Department of Clinical Chemistry,

Royal Infirmary of Edinburgh by in-house radioimmunoassays. The mean T_3 in the thyrotoxic patients was 5.9 nmol/l (SD = 2.2) and the mean T_4 was 231 nmol/l (SD = 55). Twenty one patients with Graves' disease in remission after carbimazole were studied as well as twelve cases of subclinical Graves' disease. The latter twelve patients all had flat TRH tests but high normal thyroid hormones (mean T_3 = 2.3 nmol/l, SD 0.46; mean T_4 = 142 nmol/l, SD 24) and mild symptoms. Four of the twelve had a palpable goitre.

Nineteen normal controls had no evidence of endocrine or autoimmune disease and the study also included, as disease controls, ten patients with euthyroid multinodular goitre and twenty with toxic nodular goitre. Eight hypothyroid patients with Hashimoto's thyroiditis were also studied.

ASSAYS

TSH receptor antibodies were measured as described in APPENDIX 1, using immunoglobulin concentrates prepared by precipitation from serum with polyethylene glycol. The results were expressed as a binding inhibitory index, the upper limit of normal for which was 10, in keeping with the published data of Shewring and Rees Smith (1982). Anti-microsomal and anti-thyroglobulin antibodies were detected by tanned red cell haemagglutination (Witebsky & Rose, 1956) using commercially available kits (Wellcome).

STATISTICS

Thyroid hormone and antibody data were compared using Spearman's coefficient of rank correlation, since the distribution of TSH receptor antibody measurements in the thyrotoxic patients was not

normally distributed. For trend analyses, the Jonckheere monotonic trend test for independent samples was used. This test is a non-parametric, rank correlation test and therefore suitable either where the variables are not normally distributed or where the measurements are discontinuous, as in the case of thyroid microsomal antibody titres.

THYROID AUTOANTIBODIES IN UNTREATED GRAVES' PATIENTS

TSH receptor antibodies were detected in 84 out of 138 (61%) Graves' patients with thyrotoxicosis (Median value 14.5). By contrast, none of the 19 normal controls or the ten patients with euthyroid multinodular goitre was positive (see FIGURE 2.1). Two out of twenty patients with toxic multinodular goitre were mildly positive (Binding inhibitory indices of 11.1 and 14.2). The 21 patients with Graves' disease in remission were all negative and no positive value was found in the cases of subclinical Graves' disease (see FIGURE 2.2). None of the eight patients with hypothyroidism secondary to Hashimoto's thyroiditis was positive.

There was a significant negative correlation between the age of the patient at diagnosis and binding inhibitory index ($p < 0.01$) - see FIGURE 2.3. Thyroid microsomal antibodies were detected in 46 of the 135 patients tested (34%) and there was a positive correlation between the levels of microsomal antibody and those of TSH receptor antibodies ($p < 0.02$) - see FIGURE 2.4. Antithyroglobulin antibodies were found in 19 of 135 patients (14%) and showed no correlation with TSH receptor antibodies.

FIGURE 2.1 TSH RECEPTOR ANTIBODIES IN NEWLY DIAGNOSED GRAVES', CONTROLS AND IN PATIENTS WITH NODULAR GOITRES

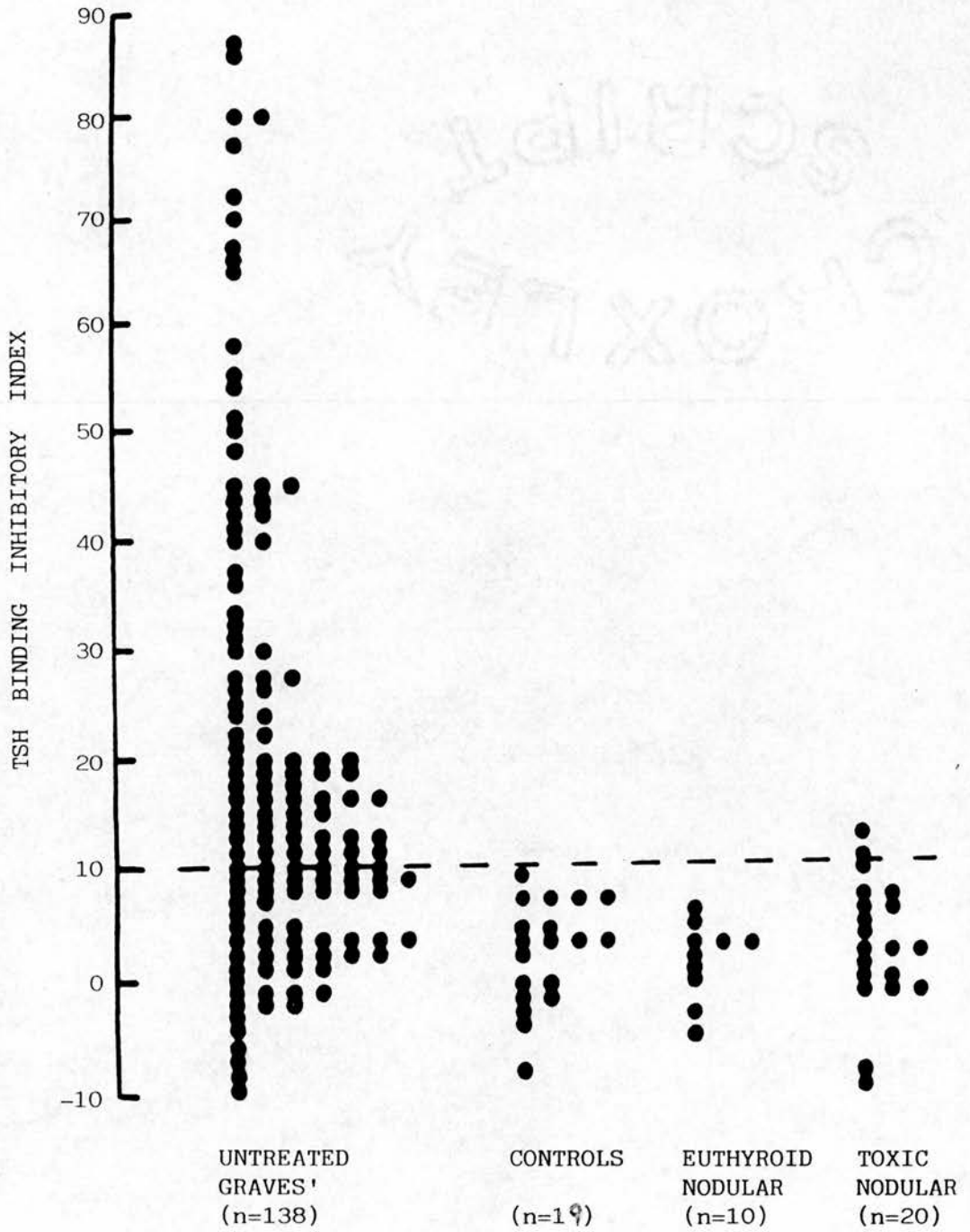


FIGURE 2.2 TSH RECEPTOR ANTIBODIES IN UNTREATED GRAVES',
EUTHYROID GRAVES' AND IN HASHIMOTO'S THYROIDITIS

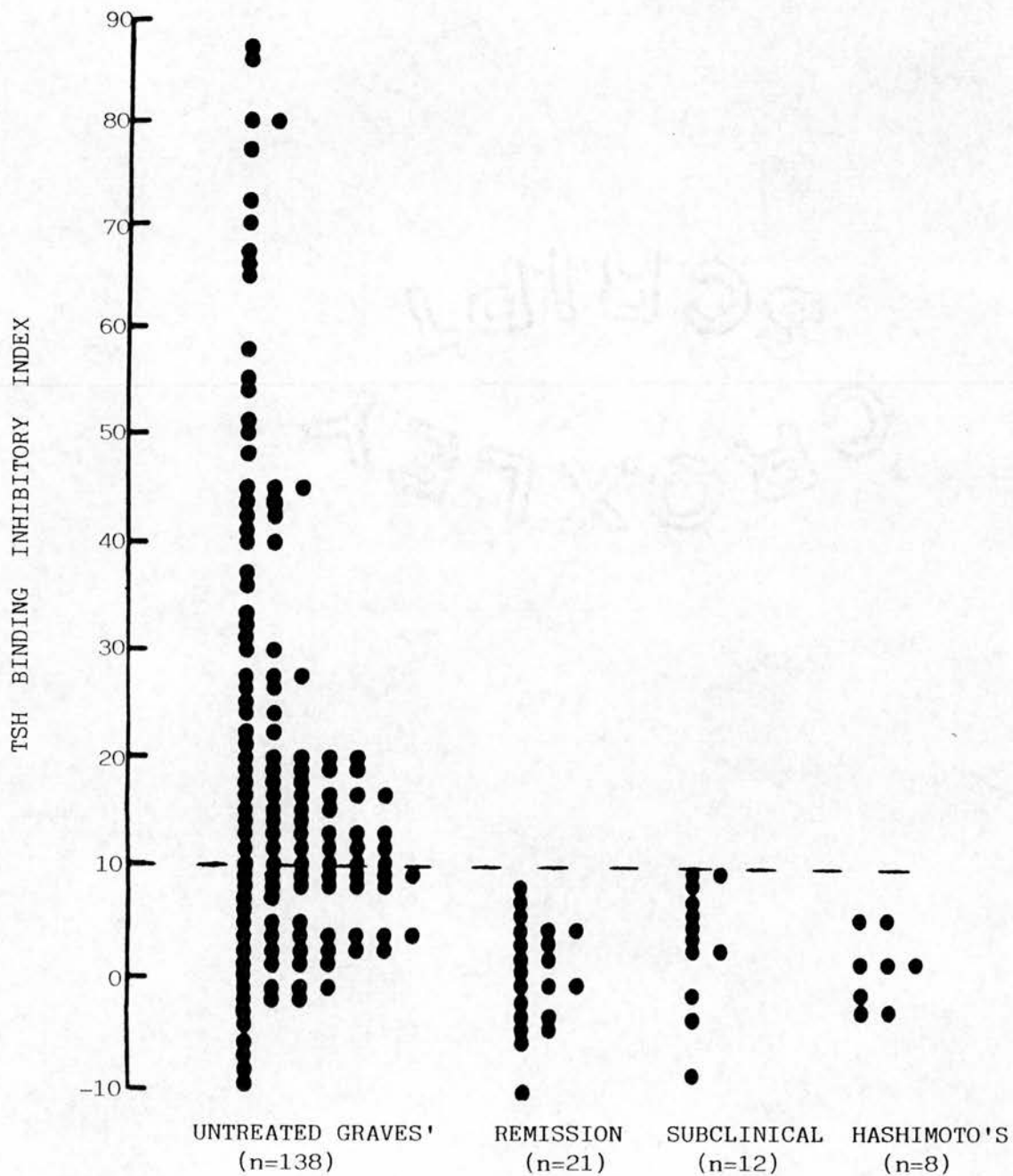
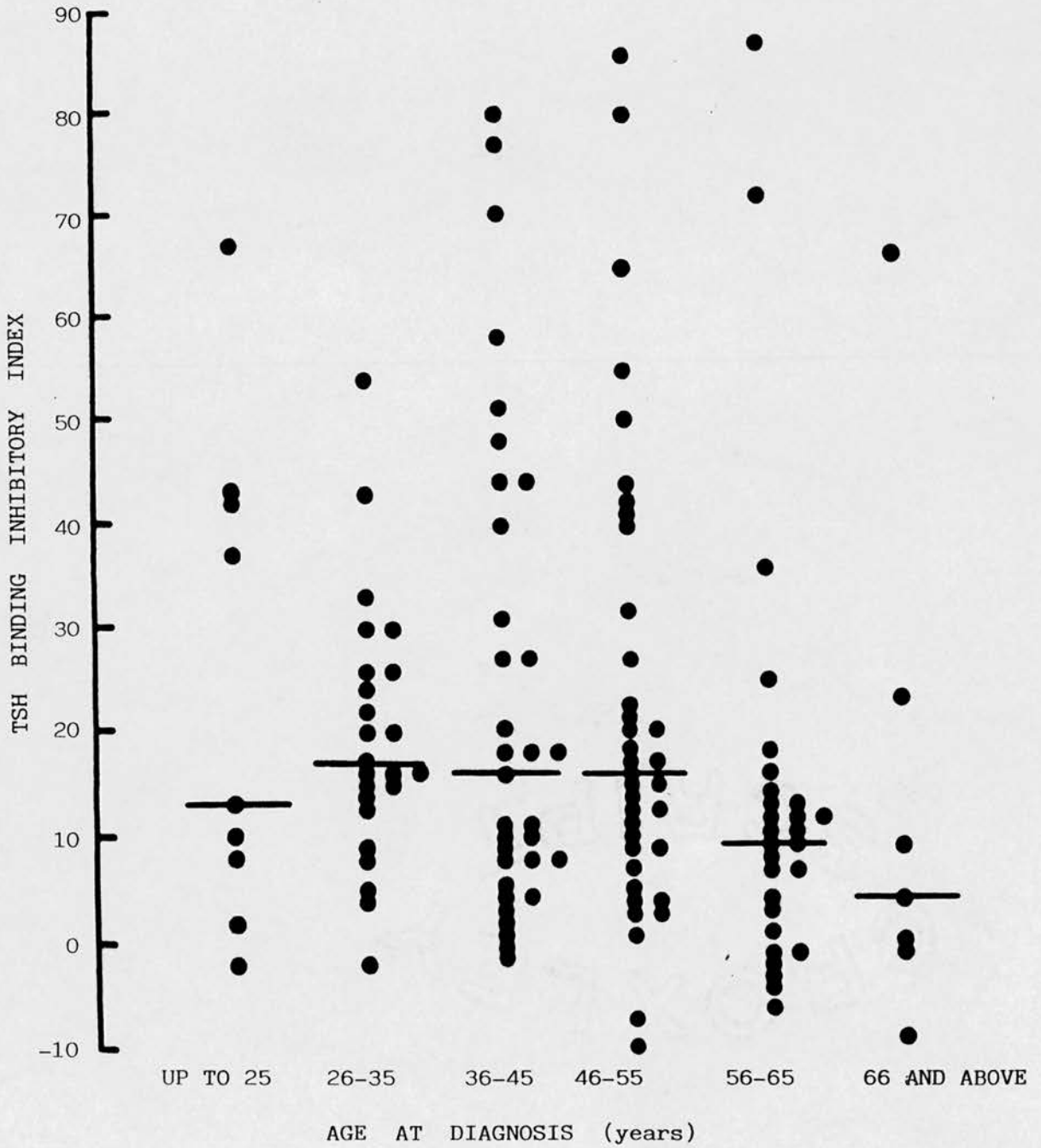
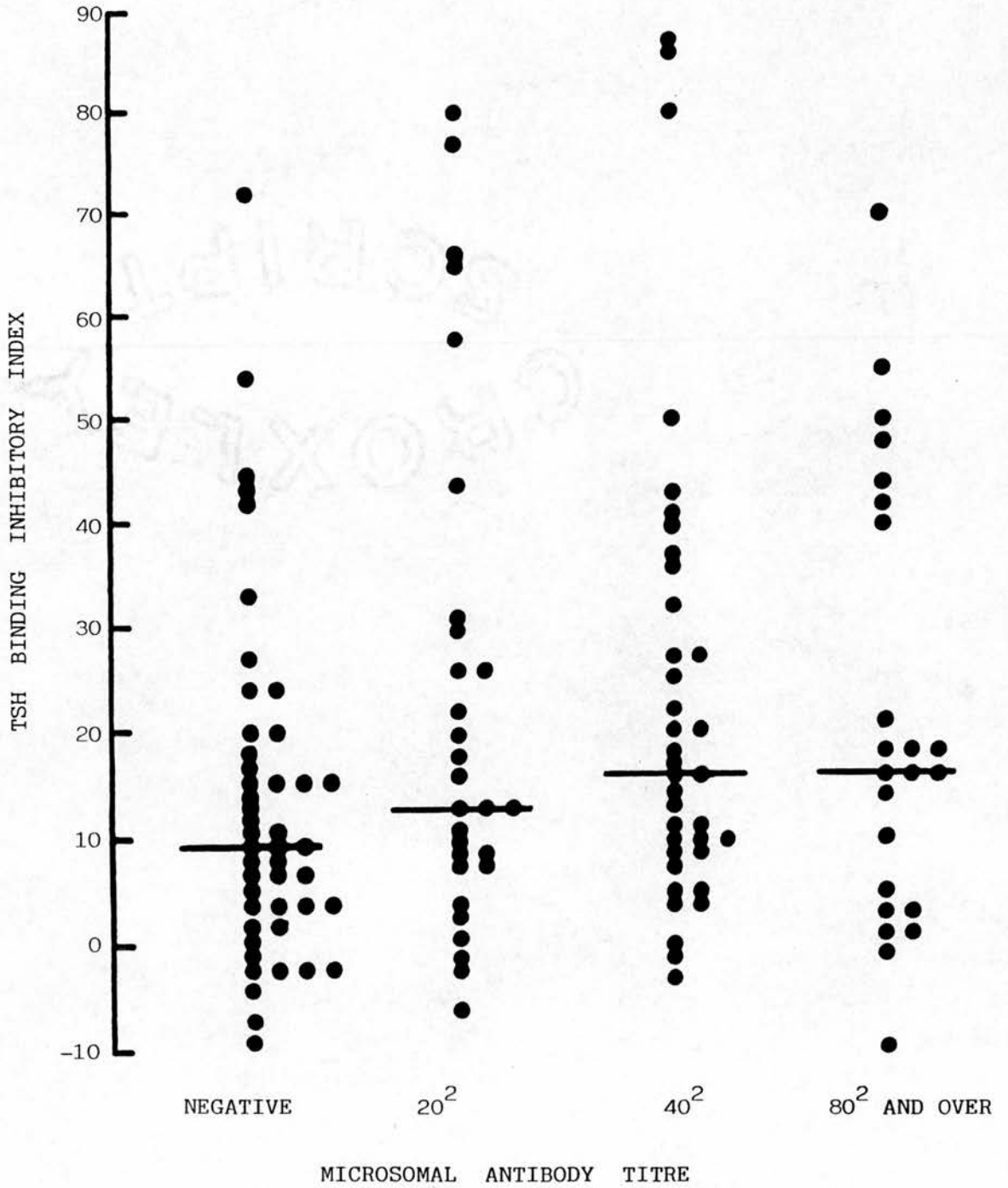


FIGURE 2.3 RELATIONSHIP BETWEEN AGE AT DIAGNOSIS AND TSH RECEPTOR/ANTIBODIES (EXTRACTED SERUM) IN UNTREATED GRAVES' DISEASE



N.B. Horizontal bars on this and other similar charts relating to TBII measurements represent the median values for the group.

FIGURE 2.4 RELATIONSHIP BETWEEN MICROSOMAL ANTIBODY TITRES AND TSH RECEPTOR ANTIBODIES (EXTRACTED SERUM)



B: Measurement of TSH Receptor Antibodies Using Neat Serum

PATIENTS AND METHODS

This study included 150 patients with thyrotoxicosis plus a diffuse goitre and/or scintiscan. There were 115 females and 35 males, overall mean age 46.8 years (SD 14.8). The diagnosis was established as for the series already described and the mean T_3 for this group was 5.7 nmol/l (SD 1.8) and the mean T_4 was 228 nmol/l (SD 46.9). TSH receptor antibodies were measured as described in APPENDIX 1 using unfractionated serum. The normal range (established by assaying serum from 98 controls) was -10 to +9. Data from patients with goitrous thyroid diseases other than Graves' is presented in APPENDIX 1. Anti-microsomal and anti-thyroglobulin antibodies were measured by tanned red cell haemagglutination using commercially available kits (Fujizoki). Statistical analyses were carried out as for the previous study.

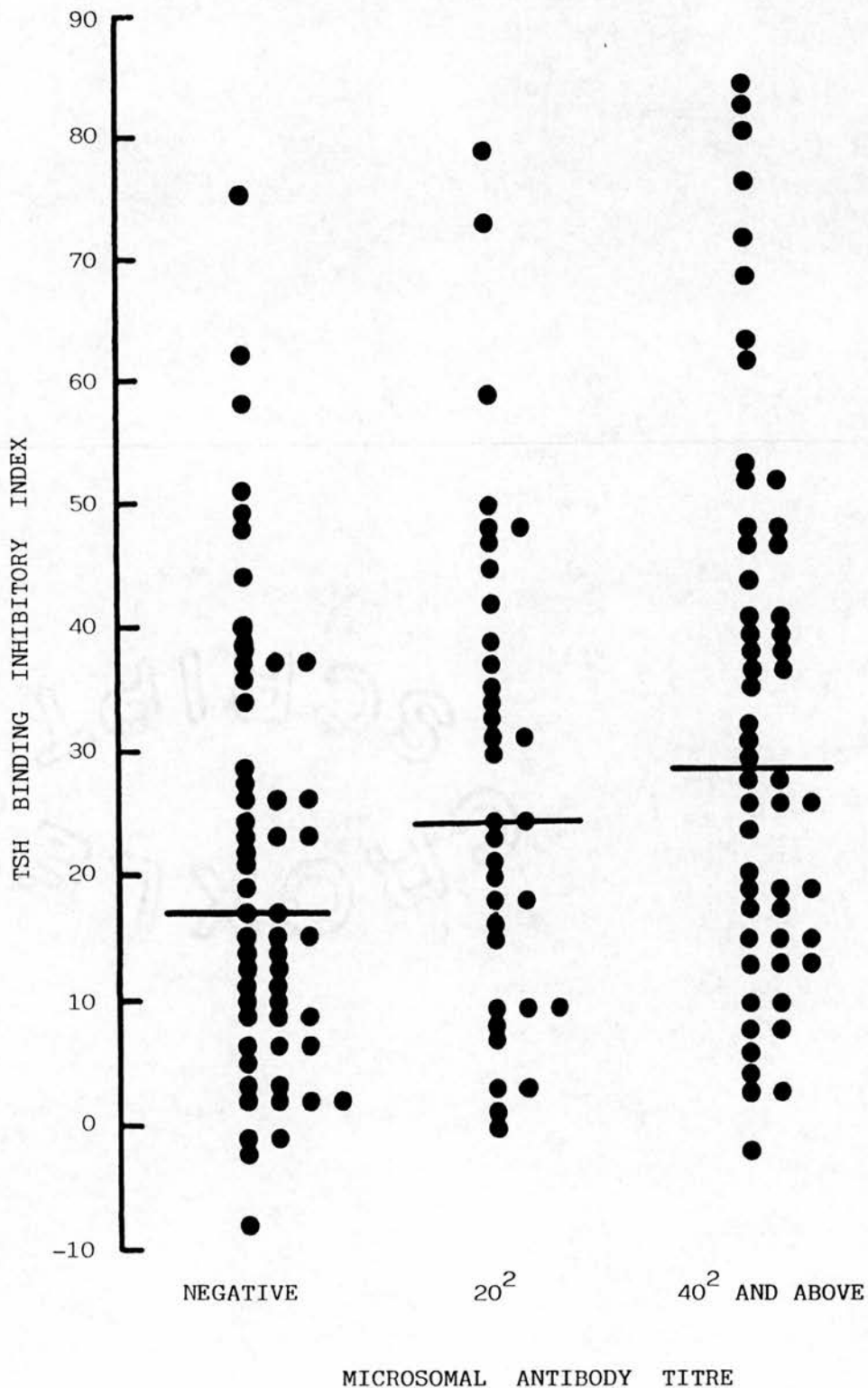
THYROID ANTIBODIES IN UNTREATED GRAVES' PATIENTS

TSH receptor antibodies were detected in 121 out of 150 patients (81%) with untreated Graves' thyrotoxicosis (see APPENDIX 1, FIGURE A1.2). Strongly positive titres of thyroid microsomal antibodies (40^2 and above) were found in 57 (38%) while anti-thyroglobulin antibodies were found in 26 (17%).

As in the previous study, there was a significant positive correlation between binding inhibitory index and microsomal antibody titre ($p < 0.001$) - see FIGURE 2.5. This trend was more marked than



FIGURE 2.5 CORRELATION BETWEEN MICROSOMAL ANTIBODY TITRE AND TSH RECEPTOR ANTIBODIES (NEAT SERUM)



in the study using immunoglobulin concentrates. In contrast to the previous study, however, there was no significant correlation between age at diagnosis and binding inhibitory index (see FIGURE 2.6). It is possible that the TBII assay using neat serum is also measuring to some extent microsomal antibody, but this does not account for the lack of age correlation: the mean age for patients in this study with microsomal antibody titres above 40^2 was 48.8 years (SD 15.0) compared to 47.4 years (SD 14.7) for patients with low or undetectable microsomal antibodies (not significant). Once again, there was no correlation between thyroid antibody measurements and biochemical tests of thyroid function.

C: Comparison of TSH Receptor Antibody Measurements Using Immunoglobulin Concentrates and Unfractionated Serum

For this study, TSH receptor antibodies were measured simultaneously by the two methods already described in 44 patients with untreated thyrotoxic Graves' disease. The mean T_3 in these patients was 6.3 nmol/l (SD 2.17), the mean T_4 was 234 nmol/l (SD 50.8) and all had flat TRH tests.

The results are shown in FIGURE 2.7. Overall, the correlation coefficient was 0.882 ($p < 0.001$). In 34 out of the 44 cases, the actual value for the binding inhibitory index was higher when neat serum was used in the assay. 28 patients (64%) were positive in both assays, while 9 (20%) were negative in both assays. Discrepancies existed in seven cases and, in all of these, the sample was negative

FIGURE 2. 6 RELATIONSHIP BETWEEN AGE AT DIAGNOSIS AND TSH RECEPTOR ANTIBODIES (NEAT SERUM)

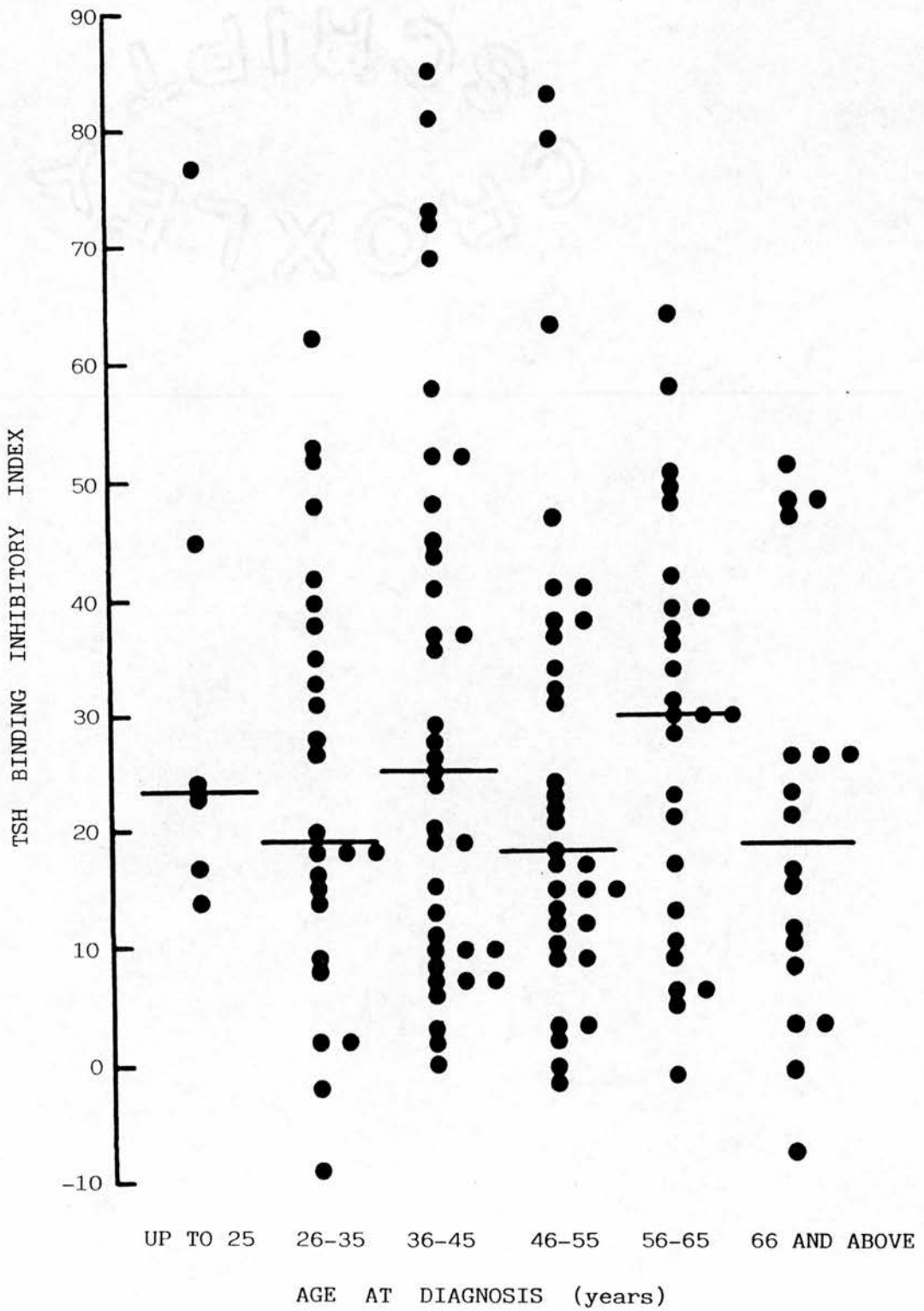
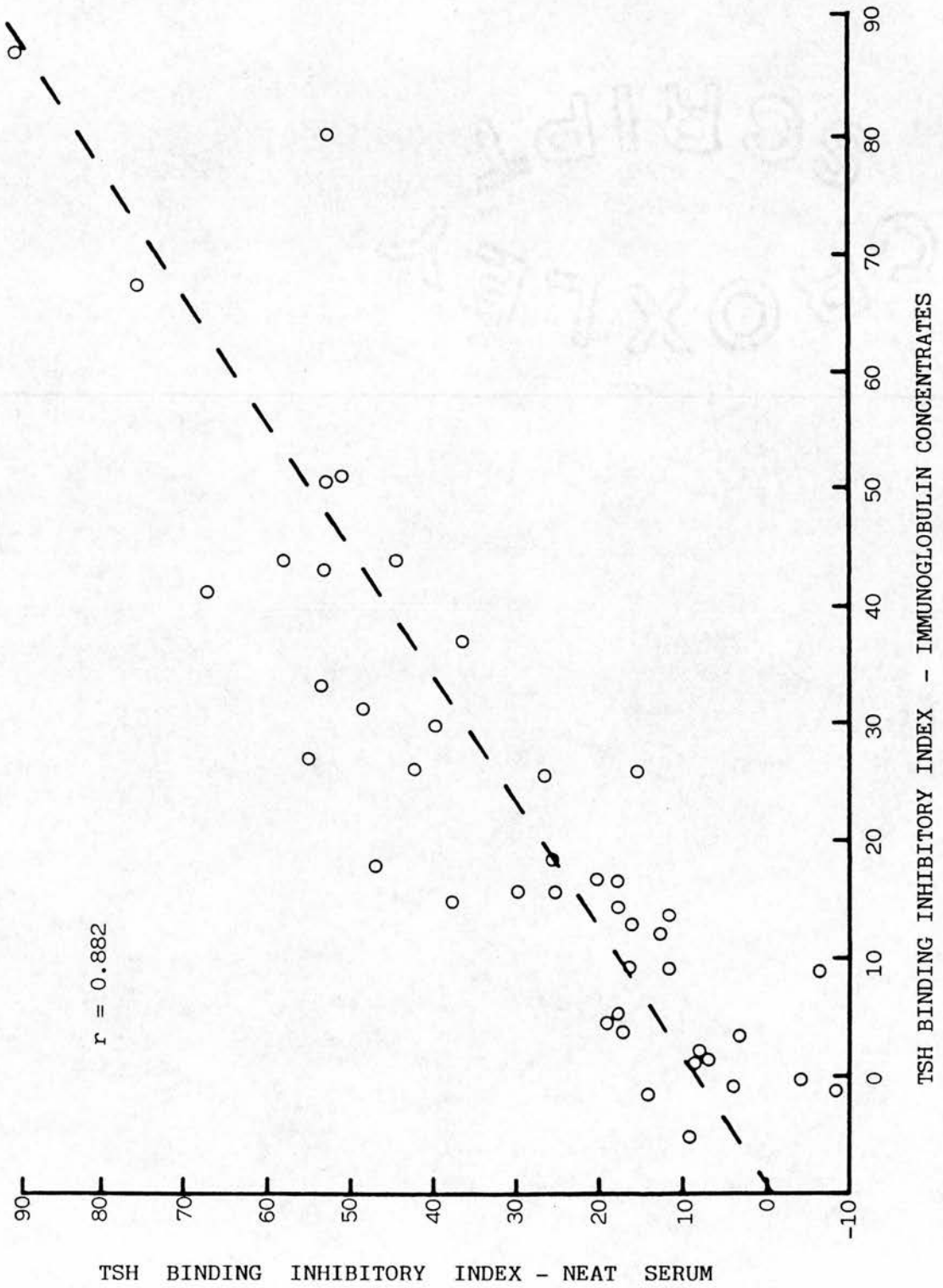


FIGURE 2.7 COMPARISON OF TSH RECEPTOR ANTIBODY MEASUREMENTS
IN IMMUNOGLOBULIN CONCENTRATES AND NEAT SERUM



in the assay using immunoglobulin concentrates but positive in the assay using neat serum, confirming the greater sensitivity of the latter method.

D: Thyroid Autoantibodies in Recurrent Graves' Disease.

Graves' is a disease of relapses and remissions where a proportion of the patients ultimately develop spontaneous hypothyroidism (Doniach, 1980). This study was undertaken to see if patients with a recurrence of their thyrotoxicosis differed immunologically from patients at first diagnosis. Fourteen patients with recurrent thyrotoxicosis were matched for age and sex with 28 newly diagnosed Graves' patients (each recurrent being matched to two new patients). The mean age in both groups was 44 years, the recurrent group consisting of 13 females and one male. The mean T_3 in the recurrent group was 5.18 compared to 5.45 in the new patients, while the four hour ^{131}I uptake in the two groups was 53.0% and 50.3% respectively. Neither of these two differences nor the difference in T_4 values was significant. TBII were measured using neat serum and microsomal antibodies using commercial kits (Fujizoki).

The difference in binding inhibitory index between the two groups is shown in FIGURE 2.8. In newly diagnosed Graves', the median value was 28 compared to 16 for the recurrent patients ($p < 0.02$). There was also a significant difference in microsomal antibody titres ($p < 0.01$) - see FIGURE 2.9. In the newly diagnosed patients, 17 out of 26 (65%) had undetectable or low levels of microsomal antibody compared to 2 out of 13 (15%) recurrent patients.

FIGURE 2.8 TSH RECEPTOR ANTIBODIES IN RECURRENT GRAVES' DISEASE

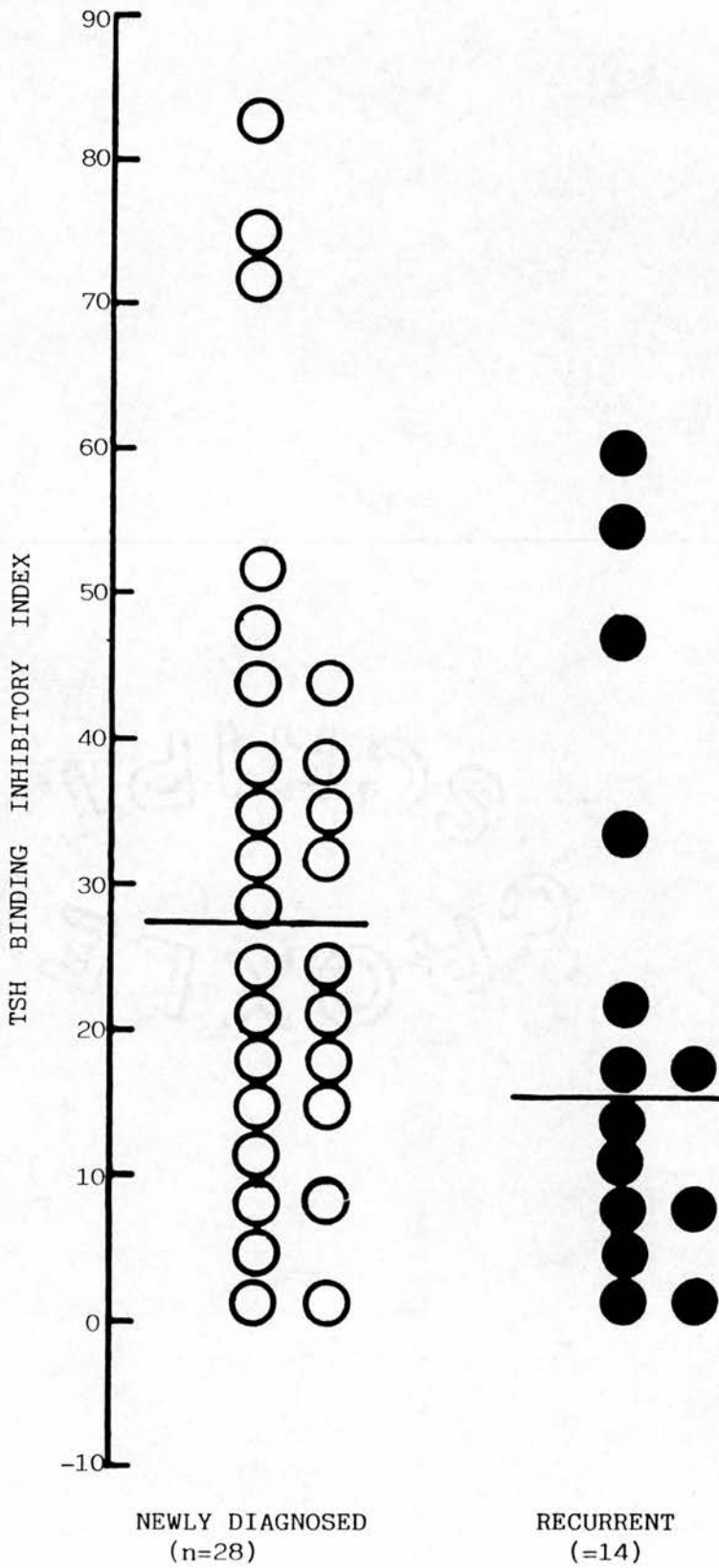
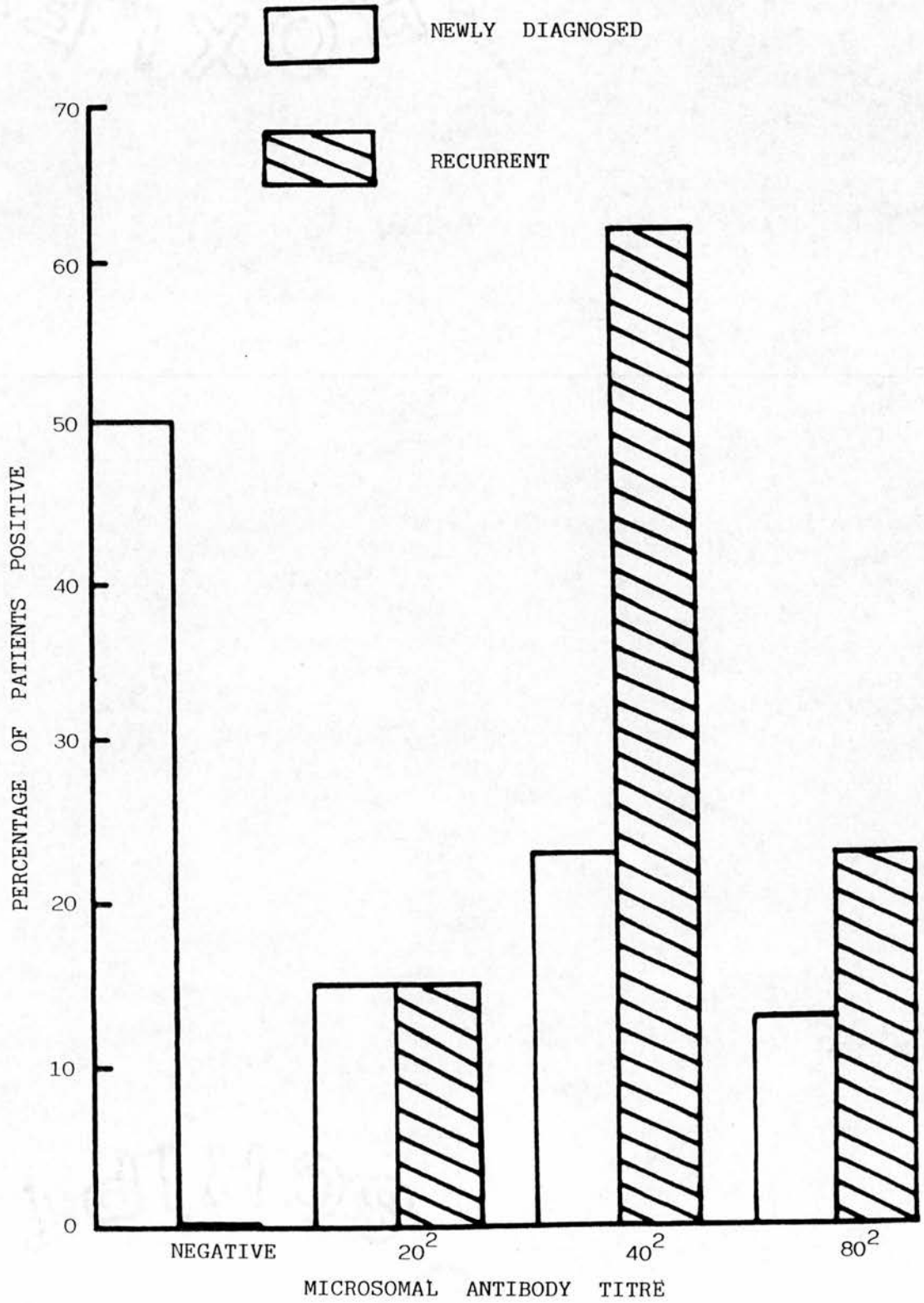


FIGURE 2.9 THYROID MICROSOMAL ANTIBODIES IN RECURRENT GRAVES' DISEASE



DISCUSSION

Part of the variability in early assays for TBII was undoubtedly due to differences in the preparation of particulate thyroid membrane fractions. The use of solubilised TSH receptors was described in 1981 by Kotulla & Schleusener. Uniform preparations of thyroid membrane could be obtained and stored long-term for standardisation of the assay. The value of solubilised receptors was confirmed by Iida et al (1982) who found it to increase assay sensitivity, largely by decreasing the inhibition of labelled TSH binding by normal serum. Shewring and Rees-Smith (1982) used an assay based on Lubrol-solubilised receptors to show that 83% of newly diagnosed Graves' patients were positive. In this study, immunoglobulin concentrates were prepared using polyethylene glycol which eliminates the dialysis step required when ammonium sulphate precipitation is used. More recently, their assay has been modified for use with unfractionated serum (Southgate et al, 1984). This modification to the assay not only makes it more convenient to apply but was also said by the authors to increase assay sensitivity. The use of the radioreceptor assay has been studied here with two large series of patients with untreated thyrotoxic Graves' disease, TBII being measured in one with immunoglobulin concentrates prepared by polyethylene glycol precipitation from serum and in the other series the measurement was performed with neat serum. The published normal ranges for these assays were confirmed. In the assay based on polyethylene glycol precipitates, 61% of patients were positive compared to 81% when neat serum was used, thus confirming the previous observation that the assay is more sensitive when neat serum is used. This may relate to the amount of immunoglobulin applied to

the receptor preparation. TSH receptor antibodies have generally been found to correlate with the presence of clinical features of Graves' disease rather than with their severity or with biochemical measures of thyroid function. This was also confirmed in the present study. Goitre size, assessed clinically, correlated well with TBII (data not shown) but patients with exophthalmos did not differ in terms of thyroid antibodies from other patients with untreated Graves' disease.

There are several possible reasons for the relatively low and variable positivity found in assays for TBII:-

1) Patient selection: patients who clinically and scintigraphically have a diffuse goitre may in fact have disseminated thyroid autonomy due to microscopic nodules (Schleusener et al, 1983). Subclinical forms of Graves' may be associated with incomplete expression of immunological markers. Patient heterogeneity is discussed further in Chapter 4.

2) Thyroid membrane preparations: solubilised TSH receptors have generally been found to be more sensitive than particulate membrane preparations (See Table 2.2). TBII are not highly species specific; porcine thyroid will bind TBII with high affinity while guinea pig and sheep thyroid bind with lower affinity (Rees-Smith & Hall, 1981; Davies, 1981). However, even with different human thyroids there may be a variation in immunoglobulin binding to TSH receptor preparations (Gossage et al, 1981).

3) Immunoglobulin preparations: there is a small amount of TBII activity in normal serum (Brown et al, 1983) and assay sensitivity is partly determined by how well this can be separated from the specific TBII in Graves' serum. The amount of immunoglobulin applied to the receptors may influence sensitivity (Endo et al, 1978), as may the method of preparing the IgG. For example, ammonium sulphate precipitation, which has been the most widely used method, may not always reflect accurately the TBII activity of serum (De Bruin et al, 1982).

4) Circulating thyroglobulin: this is elevated in patients with Graves' disease (Van Herle et al, 1973), it can interfere with TSH binding to its receptor (Hashizume et al, 1978), co-precipitates with IgG when ammonium sulphate is added to serum, and has been considered a possible source of interference in the TBII assay (Fenzi et al, 1978).

5) Immune complexes: these are present in a proportion of patients with Graves' disease and a reciprocal relationship with TBII has been suggested (Van der Heide et al, 1980). The possible role of immune complexes in modulating autoantibody expression is discussed in Chapter 5.

6) Autoantibodies to TSH: Akamizu et al (1984) have described, in two out of 154 Graves' patients, a circulating autoantibody to TSH. The antibody-TSH complex co-precipitated with the TSH receptors giving a falsely high value for TBII. A similar antibody was

reported in Graves' patients by Kajita et al (1983) and also in a normal individual (Copping et al, 1985), the latter causing a spurious positive result in the TBII assay.

7) Geographical variation: Phillips et al (1985) have recently demonstrated that the incidence of TSH receptor antibodies in thyrotoxic patients differs markedly from area to area and that there is an inverse correlation with the previous incidence of iodine deficiency. Patients with disseminated thyroid autonomy may be included in series of Graves'disease if thyroid nodules are not clinically or scintigraphically detectable.

Surprisingly little comment is made in the published literature on the correlation between TSH receptor antibodies and thyroid microsomal antibodies. Gossage et al (1983) recently reported no correlation between the two. In both assays used here, a positive correlation between TBII and thyroid microsomal antibody titre was found, although this was more marked in the assay based on neat serum ($p < 0.001$). The negative correlation between age at diagnosis and TBII found when immunoglobulin concentrates were used was not found with unfractionated serum. The latter method may have been picking up a population of antibodies to components of thyroid other than the TSH receptor. It is conceivable that such antibodies might become more frequent with age. This effect did not appear to be due to the microsomal antibodies detected by passive haemagglutination since these were not increased in frequency in patients who were older at diagnosis.

Recurrent Graves' disease differed immunologically from newly diagnosed thyrotoxic Graves' although the clinical and biochemical features of the patients compared were very similar. Fourteen patients with recurrent disease had lower TBII ($p < 0.02$) but higher microsomal antibody titres ($p < 0.01$) than age and sex matched new patients. This may reflect an increased amount of thyroiditis in patients with disease of long standing. The production of TBII by intrathyroidal lymphocytes may be sufficient to stimulate the thyroid but localisation of the immune reaction to the thyroid may account for the decreased levels of TBII.

In conclusion, the studies presented here agree with the published data on the incidence of TSH receptor antibodies in untreated Graves' thyrotoxicosis and with the observation of Southgate et al (1984) that the assay is more sensitive when unfractionated serum is used. TBII correlated with microsomal antibodies and their expression may be age related. Humoral immunity is different in recurrent Graves' compared with newly diagnosed disease.

Chapter Three

Thyroid Antibodies and the Response to Treatment in Graves' Disease

SUMMARY

TSH receptor antibodies (TBII) and other thyroid antibodies were studied before and after treatment of thyrotoxic Graves' disease with carbimazole, radioactive iodine and surgery.

A) Of 24 patients treated with carbimazole, 18 (73%) were positive for TBII at diagnosis and 17 (71%) for thyroid microsomal antibodies. While biochemical control of thyrotoxicosis was achieved within one to two months in all cases, levels of thyroid antibodies did not decline until later and were often still high at the end of a nine month follow-up period.

When TBII is normal at the end of a course of drugs, thyrotoxic relapse is usually associated with a return of TBII towards pre-treatment values. Persistent TBII at the end of a course of drugs is not invariably associated with thyrotoxic relapse (two cases are presented) and this may be due to concurrent thyroiditis. Similarly, episodes of relapse in TBII negative patients who become TBII positive may be self limited.

B) Eighteen patients were followed up after ^{131}I therapy (mean dose 7.7 mCi, range 5 - 15). The median TBII at diagnosis was 35 and, by three or four months, this rose to over 55 and did not reach normal levels until two years after therapy, although some patients still had high TBII at this stage.

TSH receptor antibody titre at diagnosis was related to the thyroid status six months after therapy in 52 patients with mild to moderate

thyrotoxicosis. In the six patients not responding to a single dose of ^{131}I , the mean TBII was 6.5, compared to 10 for the 19 euthyroid cases and 18 for the 27 hypothyroid cases ($p < 0.01$). Furthermore, antithyroglobulin antibodies appeared to have a protective role with seven of the nine patients positive for these antibodies euthyroid at six months ($p < 0.01$). Patients rendered hypothyroid also tended to show an increase in TBII after therapy ($p < 0.05$) while the euthyroid patients either decreased or showed small increases within the normal range.

24 unselected patients were tested for TBII long after ^{131}I therapy (mean period, 56.8 months, range 27 to 156). Significant levels of TBII were found in 10 cases and were strongly correlated with the presence of exophthalmos. The median TBII for the non-exophthalmic group ($n=14$) was 1.5 compared to 33.5 for the patients with exophthalmos ($n=10$, $p < 0.01$).

C) After surgery, TBII declined progressively in all eight patients tested sequentially during the first year. Persistent TBII in euthyroid patients was uncommon but two such cases were found and both had marked bilateral exophthalmos.

These studies confirm that TSH receptor antibodies have a bearing on the prognosis of Graves' treated with drugs, ^{131}I or surgery. The time course of actions with the former favours a primary antithyroid rather than an immunosuppressive action although the latter is clearly important in maintaining remission. Persistence of TBII after destructive antithyroid therapy is associated with other autoimmune diseases, particularly Graves' eye disease.

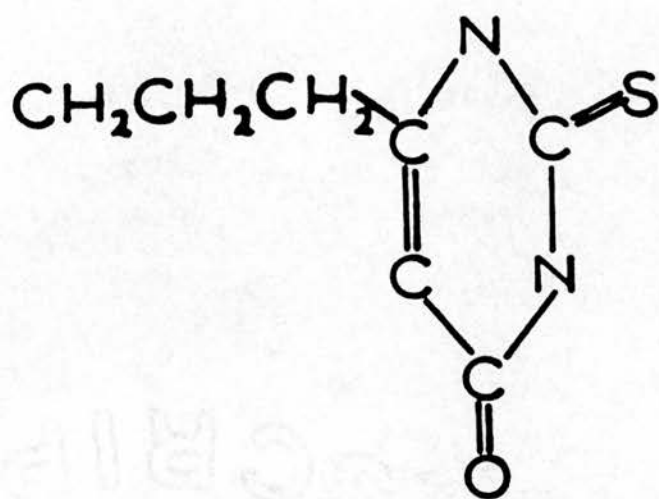
INTRODUCTION

Thyrotoxicosis in Graves' disease may be treated in one of three ways: antithyroid drugs, radioactive iodine, or surgery. Immunological tests may have a role in predicting the ultimate response to all three treatment modalities, although they have been most widely used in relation to antithyroid drug therapy. Patients with persistent evidence of the underlying immunological abnormality are much more likely to relapse after treatment, even if the biochemical control of their thyrotoxicosis is good. The aim of the work presented in this chapter is to consider the changes in thyroid autoantibodies during therapy, particularly radioactive iodine, since there is very little published work on predicting the response to this treatment.

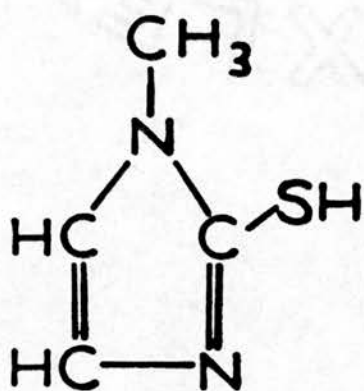
A: ANTITHYROID DRUGS

The treatment of thyrotoxicosis with thiourea and thiouracil drugs was introduced in 1943 by Astwood, who showed a prompt response of both symptoms and basal metabolic rate in three patients. Recent evidence (Davidson et, 1978) suggests that these drugs may act by trapping oxidised iodine rather than directly inhibiting the thyroid peroxidase. The structure of the common antithyroid drugs (carbimazole, its metabolite methimazole, and propylthiouracil) is shown in FIGURE 3.1. Carbimazole is the most widely used antithyroid drug in the United Kingdom. Drugs are now the treatment of choice for young patients with mild to moderate thyrotoxicosis. Antithyroid drugs will control the biochemical and clinical features of

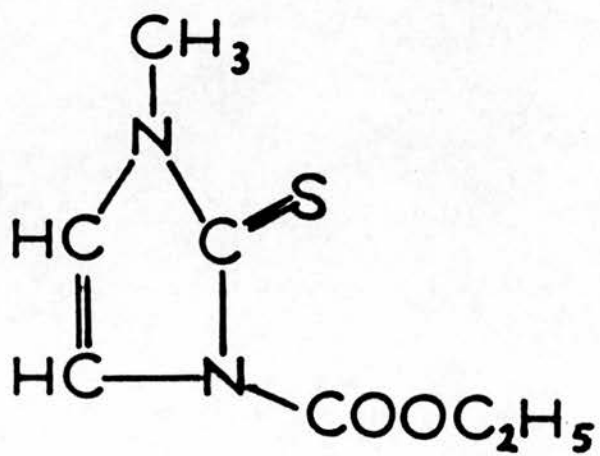
FIGURE 3.1 STRUCTURES OF THE COMMONLY USED ANTITHYROID DRUGS



PROPYLTHIOURACIL



METHIMAZOLE



CARBIMAZOLE

thyrotoxicosis in over 95% of cases, but only 45% to 70% of patients will achieve a lasting remission after a single course of drugs (Solomon et al, 1953; Manson, 1953; Trotter, 1961; Astwood, 1963; Hershman et al, 1966). There is no reliable feature at first presentation which will predict the patients most likely to relapse. Most of the recurrences are within one year of stopping therapy with a steady decline in the rate thereafter (Solomon et al, 1953; Goodwin et al, 1954; Hershman et al, 1966). Hershman (1966) suggested that the duration of symptoms prior to starting therapy may be of some relevance, with patients who have had symptoms for longer being more likely to relapse. This does not, however, agree with the studies of Manson (1953) or Goodwin (1954).

Patients with larger goitres are much more likely to relapse after therapy (McCullagh et al, 1951; Manson, 1953). A decrease in goitre size during therapy has been associated with a low rate of relapse (Solomon et al, 1953; McCullagh and Cassidy, 1953; Hershman et al, 1966). The nodularity of the goitre may also help predict relapse: in the study of McCullagh et al (1951) 62% of patients with diffuse goitre remained in remission after a course of drugs while only 34% of patients with nodular goitre did so. Aspenstrom (1953), however, in a study from an endemic goitre region, found no difference in the relapse rate between nodular and diffuse goitres. In practice, very few patients with Graves' disease have nodular goitres and undoubtedly many patients included in these studies did not have Graves' disease, which is now much easier to diagnose with the advent of scanning techniques and immunological tests. Reveno and Rosenbaum (1964) identify the patients most likely to respond to a single course of antithyroid drugs as being younger, with smaller goitres

and mild thyrotoxicosis of short duration. Patients with pure T₃ toxicosis also have a low rate of relapse (Greer et al, 1977). Another influence may be the iodine content of the diet: the relative deficiency induced by antithyroid drugs has been suggested to protect against early relapse and there may be an inverse relationship between iodine intake and the remission rate after treatment (Alexander et al, 1965; Wartofsky, 1973).

Thyroid Hormone Suppressibility and the Prediction of Thyrotoxic Relapse

In a study to determine whether or not thyrotoxicosis was pituitary dependent, Werner (1952) showed that the administration of thyroid hormone did not suppress thyroid activity. The thyroid was however suppressible in normal subjects and in patients who were in remission after a course of antithyroid drugs (Morgans et al, 1952; VanderLaan, 1957). Suppression of radioactive iodine uptake by the thyroid develops progressively after antithyroid drug therapy is instituted (Slingerland and Burrows, 1979) although this is not found in all patients. For example, in the study by Alexander et al (1966), only half the patients developed suppressibility. Furthermore, continuing disease activity was apparent in spite of adequate control of thyroid hormones (Alexander et al, 1973). There was no difference in terms of age, sex or thyroid hormone levels at diagnosis between patients who suppressed and those who did not (Alexander et al, 1967) but suppressibility did correlate with a small goitre at diagnosis and a decrease in goitre size with therapy (both factors known to influence

ultimate prognosis). Loss of suppressibility has also been documented in Graves' patients relapsing after having apparently returned to normal after a course of drugs (Alexander et al, 1968).

It was first suggested in 1960 by Cassidy and Vanderlaan that thyroid suppressibility might be useful in predicting the outcome after a course of drugs. In their study, the average 24 hour ^{131}I uptake after thyroid hormones in patients remaining in remission was 25.4% compared to 66.2% in those patients who suffered a recurrence. Alexander et al (1969) found, in a follow up study involving 93 patients (1970), that 32 out of 42 patients lacking suppressibility relapsed, compared to 16 out of 51 patients who did suppress. Similar findings were reported by Lowry et al (1971). Not all studies have confirmed the usefulness of the thyroid suppression test: although Hales et al (1969) did find a relationship with relapse, they found that only half the patients who did not suppress actually relapsed. This technique not only gives an imperfect prediction of the outcome, but also has the disadvantages of being time consuming both for the patient and for the investigator, and of requiring multiple doses of isotope to give a clear picture of trends in the underlying disease.

The Optimal Duration of Antithyroid Drug Therapy

Treatment with antithyroid drugs is generally continued for one year or more, although the rationale for this is not entirely clear. Greer et al (1977) suggested that short term therapy, i.e. treating the patients until they were symptomatically and biochemically normal (generally about four to five months) was adequate, and gave

comparable results to long term therapy. Their later study (Bourma et al, 1982) did not confirm the low incidence of relapse but pointed out that it may be a useful strategy in selected patients, while Slingerland and Burrows (1979) have demonstrated that long term therapy is not associated with an increased incidence of side effects. The original study of Greer was criticised because the basis for patient selection was not stated and there was a high incidence of T₃ toxicosis (De Groot, 1977; Burr et al, 1979). De Groot suggested that, by temporarily depleting the thyroid gland of hormone, short term therapy may produce temporary relapse without influencing the underlying disease process. Burr et al (1979) found a very high relapse rate after short term therapy. Tamai et al (1980) showed that the more prolonged the treatment, the more likely was the patient to achieve lasting remission and Sugrue et al (1982) reported a 91% relapse rate at five years in patients treated for a mean period of eleven months compared to 49% in patients treated for 3.8 years. It would seem certain then that long term therapy is generally required to ensure a high remission rate.

Beta blockade has been used as the sole agent in treating the thyrotoxicosis of Graves' disease. For example, Pimstone et al (1969) treated 27 cases and found that 13 had an adequate response clinically. This is clearly inferior to antithyroid drugs and further studies have also suggested beta blockers to be unsuitable as first line therapy except as preparation for surgery or radioactive iodine (McLarty et al 1973; Mazzaferri et al, 1976). It has been suggested by various groups of workers that antithyroid drugs may influence the natural history of Graves' disease; Lowry et al (1973) found that radioactive iodine uptake did not change at the

time of thyrotoxic relapse in patients who had already had a course of drugs, and concluded that factors other than the iodine trapping mechanism might be important. Wise and his colleagues (1973, 1979) have designed a "block-replace" regime where patients are maintained on high doses of carbimazole supplemented with triiodothyronine once the toxicosis is controlled. They feel that the decrease in ^{131}I uptake with therapy is not explicable entirely by the inhibition of iodination and that antithyroid drugs affect thyroid hyperstimulation at a pre-biosynthetic level. Wilkin et al (1979) also point out that fluxes in thyroid hyperstimulation appear to occur independently of symptoms.

TSH Receptor Antibodies and the Prediction of Thyrotoxic Relapse

TSH receptor antibodies decline progressively during treatment with antithyroid drugs and their persistence usually indicates that the underlying disease continues to be active. Thus, Hardisty et al (1984b) demonstrated a fall in LATS-P with treatment in patients who remained in remission, and recurrence of thyrotoxicosis after treatment was associated with a return of LATS-P towards pre-treatment levels. In the study of Chiovato et al (1978), there was a progressive fall in thyroid microsomal antibody titres with therapy with a later increase towards pre-treatment levels in patients who relapsed. The prognostic value of these antibodies has not been generally confirmed although these authors were using a highly sensitive radioimmunoassay, as opposed to passive haemagglutination which is less sensitive but more widely used. A progressive decline in adenylate cyclase stimulating antibodies with

drug therapy has been reported by several groups (Beck & Madsen, 1980; Karlsson & Dahlberg, 1981; Feldt-Rasmussen et al, 1982). In the first of these three studies, the rate of disappearance of antibodies was related to the age of the patient, with antibodies becoming undetectable faster in younger patients. The level of binding inhibitory immunoglobulins (TBII) also falls progressively during treatment (Chiovato et al, 1978; Fenzi et al, 1979) and this is paralleled by a fall in ^{131}I uptake (Gossage et al, 1983).

Two studies have compared changes in TSH binding inhibitory immunoglobulins and adenylate cyclase stimulating antibodies during therapy: in the first, Kuzuya et al (1979) showed a poor correlation between the two at diagnosis, the stimulatory antibodies tended to disappear faster with treatment and ultimately became undetectable in all patients. Of eight patients whose thyroids remained non-suppressible during treatment, four were persistently positive for TBII, while none of the nine suppressible patients had persistent TBII. In the second study, Bliddal et al (1982a) found that the two activities generally declined in parallel.

The concept that persistence of TSH receptor antibodies indicates that the underlying disease is still active and that patients will probably relapse on stopping therapy has led to many studies attempting to predict the ultimate response to antithyroid drugs. Although Long-Acting Thyroid Stimulator (LATS) relates well to disease activity (McKenzie & Kakarija, 1977), its use in predicting relapse may be limited since it tends to persist in many patients with exophthalmos but without continuing thyrotoxicosis (McKenzie, 1961). LATS-P is probably a better predictor of relapse (Hardisty et

al, 1982): 21 out of 24 patients with LATS-P at cessation of therapy relapsed within one year, compared to eight out of 28 patients who were LATS-P negative on stopping drug therapy.

Anti-microsomal and anti-thyroglobulin antibodies often decline during drug therapy, and fluctuations with disease activity have been reported but these antibodies have been found to be of little value in predicting the response to treatment (Wilkin et al, 1980). HLA-B8 is associated with recurrent thyrotoxicosis in Graves' disease as well as with persistence of thyroid microsomal antibodies (Irvine et al, 1977) and it would not be surprising, therefore if some relationship were to exist between microsomal antibodies and relapsing disease but this is certainly not marked enough to be of use in routine clinical practice. Zakarija, McKenzie and Barovac (1980) have measured adenylate cyclase stimulating antibodies in 28 patients after therapy: 13 patients remained in remission and all were negative for antibodies while 12 out of 15 relapsing cases were positive.

Early reports suggested that TBII were highly accurate in predicting relapse after antithyroid drugs were stopped: Davies et al (1977) found that all 16, out of a total of 30 patients, with a high TBII relapsed, while eight out of nine such patients in the study of O'Donnell et al (1978) did so. By combining HLA-DR typing with TBII measurement, McGregor et al (1980) were able to predict the ultimate outcome in 62 out of 65 cases. Since 27 out of 29 patients positive for HLA-DR3 relapsed, TBII were most useful in the DR3-negative patients. The value of TBII in predicting thyrotoxic relapse has been further confirmed (Schleusener et al, 1978; Schernthaner et al, 1980)

although the latter study did not find any difference in DR3 positivity between the relapse and remission groups. Teng and Yeung (1980) found once more that all of their patients with persistent TBII relapsed as did the patients who had become TBII negative but subsequently became positive again. A number of patients who were TBII negative relapsed, however, and this decreased the overall rate of successful prediction in this study. Docter et al (1980) found TBII to be of no value whatsoever in predicting thyrotoxic relapse. Their study was a small one, and while at odds with other published data, it does serve to underline that prediction with TBII is inconstant and therefore may be of limited use for the individual patient. A recent report by Bliddal et al (1983) has suggested that the combined use of TBII and stimulatory antibodies may give more reliable prediction.

The Immunosuppressive Action of Antithyroid Drugs

In addition to their inhibition of the organification of iodine, antithyroid drugs may directly affect the immune system (Kendall-Taylor, 1984). This action of these drugs is potentially important for several reasons. Firstly, in the planning and monitoring of drug therapy, should we be guided by the patient's biochemical status or should we be aiming to normalise immunological parameters? Secondly, if a thyroid-specific immunosuppressive action influences the natural history of the disease - and the data discussed above suggests it might - then perhaps we should be looking for more effective immunosuppressive agents. Thirdly, the potential use of antithyroid drugs, with appropriate hormone

replacement, in euthyroid or hypothyroid patients with thyroiditis may influence their ultimate prognosis as far as thyroid function is concerned.

TSH receptor antibodies, and other thyroid antibodies, decline in the course of drug therapy, their persistence indicates activity of the underlying disease and a high chance of relapse on stopping the drugs, as discussed above. A decrease in the proportion of circulating suppressor cells is well described in untreated Graves' disease (see Chapter 7) and this similarly returns to normal with therapy except in patients who relapse on drug withdrawal (Ludgate et al, 1984). Both these lines of evidence suggest that antithyroid drugs may influence the immune system and the presence or absence of this action affects prognosis. The first direct evidence was provided by Michie et al (1967), who not only confirmed that both Graves' and Hashimoto's thyroiditis were associated with an increase in thymic size but also that the size of the thymus decreased during long-term antithyroid drug treatment. Beck et al (1973) followed this up by showing that there was less lymphoid tissue in the thyroids of patients prepared for surgery with carbimazole than in those treated with propranolol alone. They also pointed out the structural similarity between carbimazole and uracil, a drug with well documented immunosuppressive actions. More recently, it has been shown that methimazole influences the degree of thyroiditis and the amount of anti-thyroglobulin antibody production in August rats immunized with thyroglobulin and that this effect is not due to changes in thyroid hormones (Keast et al, 1981; Weetman et al, 1982; Rennie et al, 1983).

Wall and his colleagues (1976) have shown that low concentrations of propylthiouracil can inhibit the mitogenic response of normal lymphocytes to the mitogens phytohaemagglutinin (PHA), concanavalin A (con A) and pokeweed mitogen (PWM) as assessed by tritiated thymidine uptake after prolonged incubation. Their findings were not, however, confirmed by Hallengren et al (1980) who found only mild inhibition of con A- and PHA- stimulated mitogenesis and only then at high doses of propylthiouracil. There was actually an enhancement of PWM stimulation and methimazole exerted no inhibitory action with any mitogen. A direct action of methimazole, inhibiting thyroid antibody production by lymphocytes in culture has been demonstrated by McGregor et al (1980) and they estimate that this occurred at doses of the drug which may prevail in the thyroid. These authors later showed that the immunosuppressive action might be specific for thyroid antibodies since it had no influence on parietal cell antibody levels (McGregor et al, 1982). A haemolytic plaque-forming assay using protein A coated sheep red blood cells and a specific plaque-forming assay using thyroglobulin coated red cells has been used by Weiss et al (1981) to assess total and specific immunoglobulin formation respectively. They found no influence of methimazole and propylthiouracil on fresh lymphocyte incubations, but on prolonged culture both exerted a significant immunosuppressive effect. Methimazole did not influence immunoglobulin production in the study of Pinchera et al (1969) when they immunized intact mice with sheep red blood cells.

Antithyroid drugs can clearly influence antibody production but this is probably not due to a direct action on lymphocytes which do not have a peroxidase and are therefore unlikely to concentrate the

drugs, unlike monocytes and polymorphonuclear leukocytes (Lam & Lindsay, 1979). It has therefore been suggested that their immunosuppressive action is mediated by an effect on accessory cells (Weetman et al, 1983). When Hashimoto lymphocytes are incubated with thyroglobulin primed accessory cells, antibody production is inhibited by the addition of methimazole. The basis for this effect may be the inhibition of oxygen radical production by monocytes which may, in turn, affect antibody presentation by these cells (Weetman et al, 1984).

Other aspects of cell-mediated immunity may also be affected by antithyroid drugs: an inhibition of antibody-dependent cellular cytotoxicity was demonstrated by Pozzilli et al (1982) when lymphocytes from eleven normal subjects were incubated with 51-chromium labelled Chang liver cells. They also showed, in six subjects, a decrease in low affinity sheep red cell rosetting cells (which possess a receptor for the Fc portion of IgG and are cytotoxic). Interleukin-2 (formerly T Cell Growth Factor) is a soluble 15,000 to 20,000 molecular weight glycoprotein found in the supernatant of stimulated lymphocyte cultures (Morgan et al, 1976) It can stimulate, for months on end, the growth of T cells in culture. A receptor for this molecule is expressed on activated lymphocytes (i.e. cells which have been exposed to an antigen in association with a major histocompatibility antigen: Larsson & Continho, 1979). This receptor, which can be detected using the monoclonal antibody TAC, is an intermediate activation antigen - it is expressed on activated T cells after the 4F2 antigen but before they express class II histocompatibility antigens (Lotner et al, 1983). Lymphocyte activation is discussed in detail in Chapter 8. Signore et al (1985)

have shown that, in lymphocyte cultures from 19 healthy volunteers, the expression of interleukin-2 receptor is inhibited by carbimazole. This was found to be a true effect on diminished production of receptor rather than a blocking effect of the drug on the interleukin-2 receptor.

Evidence against an immunosuppressive action of antithyroid drugs has been presented by Wengel and Lente (1984) who demonstrated that treatment with perchlorate produced similar changes in thyroid stimulating immunoglobulins to antithyroid drugs. Data from in vitro studies is however convincing as are observations from numerous other clinical studies: these drugs have been shown to diminish antibody production in the thyroid and this may be mediated by a metabolic action on antigen presenting cells. An effect on killer cell activity is also described as are changes in lymphocyte activation antigens.

PATIENTS AND METHODS

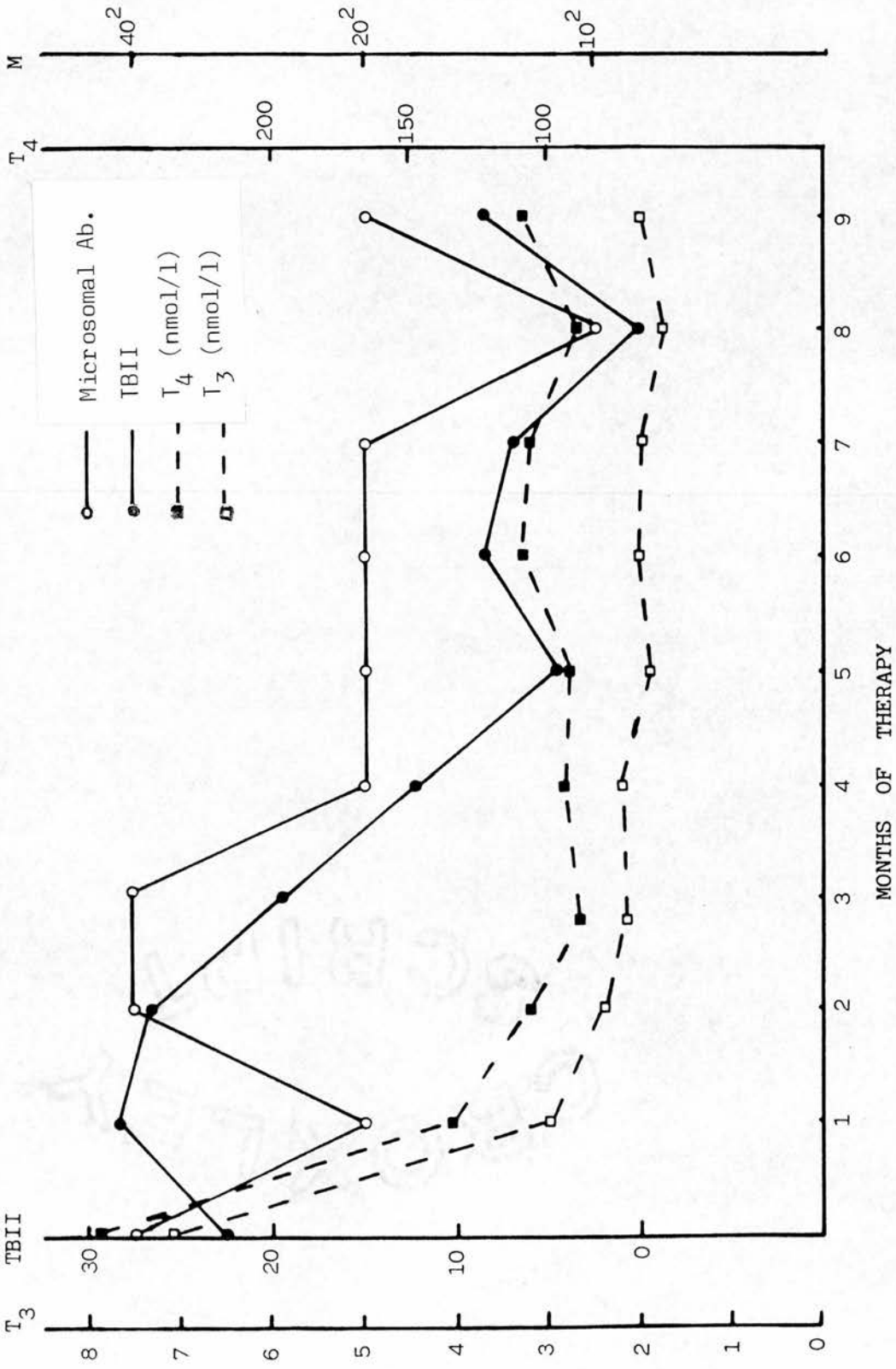
A follow-up study from the time of diagnosis was undertaken with twenty four unselected thyrotoxic Graves' patients (six male, eighteen female; mean age 31.6 years, SD 11.6). Venous blood was withdrawn from all patients prior to starting carbimazole therapy and at intervals thereafter for up to nine months (at least four samples were taken from each patient). 19 samples were studied at one month, 17 at two months and then 16, 19, 12, 13, 7, 6 and 9 samples in successive months. Thyroid hormones were measured in the Department of Clinical Biochemistry, Royal Infirmary of Edinburgh by in-house

radioimmunoassay. The upper limit of normal for T₃ was 2.8 nmol/l and for T₄, 150 nmol/l. Thyroid microsomal antibodies were measured by tanned red cell haemagglutination using commercially available kits (Fujizoki) and TSH receptor antibodies by the radioreceptor assay on neat serum as described in APPENDIX 1. Carbimazole (Neomercazole, Nicholas Laboratories Limited) was started at an initial dose of 30 to 45 milligrams per day in divided doses and the dose was subsequently reduced according to the clinical and biochemical response to a maintenance dose of five to ten milligrams per day. No patient developed worsening of their thyrotoxicosis or hypothyroidism and all were established on the maintenance dose within the first six months from diagnosis.

RESULTS

Of the 24 patients in the study, 18 (73%) were positive for TSH receptor antibodies (TBII) and 17 (71%) for thyroid microsomal antibodies at diagnosis. Thyroid hormone and antibody data on follow-up is shown in FIGURE 3.2. The mean T₃ at diagnosis was 7.1 nmol/l and the mean T₄ was 263 nmol/l. These had fallen to 3.0 nmol/l and 138 nmol/l respectively by the end of the first month of therapy and, by the second month to 2.4 nmol/l and 109 nmol/l. In spite of this rapid biochemical control and good symptomatic relief, immunological tests were abnormal for considerably longer. The initial median value for TBII was 22.6 and this was not significantly changed after one or two months of treatment with levels of 28.4 and 26.7 respectively. It was only after this point that TBII gradually declined, reaching normal by five months and remaining low for the

FIGURE 3.2 THYROID HORMONES AND AUTOANTIBODIES IN THE COURSE OF CARBIMAZOLE THERAPY



rest of the follow-up period. The modal value for microsomal antibody titre at diagnosis was 40^2 and, in most patients, showed no consistent change until after three months of therapy by which time titres were and remained lower than they had been at diagnosis.

Carbimazole treatment was generally continued for between one year and eighteen months. Recurrent thyrotoxicosis following cessation of therapy was usually accompanied by a persistence of high TBII during therapy or, in cases where the TBII had responded to treatment, by a return of TBII to values approaching those at initial diagnosis. Data from two patients, both apparent HLA-DR3 homozygotes is shown in FIGURE 3.3. Despite the normal TBII on stopping the drug, there was a fairly rapid biochemical relapse in both patients and this was associated with an increase in their TBII back into the abnormal range. Data on HLA-DR typing of Graves' patients is presented in Chapter 6. TBII usually remained negative in patients who were euthyroid after carbimazole therapy, but in two cases (see FIGURE 3.4) persistent or recurrent TBII was not associated with thyrotoxic relapse during a seven month period of follow-up. Nearly all patients in long-term remission, however, have a normal TBII as discussed in Chapter 2, but in patients with immunological recurrence the response of the thyroid may be limited by concurrent thyroiditis.

Episodes of recurrence, as shown by positive TBII, may be self limited and do not necessarily require further antithyroid therapy as demonstrated by the following case:-

FIGURE 3.3 TSH RECEPTOR ANTIBODIES AND THYROTOXIC RELAPSE IN TWO HLA-DR3 HOMOZYGOTES

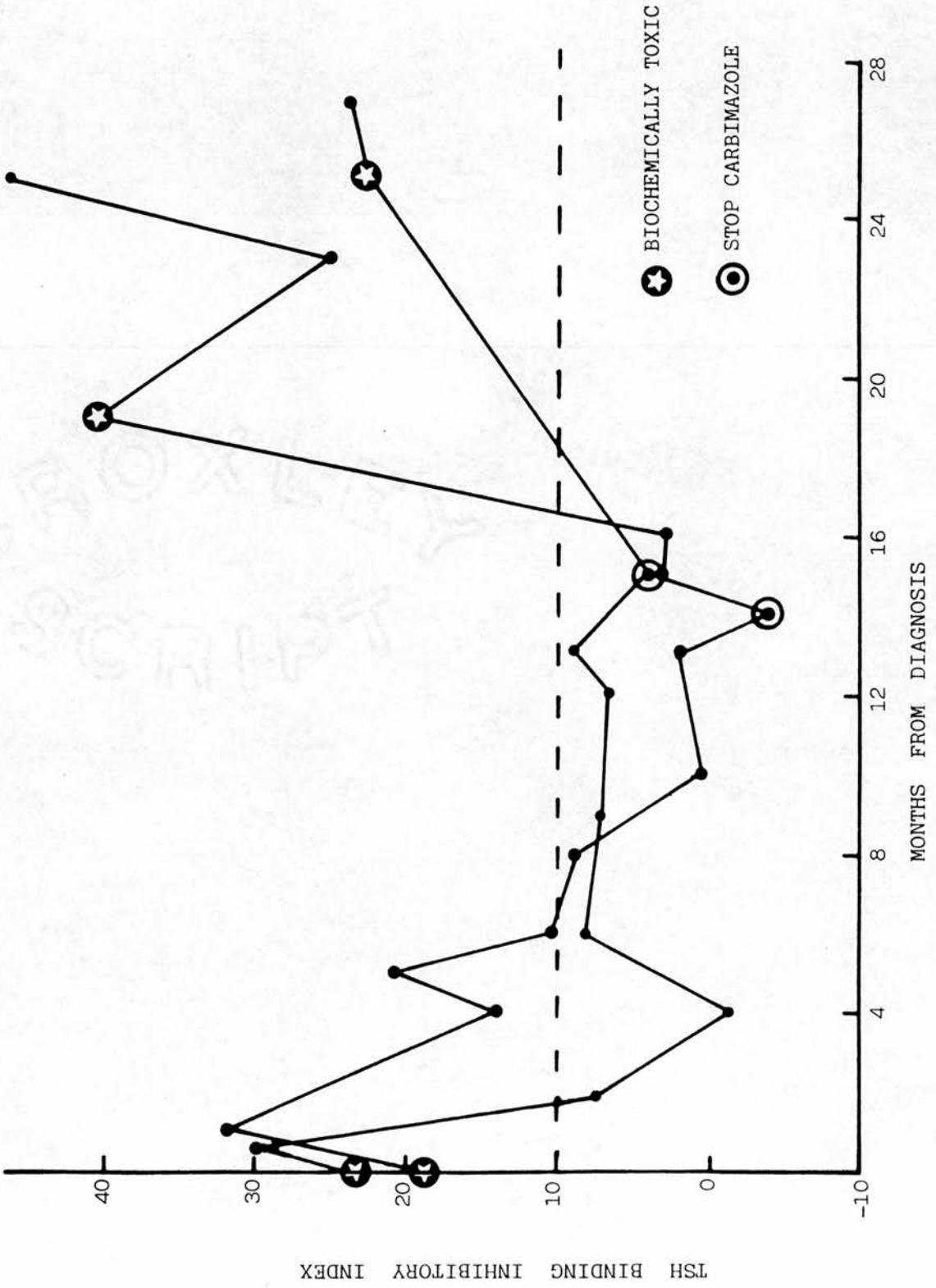


FIGURE 3.4 TSH RECEPTOR ANTIBODIES IN PATIENTS EUTHYROID AFTER A COURSE OF CARBIMAZOLE

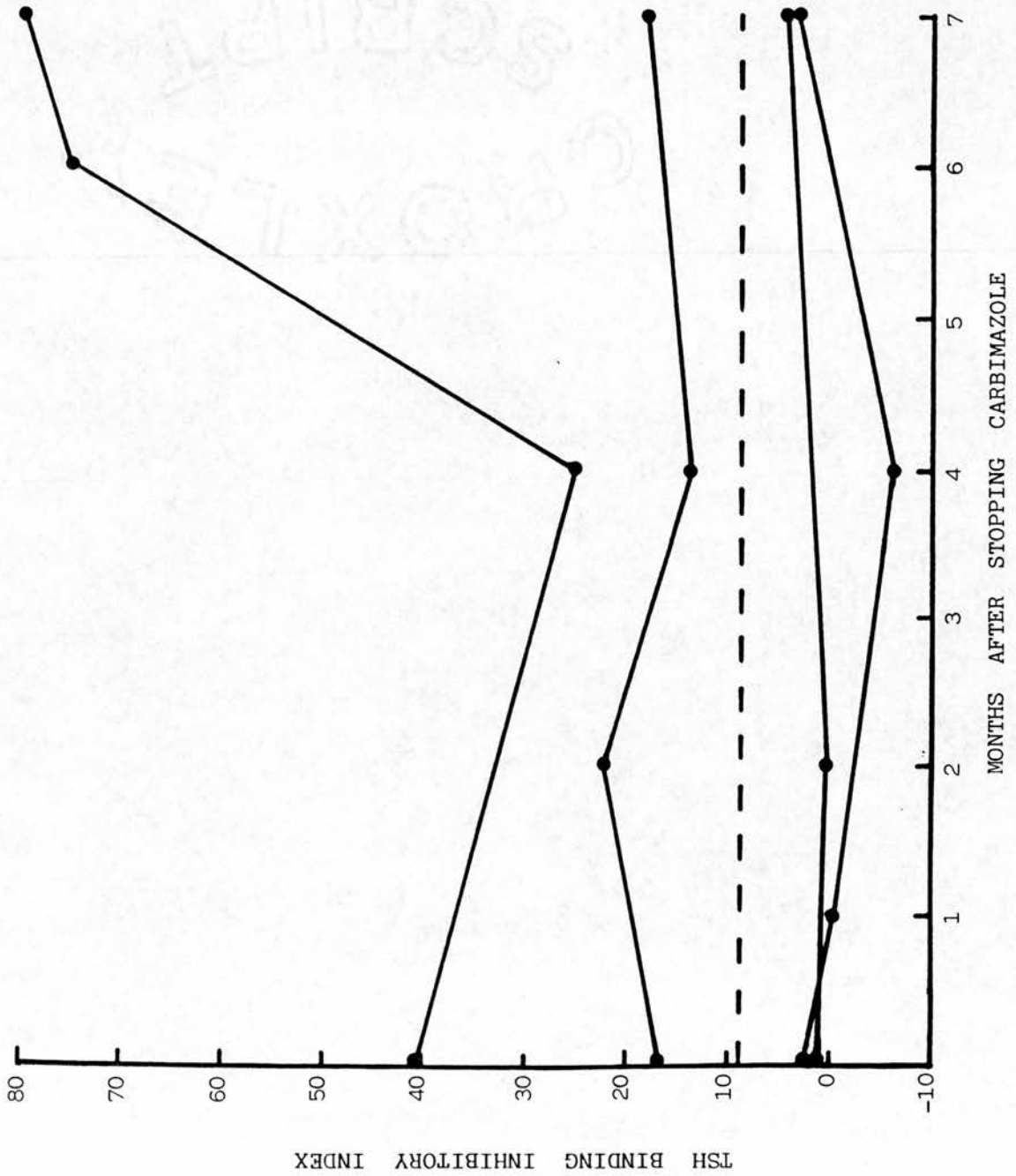


TABLE 3.1 BIOCHEMICAL AND IMMUNOLOGICAL DATA ON A PATIENT WITH A SELF-LIMITED RELAPSE OF THYROTOXICOSIS AFTER CARBIMAZOLE

	MONTHS AFTER STOPPING CARBIMAZOLE								
	0	2	4	6	7	8	7	10	18
T ₃ (nmol/l)	-	2.5	-	5.2	4.3	3.2	2.6	2.7	2.1
T ₄ (nmol/l)	-	122	125	150	160	143	130	125	135
MICROSOMAL Ab.	10 ²	10 ²	40 ²	40 ²	20 ²	40 ²	20 ²	40 ²	-
TBII	3.1	12.1	25.4	31.9	26.1	25.1	18.2	19.8	5.2

TABLE 3.2 BIOCHEMICAL AND IMMUNOLOGICAL DATA ON A PATIENT RELAPSING AFTER LONG-TERM CARBIMAZOLE TREATMENT

	MONTHS AFTER TREATMENT STOPPED				
	0	2	5	9	11
T ₃ (nmol/l)	1.7	1.8	3.1	2.5	4.7
T ₄ (nmol/l)	90	115	150	150	240
MICROSOMAL Ab.	40 ²	-	80 ²	40 ²	40 ²
TBII	-1.6	-2.3	17.3	31.6	19.7
OTHER Abs.	PC/ANF	-	PC/ANF	PC/ANF	PC/ANF

PC = Parietal cell antibody

ANF = Antinuclear factor

CASE HISTORY 1.

A 27 year old lady presented in October 1981 with mild symptoms of thyrotoxicosis and a small goitre but no exophthalmos. Her thyroid hormones were elevated (T_3 5.2 nmol/l; T_4 184 nmol/l) and the TRH test was flat. She was taking no medications and there was no family history of autoimmune disease. Technetium scanning confirmed a diffuse goitre and the four hour ^{131}I uptake was elevated at 70% (normal up to 30%). Thyroid microsomal antibodies were detected at a titre of 20^2 and TBII was 35.0. She was HLA-DR 3+7. Carbimazole was started using the regime described above and treatment was continued until March 1983 (a total of 15 months). On stopping therapy, she was both biochemically and clinically euthyroid. Her hormone and immunological data on follow-up thereafter is shown in TABLE 3.1. Her TBII rose gradually to nearly pre-treatment levels and she suffered a mild biochemical relapse although she had no real symptoms during this and antithyroid drugs were not therefore restarted.

The controversy surrounding the duration of antithyroid therapy was highlighted in the introduction to this chapter. The case history below demonstrates that, even with prolonged therapy, where thyrotoxicosis was readily controlled by a small maintenance dose of carbimazole, relapse may still occur promptly on stopping therapy:-

CASE HISTORY 2.

A twelve and a half year old girl presented in February 1979 with symptoms and signs of hyperthyroidism. She had a small diffuse goitre and mild bilateral exophthalmos. Thyrotoxicosis was easily controlled with carbimazole and she was rapidly established on a maintenance

dose of 5 mg. per day. Because of her age, this treatment was continued until after puberty. Treatment was stopped in September 1983 (a total of four years and six months). Immunological data is not available from her first presentation. She was homozygous for the HLA-DR3 phenotype. Data in TABLE 3.2 shows her course after stopping carbimazole. She ultimately underwent subtotal thyroidectomy.

B: THYROID ANTIBODIES AFTER DESTRUCTIVE ANTITHYROID THERAPY

I) RADIOACTIVE IODINE THERAPY

The treatment of thyrotoxicosis with radioactive iodine was introduced in the late 1940s. While it will control the thyrotoxicosis in over 97% of cases, it has the disadvantages of occasionally temporarily worsening the thyrotoxicosis biochemically, there is often a delay in its onset of action and there is a high incidence of hypothyroidism following treatment. With conventional therapeutic doses of ^{131}I , hypothyroidism is commonest in the first year (Dunn & Chapman, 1964) and the incidence of this early hypothyroidism is dose dependent (Smith & Wilson, 1967). Thereafter, there is a steady cumulative rise in the prevalence of hypothyroidism amounting to about 1 - 6% per year and this does not appear to diminish, even with prolonged follow-up (Belling & Einhorn, 1961; Dunn & Chapman, 1964; Nofal et al, 1966). Temporary changes in thyroid function are common following therapy, with either a low T_4 alone or in association with elevated TSH (Bellarba, Benard &

Langlois, 1972). These changes do not necessarily herald permanent hypothyroidism. A high TSH is common long after therapy, even in apparently euthyroid patients (Slingerland et al, 1972; Gordin, 1973; Gordin et al, 1973) and, while this obviously does help to predict permanent hypothyroidism, it may take many years to develop (Toft et al, 1975).

In view of the high incidence of hypothyroidism, attempts have been made to reduce the dose of radiation while still controlling the thyrotoxicosis. The incidence of hypothyroidism in the early years after therapy is undoubtedly reduced (Hagen, Ouelette & Chapman, 1967; Smith & Wilson, 1967) but there may be a marked decrease in the proportion of patients responding to a single dose of ^{131}I (Goolden & Fraser, 1969; Rapoport, Caplan & DeGroot, 1973). It may be a reasonable strategy to treat selected patients where rapid control is not crucial with low dose therapy, but the response rate of Rapoport et al, for example, where 46 out of 85 patients required at least one further dose is clearly unacceptable. Also, it is by no means clear that low dose therapy alters the long-term prognosis as far as hypothyroidism is concerned: Glennon et al (1972) found 48% of their patients to be hypothyroid 17 years after low dose therapy (3 mCi) while Cevallos et al (1974) showed that the annual incidence of late hypothyroidism may actually be increased with low dose therapy (presumably because fewer of the cases develop in the immediate post-treatment period). Roudebush, Hoyle and Degroot (1977) have described a compensated low dose regime where the dose of radiation is closely matched to the mass of tissue in the gland. 66% of their patients were euthyroid at one year with only 10% being hypothyroid.

It has been suggested that two mechanisms may operate in post-irradiation hypothyroidism (Malone & Cullen, 1976): In the early months, direct damage to the thyroid cells occurs and the extent of this is related to the dose of radiation. The iodine trapping capacity of the normal gland is not, in fact, unduly sensitive to irradiation but the high mitotic index of toxic glands may render them more sensitive (Greig et al, 1965). By contrast, late hypothyroidism is not so clearly related to the dose of ^{131}I and may relate more to the natural history of Graves' with autoimmunity playing a part. It is by no means certain however, that early hypothyroidism is only due to irradiation changes which are often unimpressive when the glands are examined histologically (Dailey, Lindsay & Miller, 1953) and immunological factors may again be important. Certainly, increases in the titres of antithyroid antibodies after ^{131}I are well documented and may influence the ultimate prognosis.

Increased levels of thyroid cytoplasmic antibodies after ^{131}I therapy have been documented by several groups (Buchanan et al, 1962; O'Gorman, Staffurth & Ballentyne, 1964; Einhorn et al, 1965). The latter authors found no difference in the titre of anti-thyroglobulin antibodies, although this has been reported by at least two other groups (Burke, 1967; Kriss et al, 1967). Thyroid antibodies did rise after therapy in the study of Jonsson et al (1968), there was no increase in parietal cell antibodies, antinuclear factor or antibodies to gamma globulin and irradiating the uterus produced no change in antithyroid antibodies. In the study by Irvine et al (1964), the major rise in the average titre of antithyroid antibodies in a series of thyrotoxic patients treated with ^{131}I was due to those

patients who were already thyroid antibody positive before therapy. The toxic gland may be more sensitive to the effects of irradiation but rises in both anti-cytoplasmic and anti-thyroglobulin antibodies were found when euthyroid subjects were given ^{131}I for nodular goitre, cardiac disease or residual carcinoma after surgery (Einhorn, Fagraeus & Jonsson, 1966). In an in vitro study, Einhorn et al (1971) have shown that lymphocyte stimulation by thyroglobulin is enhanced at six to twelve weeks after ^{131}I . When lymphocytes from Hashimoto patients are cultured with previously irradiated lymphocytes, there is an enhanced antibody production, even though the irradiated cells on their own show diminished antibody secretion (McGregor et al, 1979b; 1979c). This may be due to selective depletion of suppressor lymphocytes by the radiation.

Several groups have reported increases in LATS after radioactive iodine therapy (Pinchera et al, 1965; Kriss et al, 1967; Pequegnat et al, 1967) although these rises are usually transient (Lipman, 1967) and are not found in patients treated for toxic adenomas (Pinchera et al, 1969). Wasnich et al (1969) reported seven patients developing exophthalmos after external neck irradiation for non-thyroidal neoplasms. All had residual thyroid function, indeed four became thyrotoxic and all developed thyroid autoantibodies (including LATS in three cases). None of these patients had previous evidence of thyroid dysfunction. Volpe et al (1969) did not detect any increase in LATS in thirteen patients treated with large doses of radioactive iodine, but they had all been treated with ^{131}I one to eight years previously and were selected for the study because of their persistently elevated LATS following this first treatment. Martin et al (1972) similarly did not find any difference in LATS after

therapy, in agreement with studies by McKenzie (1965; McKenzie & McCullagh, 1968) although in the latter studies persistent LATS long after therapy was found to be quite common. Increases in both the level and the incidence of TSH binding inhibitory antibodies have been shown to occur after ^{131}I therapy (Fenzi et al, 1979; Teng et al, 1980), while Beck & Madsen (1980) showed that adenylate cyclase stimulating antibodies increased, with some patients who were initially negative becoming positive.

Compared to the work done on predicting the response to antithyroid drug therapy, relatively little has been published relating the relapse or hypothyroid rate after ^{131}I to immunological tests. Greer and Wilson (1964) found no correlation in their large series between the ultimate outcome and serological tests or their changes with therapy. Einhorn et al (1965), on the other hand, did find a relationship between the expression of thyroid cytoplasmic antibodies after therapy and the occurrence of permanent hypothyroidism. In 1973, Lundell and Jonsson reported that, in a follow-up study of 188 patients, hypothyroidism occurred in 52% of patients with cytoplasmic antibodies compared to only 21% of those without antibodies. An increase in cytoplasmic antibodies was frequently recorded at two to twelve months after treatment but this did not apparently relate to the development of hypothyroidism except in those patients who were initially negative for antibodies. Anti-thyroglobulin antibodies were also associated with hypothyroidism after ^{131}I but hypothyroidism was not more common in those who only expressed these antibodies after treatment. A survey of thyroid function in over 5000 adults in east Finland actually suggested thyroglobulin antibodies may be protective as far as the effects of ^{131}I on the thyroid are concerned (Einhorn et

al, 1966), since in euthyroid patients after treatment, the mean TSH was lower in patients with anti-thyroglobulin than in those without. This was not however borne out by a later study published by the same authors (Einhorn et al, 1966b) where 72 patients were examined at one to ten years after therapy: five out of nine patients with a raised TSH were anti-thyroglobulin antibody positive compared to only nine of the remaining 63 with normal TSH.

Direct evidence that TSH receptor antibodies are related to the prognosis after ^{131}I was provided by the study of McGregor et al (1979a). Most of their patients showed an increase in TBII after therapy and also responded biochemically within three months. The three patients who had increased TBII and were still toxic at three months were controlled without further treatment by six months. Of the five cases still toxic at six months, four showed a decrease in TBII while the level was unchanged in the fifth. Beck et al (1982) demonstrated that the binding inhibitory antibodies produced after ^{131}I might be non-stimulatory to the thyroid by showing that a dissociation existed after therapy between TBII and adenylate cyclase stimulating activity. TBII were higher both before and after treatment in patients developing hypothyroidism. Davies, Platzer and Farid (1982) found a strong relationship between TBII and the response to ^{131}I with HLA-DR typing also helping to predict the response: 23 out of 43 patients studied were TBII positive and eighteen of them had responded to therapy by three months, compared to only 4 out of 20 TBII negative patients. Ten of the 16 (62%) TBII negative patients who failed to respond were HLA-DR3 positive compared to 25% of the general population.

If autoantibody production can be causally linked to the response to radioactive iodine, then pretreatment with antithyroid drugs might be expected to reduce the response rate in view of their immunosuppressive actions. Early studies with methylthiouracil did indeed show a reduced one dose cure rate even though the effect on iodide trapping by the gland was similar to that which could be achieved with potassium perchlorate although the latter drug did not affect the response rate. This effect was not seen with carbimazole pretreatment (Goolden & Fraser, 1969) and it was suggested that the SH group in methylthiouracil might act as a free radical scavenger. However, propylthiouracil which does not contain a sulphhydryl group also reduces the response rate when it is given prior to ^{131}I (Einhorn & Saterborg, 1962). In spite of the reduction in the one dose cure rate with some antithyroid drugs, their use has not been associated with a reduction in the incidence of hypothyroidism and the effect of antithyroid drugs given after radioactive iodine on the response would make an interesting study.

II) SURGERY

Partial thyroidectomy is an acceptable form of treatment for many patients with Graves' disease. There is a low incidence of complications and a low recurrence rate (Michie, 1975; Blichert-Toft et al, 1977; Toft et al, 1978). The most important factor in the development of post-operative hypothyroidism is the amount of functioning thyroid tissue left after surgery. the incidence of hypothyroidism after partial thyroidectomy varies in published series between 6% and 17% (McNeill & Thompson, 1968; Griffiths et al, 1974;

Evered et al, 1975; Michie, 1975; Toft et al, 1978). A higher incidence has been reported in two series, however: Hedley et al (1970) found 36% of their patients to be hypothyroid after surgery while Michie, Pegg and Brewster (1972) reported an incidence of 49%. The former study was a retrospective one covering a long period of time and many of the operations had been performed before the importance of remnant size was recognised. In the second study, this factor became obvious in the course of the project and clinical practice was modified accordingly.

Thyroid histology also has an influence on the outcome after operation. Patients with nodular goitres have less tendency to become hypothyroid. For example, Lundstrom et al (1977) found a negligible incidence of hypothyroidism after surgery for nodular goitre, compared to 15% in patients with diffuse goitre, while Heiman et al (1962) had reported the incidence of post-operative hypothyroidism in these two groups to be 1% and 5% respectively. Presumably areas of the gland which are suppressed in the presence of autonomous nodules respond to the high TSH after operation and recover functionally.

Temporary hypothyroidism in the months after operation is extremely common (Toft et al, 1978). Even if the patient is not clinically hypothyroid, elevation of the TSH and decrease of thyroid hormones is frequently found. Evered et al (1975) discovered 65% of their euthyroid patients after operation to have a high TSH. These changes usually resolve and relate poorly to the development of permanent hypothyroidism (Lundstrom et al, 1978) although the high TSH may persist for many years in clinically euthyroid patients (Hedley et

al, 1971). There is not a cumulative increase in the prevalence of hypothyroidism in the years after treatment as is found with radioactive iodine therapy.

There is a well recognised association between inflammatory changes in the thyroid and post-operative hypothyroidism (Whitesell & Black, 1949). In an impressive study, Green (1950) demonstrated lymphocytic infiltration in 44 out of 161 thyroidectomy specimens. Twenty of these patients became hypothyroid after operation while none of the 117 patients with no lymphocytic infiltration did so. Similar findings have been reported by Levitt (1951) and by Young, Beck and Michie (1975). It is well known that lymphocytic infiltration of the thyroid, particularly when germinal centres have formed, is associated with circulating anti-thyroid antibodies (Goudie et al, 1959; Schade et al, 1960). Irvine and his colleagues (1962; 1967) have reported on the value of circulating antithyroid antibodies in predicting post-operative hypothyroidism even though they appear to have no comparable value after ^{131}I . Finally, van Welsum et al (1974) also found circulating cytotoxic antithyroid antibodies to be more common in those patients who became hypothyroid after surgery and their presence related to plasma cell infiltration of the gland but there was no difference in the extent of lymphocytic infiltration of the gland in hypothyroid and euthyroid subjects following thyroidectomy. They concluded that B cells and the humoral immune response were important in the development of post-operative hypothyroidism.

Although both anti-cytoplasmic and anti-thyroglobulin antibodies may persist in the serum after operation, they both generally decline within one year of treatment (Einhorn et al, 1965; Gordin, 1973). LATS may persist for many years after surgery (McKenzie, 1965) but this is probably related to underlying disease activity, with thyrotoxicosis being prevented by the reduced mass of functioning thyroid tissue, rather than to destruction of tissue caused by the operation. LATS-Protector usually declines to normal within one year of operation and rises in this activity are not seen following operation (Hardisty, Talbot & Munro, 1981). The incidence of binding inhibitory antibodies in the year following operation is low (17% in the series of Mukhtar et al, 1975) when compared to the incidence in untreated cases or those in the months after radioactive iodine therapy. This finding was confirmed by Teng et al (1980) who found three out of seven TBII-positive patients developed recurrent thyrotoxicosis. Clearly, TSH receptor antibodies after surgery better reflect the activity of underlying disease than they do after ^{131}I and can thus be reliably used to predict the recurrence of thyrotoxicosis.

RESULTS

A: CHANGES IN TBII FOLLOWING RADIOACTIVE IODINE THERAPY

Eighteen patients with Graves' disease were followed up at intervals after ^{131}I therapy for up to two and a half years. There were two men and sixteen women in the study and the mean age was 46.8 years (SD

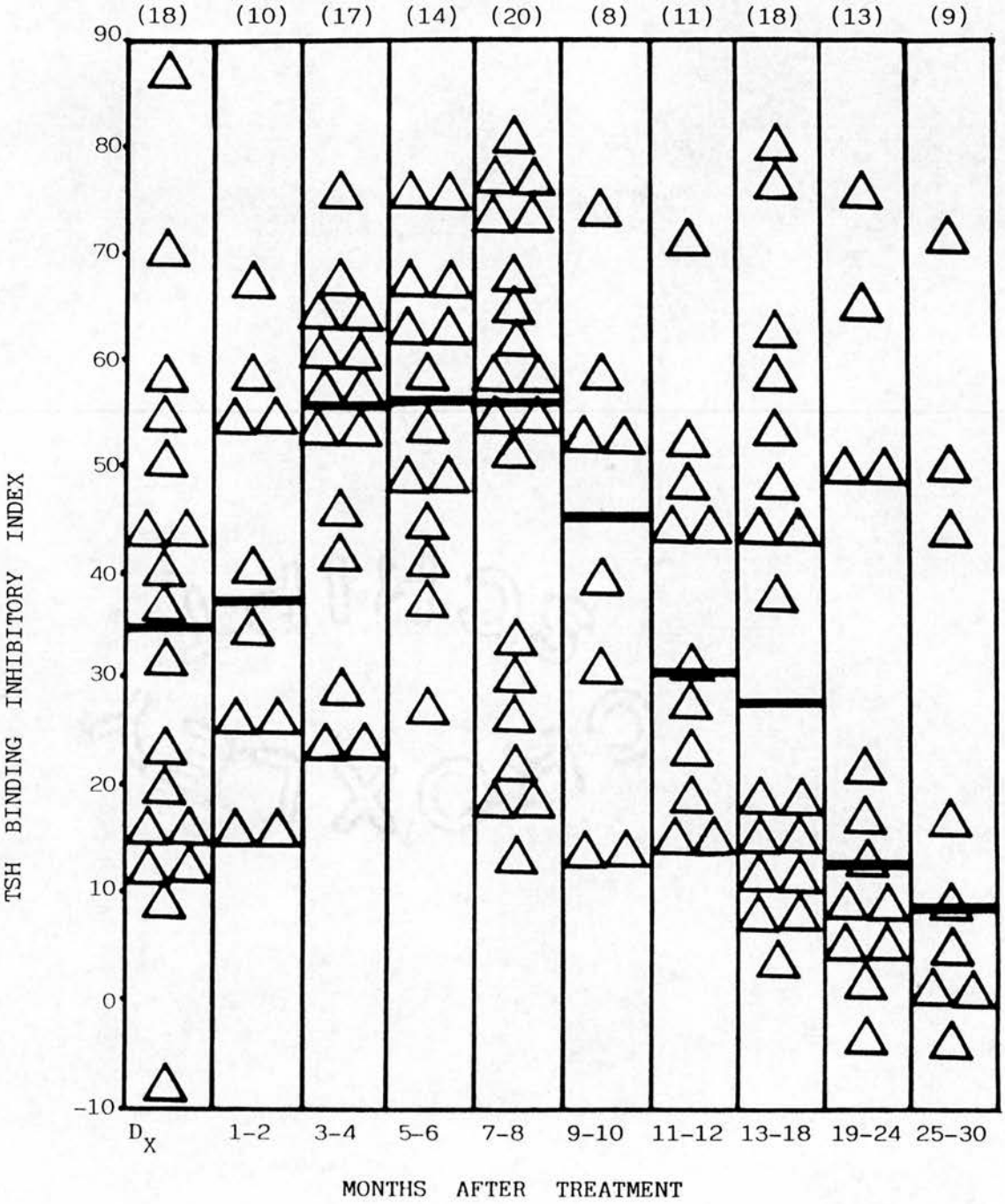
7.1). Mean T_3 was 6.9 nmol/l (SD 2.18) and the mean T_4 was 236 nmol/l (SD 61.1). Blood was withdrawn from each patient prior to radioactive iodine therapy and thereafter at least five sequential samples were collected from each patient. TSH receptor antibodies were measured with the radioreceptor assay on neat serum. The dose of ^{131}I varied from patient to patient depending on goitre size, radioactive iodine uptake and the severity of thyrotoxicosis, the mean dose for the group being 7.7 mCi (range 5 to 15 mCi).

The results are shown in FIGURE 3.5 along with the number of samples collected at each time interval. The median TBII at diagnosis was 35.0, and this was not markedly different at 1 - 2 months (37.5) but had risen to over 55 by 3 - 4 months from treatment. This rise in the median TBII was sustained until after eight months when it began to decline although it did not reach normal levels until two years after treatment.

B: TSH RECEPTOR ANTIBODIES AND THE RESPONSE TO RADIOACTIVE IODINE

A study was undertaken of 52 consecutive patients with mild to moderate hyperthyroidism who were treated with ^{131}I to see whether immunological tests performed at diagnosis had any bearing on the ultimate outcome. All patients had a diffuse goitre and/or scintiscan. Radioactive iodine uptake was measured four hours after an oral dose of five microCuries ^{131}I . Blood was withdrawn in all cases prior to treatment. Thyroid hormones were measured by in-house radioimmunoassay, TBII by the radioreceptor assay on immunoglobulin concentrates as described in APPENDIX 1 and other thyroid antibodies

FIGURE 3.5 CHANGES IN TSH RECEPTOR ANTIBODY LEVEL FOLLOWING RADIOACTIVE IODINE THERAPY



were assayed by tanned red cell haemagglutination using commercially available kits (Wellcome). Six patients were excluded from the study because they had severe thyrotoxicosis (mean T_3 8.4 nmol/l), large goitres and a high uptake of ^{131}I (mean uptake 77%). The criterion for exclusion was a four hour radioactive iodine uptake above 70%. Such patients are well known to respond less well to radioactive iodine, and since they would in any case be closely followed up prediction of their outcome is of less importance. For the 52 patients included in the study, only their thyroid status at the end of the six month follow up period was considered. Temporary hypothyroidism occurring in the few months after therapy was disregarded and only biochemically confirmed hypothyroidism after six months was deemed to be permanent. Where patients did not respond to the initial dose of ^{131}I , they were treated with a further dose but their subsequent response was not included in the study. The dose of radioactive iodine varied from patient to patient as described above and depending on the need for other medical reasons to control thyrotoxicosis urgently. The dose varied between five and fifteen milliCuries.

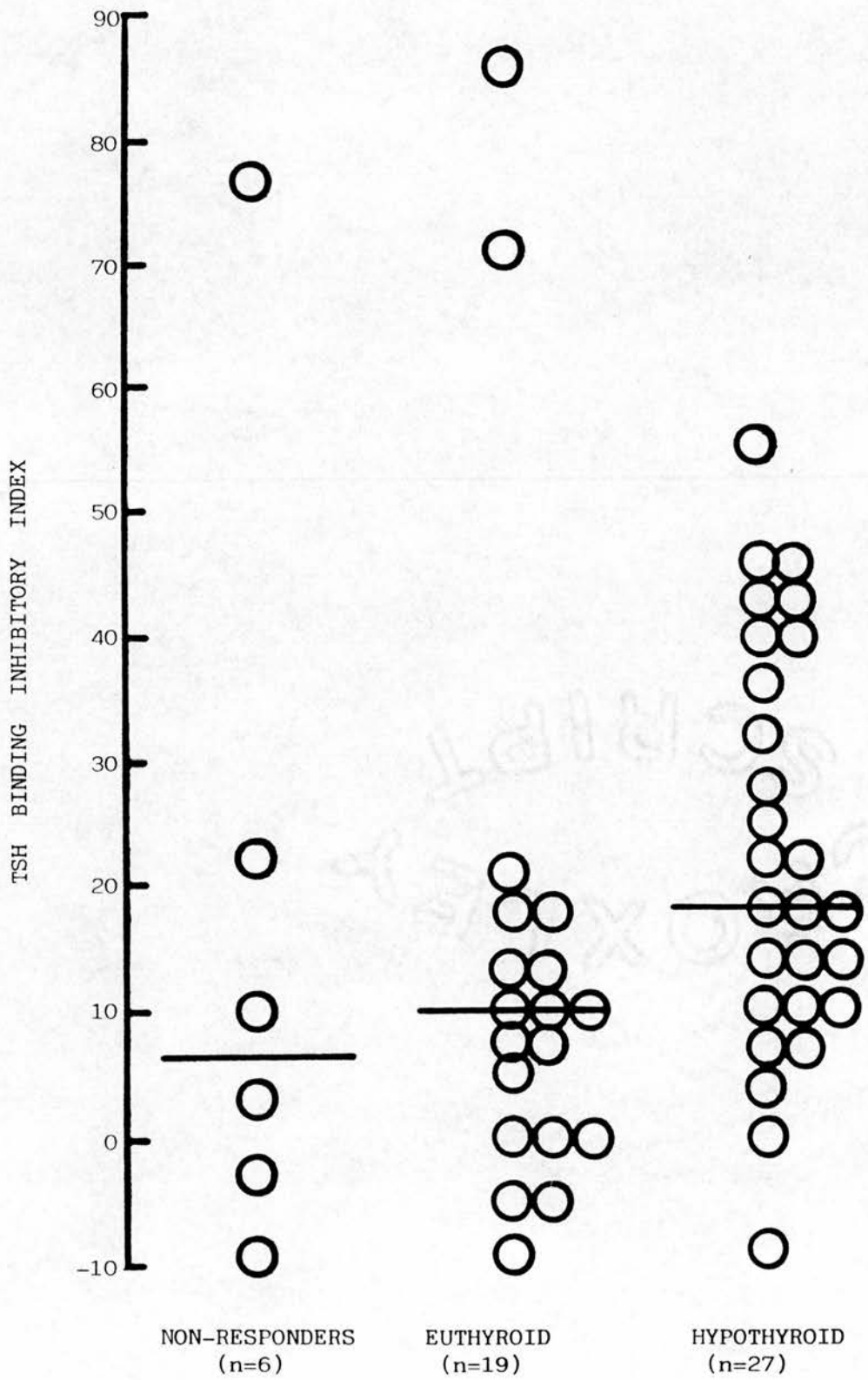
Of the 52 patients in the study, six did not respond to their initial dose of ^{131}I and required further treatment, while 19 of the remainder were euthyroid and 27 hypothyroid at the end of six months. The mean dose of ^{131}I in the three groups was 8.75 mCi, 7.0 mCi and 7.1 mCi respectively (none of these differences was statistically significant). The mean T_3 for the responders was 4.8 nmol/l compared to 5.8 nmol/l for the euthyroid patients and 5.4 nmol/l for the hypothyroid patients (nil significant), while the T_4 in the three groups was 232, 237 and 208 nmol/l ($p < 0.05$ for the euthyroid Vs

hypothyroid). Four hour ^{131}I uptake for the three groups was 50%, 51% and 46% respectively (nil significant). The levels of TSH binding inhibitory immunoglobulins in the non-responders, euthyroid and hypothyroid patients is shown in FIGURE 3.6. The median value for the six non-responders was 6.5, compared to 10 for the euthyroid cases and 18 for the hypothyroid cases. Only the difference between the euthyroid and the hypothyroid cases was significant ($p < 0.01$, Wilcoxon Rank Sum Test) and there was a significantly progressive rise of TBII with increasing response to radioactive iodine ($p < 0.01$, Jonckheere Monotonic Trend Test for Independent Variables).

Thyroid microsomal antibodies were found in all six of the patients who did not respond to their first dose of ^{131}I , in 14 of the 19 patients who were euthyroid at the end of six months and in 15 of the 27 patients hypothyroid after six months (not significant). All of the eight hypothyroid patients who were TBII negative were also negative for thyroid microsomal antibody ($p < 0.001$, Chi Squared Test with Yates' Correction). Anti-thyroglobulin antibodies were found in nine patients overall, and seven of them were euthyroid at the end of the six month follow up period ($p < 0.01$). Of the six patients excluded from the study because of the severity of their thyrotoxicosis, three were euthyroid after six months, two hypothyroid while one required yet a further dose. All three euthyroid patients had circulating antithyroglobulin antibody but none of the other three patients was positive.

The difference in the response of TBII to ^{131}I between nine consecutive patients becoming hypothyroid and seven who were euthyroid after six months was studied. The hypothyroid group

FIGURE 3.6 TSH RECEPTOR ANTIBODIES AND THE RESPONSE TO THERAPEUTIC DOSES OF RADIOACTIVE IODINE

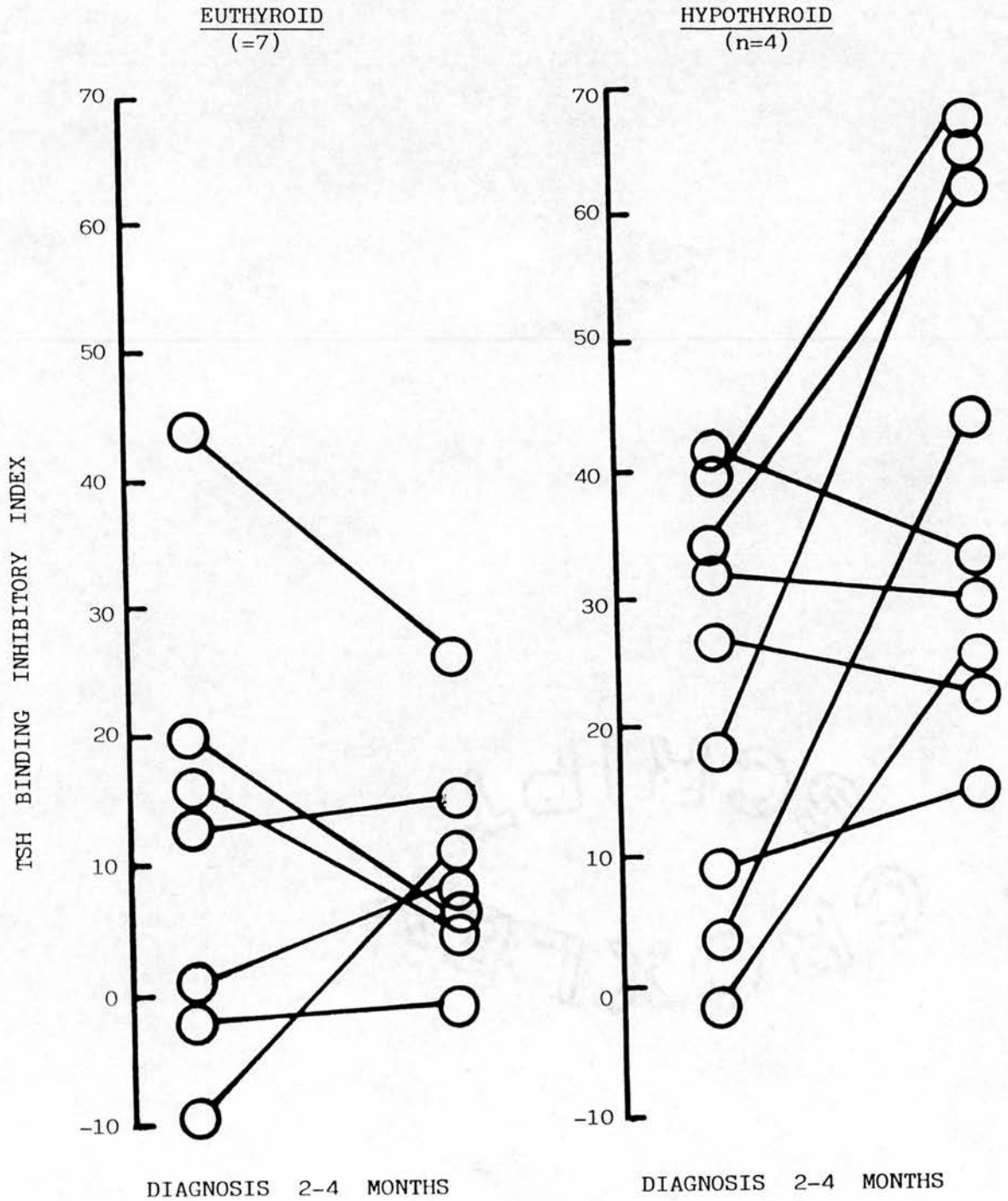


consisted of eight women and one man, mean age 45.2 years (SD 5.9) with a mean T_3 at diagnosis of 5.9 nmol/l (SD 0.7) and a mean T_4 of 213 nmol/l (SD 40.4). The euthyroid group were two men and five women, mean age 46.0 years (SD 10.7) and a mean T_3 of 5.2 nmol/l (SD 1.2) and a T_4 of 218 nmol/l (SD 46.8). Samples of serum were studied before treatment and at two to four months after therapy. All measurements were performed as described above.

The changes in the two groups with therapy are shown in FIGURE 3.7. In the euthyroid patients, the median TBII at diagnosis was 13.4 decreasing to 8.6 after ^{131}I (not significant). Three patients showed a marked decrease in TBII, two increased but not outwith the normal range and the other two did not change substantially. The median TBII in the hypothyroid group was 26.7 at diagnosis rising to 33.1 after ^{131}I ($p < 0.05$, Wilcoxon Rank Sum Test for Paired Samples). Six of these patients showed a marked increase in TBII and the other two did not change although they had initially been very high. After therapy, the titre of microsomal antibodies increased in seven of the hypothyroid patients and was the same in two as it had been before treatment. The euthyroid group's response was much more variable with two increasing, three decreasing and two staying the same.

Two patients who had previously been treated with carbimazole but developed a recurrence were treated with radioactive iodine. Both had high levels of TBII at diagnosis but remained euthyroid after ^{131}I , thus not conforming to the pattern described above for newly diagnosed Graves' patients:-

FIGURE 3.7 TSH RECEPTOR ANTIBODIES FOLLOWING RADIOACTIVE IODINE
IN PATIENTS RENDERED EUTHYROID AND HYPOTHYROID



CASE HISTORY 3.

A 42 year old lady was treated with carbimazole between June 1979 and May 1981. She was euthyroid thereafter until August 1982 when she developed recurrent thyrotoxicosis with a T_3 of 4.2 nmol/l, a T_4 of 164 nmol/l and a flat TRH test. She had a large goitre and the four hour ^{131}I uptake was markedly elevated at 63%. Ten milliCuries of ^{131}I were administered and she became biochemically and clinically euthyroid and remained so until June 1983 when she was lost to follow up. At the time of her recurrence, TBII was 10.9 and microsomal antibodies were detected at a titre of 40^2 . Three months after therapy, TBII had risen to 50.2 and microsomal antibodies to 160^2 .

CASE HISTORY 4.

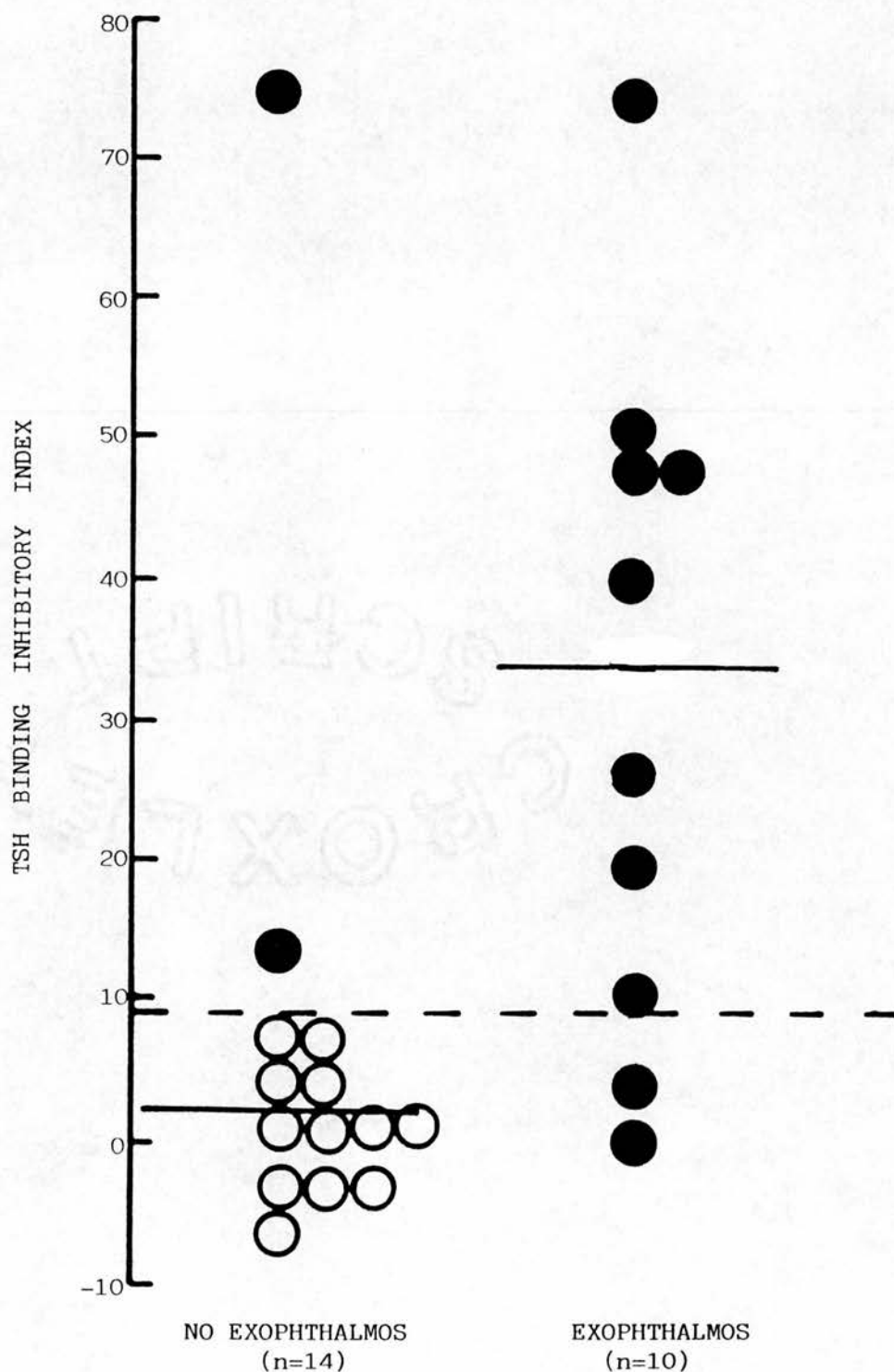
A 43 year old lady relieved carbimazole between June 1978 and June 1979. In January 1980, she had a mild recurrence of thyrotoxicosis and was treated with 5 mCi of ^{131}I . She was euthyroid after this until September 1982 when thyrotoxicosis again developed (T_3 4.1 nmol/l, T_4 200 nmol/l and ^{131}I uptake 68%). At the second recurrence she had a small goitre and moderately severe bilateral exophthalmos, the TBII was 69.7 and the microsomal antibody titre was 80^2 . Four months later, the TBII was 63.8 and the microsomal antibodies 320^2 . She was still euthyroid and had high TBII in October 1984, 25 months after the second dose of radioactive iodine.

C: PERSISTENCE OF TSH RECEPTOR ANTIBODIES LONG AFTER ^{131}I THERAPY

It is clear from the data presented in FIGURE 3.5 that TSH receptor antibodies not only rise after radioactive iodine therapy but may persist in the circulation for a considerable time. TBII were measured on neat serum from 24 unselected patients followed up long term after ^{131}I therapy for Graves' thyrotoxicosis (mean period 56.8 months, range 27 to 156 months). Significant levels of TBII were found in ten cases and the persistence of TBII was strongly correlated with the presence of exophthalmos at the time of follow-up (see FIGURE 3.8). Ten patients in the study had clinically detectable exophthalmos (two men and eight women treated an average of 52 months previously with a mean dose of 7.0 mCi, range 5 to 15 mCi). The fourteen patients without exophthalmos consisted of four men and ten women treated on average 60.1 months before with a mean dose of 10.5 mCi (range 5 to 30 mCi).

TSH receptor antibodies were detected in eight of the ten exophthalmic patients with a ^{median} value of 33.5 while only two of the patients without exophthalmos had TBII, the median value for this group being 1.5 ($p < 0.01$, Mann-Whitney U Test). The two patients who were TBII positive but did not have exophthalmos both had evidence of other autoimmune disease: the first was a man in his late 50s who was treated with 15 mCi in November 1980 and followed up until 1984 by which time he had developed seropositive rheumatoid arthritis and had a TBII of 12.7. The second was a lady treated with 5 mCi in January 1976 (then aged 52) and was followed up until June 1983 (TBII 74.1). During the follow-up period she developed an illness with

FIGURE 3.8 PERSISTENCE OF TSH RECEPTOR ANTIBODIES LONG AFTER RADIOACTIVE IODINE THERAPY: RELATION TO EXOPHTHALMOS



N.B. Filled circles in patients without exophthalmos indicates the presence of other autoimmune disease (see text).

polyarthralgia and severe Raynaud's phenomenon, associated with high ESR and high levels of circulating immune complexes (C1q solid phase).

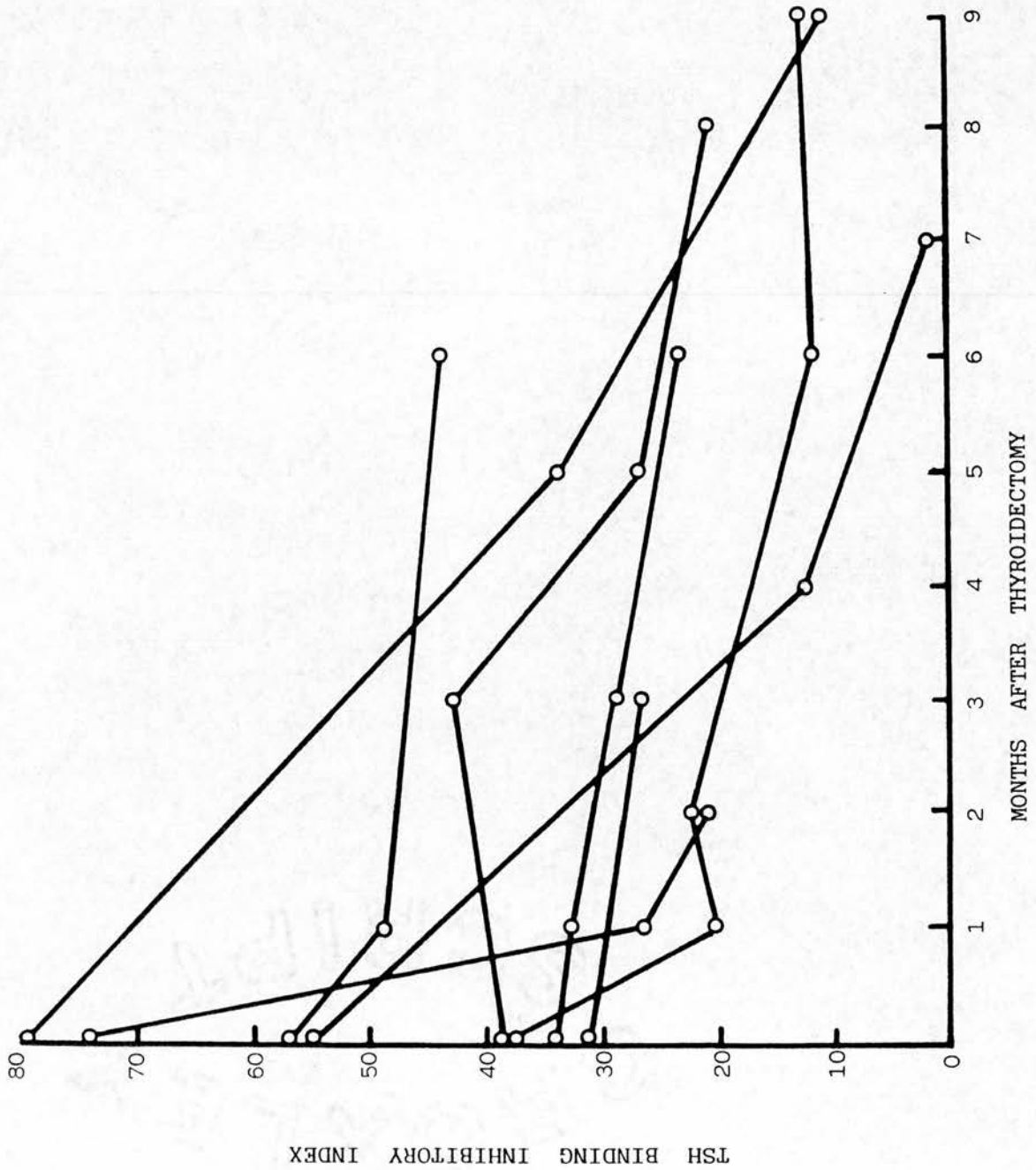
D: TSH RECEPTOR ANTIBODIES FOLLOWING PARTIAL THYROIDECTOMY

TBII data from eight female patients (mean age 29.1 years, SD 5.95, range 18 to 40 years) following partial thyroidectomy is shown in FIGURE 3.9. In all eight cases there was a decline in TBII from pre-operative levels within a few months. All patients had been rendered euthyroid prior to operation with carbimazole and all were treated with potassium iodide and propranolol in the immediate pre-operative period. They were all euthyroid during the nine month follow-up period shown in Figure 3.9. One further patient (a twenty year old female discussed in detail in Chapter 6) was negative for TBII prior to operation but TBII appeared in the serum subsequently and was associated with thyrotoxic relapse. Where data was available, TBII declined to normal usually within one to two years after operation but persistent TBII were found in two cases:-

CASE HISTORY 5.

A 39 year old lady presented in September 1981 with thyrotoxicosis having had a small diffuse goitre for five years previously. Carbimazole was started, but the goitre increased markedly in size and she therefore underwent thyroidectomy in January 1982. Six months later she developed severe bilateral exophthalmos and TBII was

FIGURE 3.9 TSH RECEPTOR ANITBODIES IN THE MONTHS AFTER PARTIAL THYROIDECTOMY



positive at 52.1 (immunological tests had not been performed at initial diagnosis). TBII was still positive two years after operation at 10.9. She is HLA-DR 2 positive.

CASE HISTORY 6.

A 41 year old lady had a partial thyroidectomy in November 1976 having been treated with carbimazole for fourteen months previously. there was a moderate sized diffuse goitre before surgery and marked bilateral exophthalmos. TBII was 40.8 prior to operation and she was HLA-DR 3+4. Over eight and a half years later in February 1984, she was still TBII positive at 33.5 and her severe exophthalmos persisted.

DISCUSSION

Carbimazole treatment for Graves' thyrotoxicosis produces symptomatic and biochemical control within one to two months. It also lowers TSH receptor antibody and thyroid microsomal antibody levels although the time course of this is much slower, suggesting that the primary action of the drug is antithyroid rather than immunosuppressive. One of the problems in accepting that the immunosuppressive action is important in vivo relates to the concentration of the drug required to produce these effects. The thyroid is the major site of thyroid autoantibody production (McLachlan et al, 1979) and antithyroid drugs are concentrated in the thyroid. When ³⁵S-labelled carbimazole is administered, its metabolite methimazole accumulates in the thyroid (Marchant et al, 1972) and, similarly, methimazole

itself and propylthiouracil accumulate in the thyroid when given systemically (Lazarus et al, 1975). These studies showed a fairly low plasma to tissue ratio of the drug, but in an even more recent study using a highly sensitive gas chromatography-mass spectroscopic analysis which showed that the ratio could be as high as 60:1 (Jansson et al, 1983), the estimated concentration in the thyroid was not as high as that required to produce some of the in vitro immunosuppressive effects. The major effect of antithyroid drugs on the immune system may be mediated via accessory cells which could become depleted in the preparation of mononuclear cells for in vitro studies. The effect of these drugs on antigen presentation by the thyroid itself, which is after all the major site of drug accumulation, has not yet been considered in the published literature.

By the end of a twelve to eighteen month course of drug treatment, TBII is usually normal and if thyrotoxicosis recurs it is generally associated with a further increase in TBII. Persistently high TBII in the months after a course of carbimazole is not invariably associated with thyrotoxic relapse - two such cases have been presented where they remained euthyroid. The response of the thyroid to stimulatory antibodies may have been limited by concurrent thyroiditis causing destruction of functioning gland tissue or, alternatively, the antibodies detected in the radioreceptor assay in these patients' sera may have been receptor blocking antibodies rather than stimulatory ones. A patient was described with a self-limited recurrence of thyrotoxicosis which did not require therapy. The TBII were very high in this case, yet the response of the thyroid was clearly limited. The optimal duration of drug therapy is

controversial, but in one case treated for four and a half years, thyrotoxicosis recurred immediately carbimazole was stopped. This patient was a DR3 homozygote and such patients frequently require definitive treatment in the form of thyroidectomy or radioactive iodine although the role of HLA typing in predicting the likelihood of relapse has recently been challenged (see Chapter 6). It seems however that immunological factors are certainly important in determining the natural history of Graves' even if DR phenotype is a poor marker: in toxic multinodular goitre, where the thyrotoxicosis is probably not immunologically mediated, the therapeutic response to antithyroid drugs is good but there is a high relapse rate on stopping the drug in contrast to cases of thyrotoxic Graves' disease where the immunological abnormalities have normalised during drug treatment.

After therapeutic doses of radioactive iodine there was a transient rise in TBII, in keeping with published data. The median value for TBII in a follow up study of eighteen patients took two years to return to normal and, even then, some patients still had high values. Persistent TBII was found in ten out of 24 patients tested at random 27 to 156 months after therapy although none of these patients was thyrotoxic. The persistence of TBII after ^{131}I therapy strongly correlated with the presence of exophthalmos or other autoimmune diseases. In untreated Graves' patients, the levels of TBII do not correlate with the presence or severity of eye disease although persistent LATS after ^{131}I therapy has previously been associated with exophthalmos (Lipman, 1967). A role for LATS in the pathogenesis of Graves' eye disease has been suggested: for example, the study of Wasnich et al (1979) where seven patients became exophthalmic and

developed LATS after external neck irradiation for non-thyroidal neoplasms. Generally, however, thyroid stimulating antibodies are not thought to have any direct role in the production of eye changes in Graves' disease. In spite of the rise in TBII after ^{131}I , there is no difference between the incidence of exophthalmos after this form of therapy and that after surgery (Hamilton et al, 1967).

TBII may have a part to play in predicting the response to radioactive iodine. In a six month follow-up study of 52 patients after therapeutic doses of ^{131}I , the median TBII was 6.5 in six non responders compared to 10 in 19 euthyroid patients and 18 in 27 hypothyroid patients. TBII positive patients are more likely to respond to ^{131}I and are more likely to develop hypothyroidism. TBII negative patients who became hypothyroid were also negative for microsomal antibodies suggesting that factors other than autoimmunity are involved or that these patients may have had a non-immunologically mediated thyrotoxicosis. Seven out of the nine patients with anti-thyroglobulin antibodies remained euthyroid after therapy ($p < 0.01$). This may reflect higher levels of thyroglobulin in these patients - thyroglobulin can interfere with the binding of TSH to its receptor as discussed in Chapter 2, this may prevent a pathogenetic effect of receptor antibodies in post-iodine hypothyroidism.

The response of TBII to ^{131}I was different in patients becoming hypothyroid to those who remained euthyroid, the former patients showing a greater rise in TBII after therapy, suggesting that autoantibodies to the TSH receptor might cause hypothyroidism. Radioactive iodine therapy thus might be an important model for the

study of immunologically mediated thyroid damage. Where the thyrotoxicosis treated with radioactive iodine was recurrent, having been previously treated with carbimazole, TBII was high in two patients after ^{131}I although both remained euthyroid after ^{131}I . TSH receptor blocking antibodies would be of interest to study in these patients since such antibodies might protect the thyroid cell from an immunological reaction directed at the TSH receptor.

After surgery, TBII declined in all eight patients studied during a nine month follow-up period. Values for TBII were nearly always normal in patients studied within one to two years after operation (data not presented) but two cases are reported where persistent TBII long after operation were associated with Graves' eye disease. It would seem that patients with multiple autoimmune diseases are more likely to become sensitised immunologically to thyroid cell damage caused by destructive antithyroid therapy.

Chapter Four

Heterogeneity

Within Graves' Disease

SUMMARY

A series of 139 consecutive thyrotoxic patients with Graves' disease (flat TRH test, raised T_3 and/or T_4 , diffuse goitre and/or scintiscan) was subdivided as follows: Group A - 89 patients with goitre and high ^{131}I uptake (over 40% at four hours). Group B - 21 patients with goitre but lower ^{131}I uptake (under 40%). Group C - 29 patients with no detectable goitre. The mean age in Group A was 41.0 years at diagnosis compared to 51.6 and 52.6 years respectively for Groups B and C. The mean T_3 for Group A was 6.6 nmol/l compared with 4.2 nmol/l ($p < 0.001$) for Group B and 5.4 nmol/l ($p < 0.001$) for Group C. The four hour ^{131}I uptake was lower for the patients without a goitre (Group C) than for those patients with goitre (Groups A and B together) with values of 45% and 56% respectively ($p < 0.001$).

TSH receptor antibodies were detected using the binding inhibitory assay in 64/89 (72%) of Group A, 9/21 (43%) of patients in Group B and 9/29 (31%) of Group C. The median inhibition index in the three groups was 24.8, 12.0 and 8.4 respectively. Titres of thyroid microsomal antibody were significantly lower in Group C (no goitre) than in group A ($p < 0.02$). In Group B, 9/21 patients had parietal cell antibodies (42.9%) compared to only 4/89 (10.1%) of group A ($p < 0.001$). The total number of patients in group B with non-thyroidal antibodies was 12/21 (57%) as against 14/89 (15.7%) for group A.

It is concluded that patients with Graves' disease but no goitre differ from the majority of Graves' patients in that they are older, they have less severe thyrotoxicosis and lower titres of thyroid antibodies including TSH receptor antibodies. Patients with lower four

hour ^{131}I uptake but with a goitre are also older and less toxic but have a remarkable incidence of non-thyroidal autoantibodies. These subgroups may represent different disease states or simply an incomplete expression of Graves' disease, possibly due to age-related changes in autoimmune phenomena.

INTRODUCTION

There is evidence for clinical and immunological heterogeneity in a number of autoimmune diseases and in the polyendocrine deficiency syndromes. For example, in Addison's disease (primary adrenocortical failure) due to autoimmune destruction of the adrenal glands, not all patients have circulating anti-adrenal antibodies and not all patients show the recognised association with the HLA B8/DR3 axis (see Chapter 6). On the basis of the association with other organ specific autoimmune diseases, it has been suggested that two different subgroups of Addison's patients may exist (Neufeld et al, 1979) and from this observation has developed the classification of the polyendocrine deficiency syndromes (for review, see Loriaux, 1985). In Type I syndrome, adrenocortical failure is associated with hypoparathyroidism and mucocutaneous candidiasis and, in a lesser proportion of patients, with alopecia, pernicious anaemia, malabsorption and chronic active hepatitis. The syndrome typically begins in late childhood and has no recognised HLA association. In Type II syndrome (Schmidt's syndrome), Addison's disease is found in association with autoimmune thyroid disease and insulin dependent

diabetes mellitus. Patients show an increased incidence of HLA-B8 (Eisenbarth et al, 1978). Gonadal failure and vitiligo may occur in both types of polyendocrine deficiency syndrome.

The clinical syndrome of myasthenia gravis occurs in patients with differing thymic pathologies and this relates to the expression of autoantibodies and to the presence of HLA markers (Newsom-Davis et al, 1982). The small group of patients with thymoma typically presents in the fourth or fifth decade of life and there is an equal sex incidence. High circulating levels of acetylcholine receptor antibody are found and there is antibody to striated muscle in 90% of cases but no clear HLA association exists. Amongst patients with thymic hyperplasia, females predominate and the disease usually comes on before the age of forty years, there are moderately high levels of acetylcholine receptor antibody and an association with the HLA-B8/DR3 axis (Pirskanen, 1976). Patients with thymic involution usually present in later life, with males and females equally affected, low levels of acetylcholine receptor antibody in the circulation and a possible association with HLA-DR2.

In type I diabetes mellitus, there is no definite evidence for disease heterogeneity based on HLA typing studies (for review, see Farid & Bear, 1981) except that those patients with coincident thyroid disease show a stronger than expected association with HLA-B8, marked female preponderance, onset in later life and a strong family history of autoimmune disease (Bottazzo et al, 1978). Cudworth suggested that heterogeneity may exist with respect to aetiology: Patients with purely virus induced insulinitis would be younger, with equal sex distribution and only transient islet cell antibody

production around the time of onset, while those with a predominant autoimmune aetiology who would have a wider age distribution at diagnosis, be mainly female, tend to have persistent islet cell antibodies and have a strong association with other autoimmune diseases (Cudworth et al, 1980). Undoubtedly there is a spectrum with some patients having entirely virus induced disease at one end and, at the other end, patients in whom the islet cell damage is due entirely to a primary autoimmune attack. In many patients it may well be a combination of the two which gives rise to diabetes mellitus.

There is only very limited evidence for environmental factors being important in the induction of Graves' disease. Joassoo et al (1975) have found an increased incidence of antibodies to influenza B and mumps viruses in Graves' patients and *Yersinia enterocolitica* type 3 infection has also been suggested as a possible trigger for the disease (Shenkman & Bottonne, 1976; Bech et al, 1977). It has been postulated by Volpe's group that environmental stress, by decreasing suppressor cell activity may, initiate some cases of autoimmune thyroid disease (Strakosh et al, 1982). Doniach (1960) has reported three cases of thyroiditis developing after mumps. If aetiological or genetic heterogeneity were to exist in Graves' disease, it may account for the variable expression of immunological markers and recognition of this could increase the usefulness of immunological tests in choosing the most appropriate therapy for a given patient and in monitoring the response to that therapy.

Two recent studies have suggested that heterogeneity may indeed exist in Graves' disease: Schleusener et al (1983) have divided 150 consecutive patients with thyrotoxicosis and a diffuse goitre into two groups. Group A consisted of 101 patients with either ophthalmopathy or circulating TSH receptor antibodies, while Group B had 49 patients with neither. Within Group A, there was the expected association with HLA-DR3 particularly in those patients with relapsing hyperthyroidism. Group B patients, however, had no increased incidence of HLA-DR3 but did have increased DR5 and in this group DR5 was found particularly in patients with a tendency to relapse after medical therapy. HLA antigens and TSH receptor antibodies may thus be completely independent markers for Graves' disease and there may be a genetically predisposed subgroup of Graves' for which TSH receptor antibodies as detected by the radioreceptor assay do not serve as a disease marker.

Stenszky et al (1983) used the mathematical technique of cluster analysis to look at 49 clinical and immunological characteristics in 196 patients with Graves' disease. They did not measure TSH receptor antibodies and only performed HLA-A and B typing but they did have information from thyroid biopsies. Patients in this study could be divided into three groups: the first group consisted of 79 patients with small goitres, low indices of autoimmunity including lymphocyte proliferative responses to thyroglobulin and a tendency to remit with medical therapy. The second group was of 29 patients with "Hashitoxicosis", having lymphocytic infiltration of the thyroid and high titres of anti-thyroglobulin antibodies. The third, and largest group, was of 84 patients with high indices of autoimmunity, high

incidence of ophthalmopathy, strong familial tendency and a high rate of relapse after medical therapy. The incidence of HLA-B8 in these three groups was 9%, 21% and 87% respectively.

The relationship between Graves' disease and Hashimoto's thyroiditis is a complex one. Both occur in patients with a similar genetic background and both share many immunological features (for review, see Kidd et al, 1982). Autoimmune ophthalmopathy may occur in Graves', in pure Hashimoto's thyroiditis or indeed in patients with entirely normal thyroids (Solomon et al, 1977). It may well be wrong, in fact, to regard Hashimoto's and Graves' as separate disease entities and perhaps they should be seen as different aspects of the same disease process causing a spectrum of clinical conditions with pure thyroid stimulation at one end and thyroid destruction by cytotoxic antibodies and T cells at the other end. In terms of treatment, it may be important to recognise the presence of thyroiditis in a patient with Graves' thyrotoxicosis since it may affect the response to radioactive iodine (Lundell & Johnson, 1973) and to surgery (Whitesell & Black, 1949; Green, 1950; van Welsum et al, 1974).

The coexistence of thyroiditis with LATS positivity has been postulated to account for those patients with exophthalmos or dermopathy and who have circulating LATS but do not become hyperthyroid (Kriss et al, 1964; Liddle et al, 1965; Adams et al, 1969). Thyroiditis was demonstrated histologically in three thyrotoxic patients with high levels of circulating antithyroid antibodies by Buchanan and colleagues (1961). These authors also described five patients with goitres and mildly elevated radioactive

iodine uptakes who were biochemically euthyroid and had chronic lymphocytic thyroiditis. Another possible source of confusion in diagnosis is subacute thyroiditis with a presumed viral aetiology where patients may develop transient mild thyrotoxicosis but soon return to euthyroidism and occasionally ultimately become hypothyroid. The ^{131}I uptake in these cases is characteristically very low. Fatourechí et al (1971) studied 24 Graves' patients with histological Hashimoto's thyroiditis and found them to be older than the average Graves' patient and they also had a particularly high incidence of post-operative hypothyroidism. Ten of these patients had either a personal or a family history of diabetes mellitus. The identification of a subgroup of Graves' associated with HLA-DR5 by Schleusener is relevant here since this marker is also known to be associated with Hashimoto's thyroiditis (Farid et al, 1980).

That hypothyroidism may develop spontaneously in Graves' disease was first recognised as long ago as 1895 by Baldwin, while Eason in 1928 demonstrated histologically that the two conditions can coexist in the same gland. The possibility of progressive thyroiditis as an important factor in the natural history of Graves disease was well demonstrated in a pathological study of over 2000 thyroids by Levitt in 1951. It is currently thought that, without destructive antithyroid therapy, about 10% of Graves' patients will become spontaneously hypothyroid (Doniach, 1981). Primary hypothyroidism is generally a permanent state but spontaneous remission has been described (Nelson & Palmer, 1975; Yamamoto & Sakamoto, 1978) and there are now a number of cases reported where primary hypothyroidism has been succeeded by thyrotoxicosis. In 1959, Joplin & Fraser reported a case in which hyperthyroidism developed in a patient with

focal thyroiditis but the first cases of thyrotoxicosis following biochemically confirmed hypothyroidism were reported in 1971 (James, 1971; Goolden et al, 1971). Since then further case reports have appeared in the literature (Gavras & Thomson, 1972; Williams et al, 1974; Bremner & Griep, 1976; Olczak et al, 1978). In some of these cases, for example, the two reported by Irvine and his colleagues, the thyrotoxicosis was quite resistant to therapy. Presumably where hypothyroidism remits spontaneously or progresses to thyrotoxicosis there is either regeneration of destroyed thyroid tissue under a trophic stimulus or the hypothyroidism was in the first place due to TSH receptor blocking antibodies rather than to autoimmune tissue destruction.

It is well recognised that thyrotoxicosis may occur in the absence of a goitre but standard textbooks of endocrinology usually state this to be a relatively uncommon occurrence although its frequency may increase with age. (Burgi & Labhart, 1974; Montgomery & Wellbourn, 1975; Ingbar, 1985). Hegedus et al (1983a) have developed the use of ultrasound to determine thyroid gland volume and have found it to relate to age, sex and body weight in normal subjects. When they studied 90 consecutive thyrotoxic patients (Hegedus et al, 1983b), they found the gland to be impalpable in 28 (31%) and normal thyroid volume was confirmed by ultrasound in 21 (23%). Thyroid volume in this study did not correlate with any measure of thyroid function or with the age of the patient. The high frequency of thyrotoxicosis with an impalpable thyroid gland has recently been confirmed by Greenwood et al (1985) who reviewed 594 consecutive cases of thyrotoxicosis. A diffuse goitre was found in 374 (63%), a nodular goitre in 110 (19%) and no goitre in 96 (16%). The proportion of

patients with nodular goitres or impalpable glands increased with age. The frequency of antithyroid antibodies was similar in all three groups and patients with no goitre had the same incidence of exophthalmos as those with diffuse goitre although there was a very low incidence in patients with nodular goitre. The frequency of post-partum thyrotoxicosis and hypothyroidism in women with subclinical autoimmune thyroid disease has recently become appreciated and many of these patients do not have goitre at the time of presentation (Amino et al, 1982). Some patients, at least, with thyrotoxicosis but no goitre therefore have evidence of an underlying autoimmune disease although they clearly lack the usual stimulus to thyroid cell growth which is found in classic Graves' disease.

The aim of the work presented in this chapter is to consider humoral immune changes in a large consecutive series of patients with thyrotoxic Graves' disease in the light of possible disease heterogeneity. In addition to patients with classical Graves' disease (goitre plus high ^{131}I uptake), two possible subgroups have been considered: Firstly, those patients without grossly elevated four hour ^{131}I uptakes since this group may include patients with coincident thyroiditis which could be of considerable prognostic importance. Secondly, patients with no palpable goitre, a presentation of Graves' disease which is now considered to be relatively common. These patients need to be included in epidemiological studies of thyrotoxicosis, and some clearly have autoimmune disease since they have an appreciable incidence of exophthalmos (Greenwood et al, 1985) but they are often excluded from studies on Graves' disease.

PATIENTS AND METHODS

PATIENTS

The study included 139 consecutive patients presenting to the Royal Infirmary, Edinburgh with thyrotoxicosis and a diffuse uptake of isotope on a technetium scintiscan. There were 124 females and 15 males with ages ranging from 15 to 78 years (mean 44.9 years). Graves' disease was diagnosed on the basis of raised T_3 and/or T_4 , a flat TRH test and a diffuse goitre and/or scintiscan. Rectilinear scanning was performed twenty minutes after the injection of one microCurie of ^{99}Tc sodium pertechnetate. ^{131}I uptake was measured four hours after an oral dose of five microCuries. The presence of goitre was assessed clinically and all patients without goitre irrespective of their ^{131}I uptake were assigned to Group C. Radioactive iodine uptake was also measured in 41 controls who were unselected clinic patients in whom endocrine and autoimmune disease had been excluded.

AUTOANTIBODY MEASUREMENT

Thyroid microsomal and anti-thyroglobulin antibodies were measured using standard, commercially available haemagglutination kits (Wellcome). For the former, a titre of 20^2 was considered to be weakly positive, 40^2 and above to be definitely positive and for the latter a titre of 320 or above was positive. Indirect immunofluorescence assays were performed on seven micrometer sections of human thyroid, rat stomach and kidney. TSH receptor antibodies were measured by the radioreceptor assay on immunoglobulin concentrates as described in APPENDIX 1.

STATISTICS

Data on radioactive iodine uptake, age at diagnosis and thyroid hormone levels were analysed using Student's t test. TSH receptor antibody values were clearly not normally distributed and were therefore compared using the Wilcoxon rank Sum Test. All other probability values were derived using the Chi-squared test with Yates' correction.

RESULTS

The series of 139 patients with thyrotoxicosis were divided into three groups:-

Group A - 89 patients (64%) with goitre and high radioactive iodine uptake (over 40%)

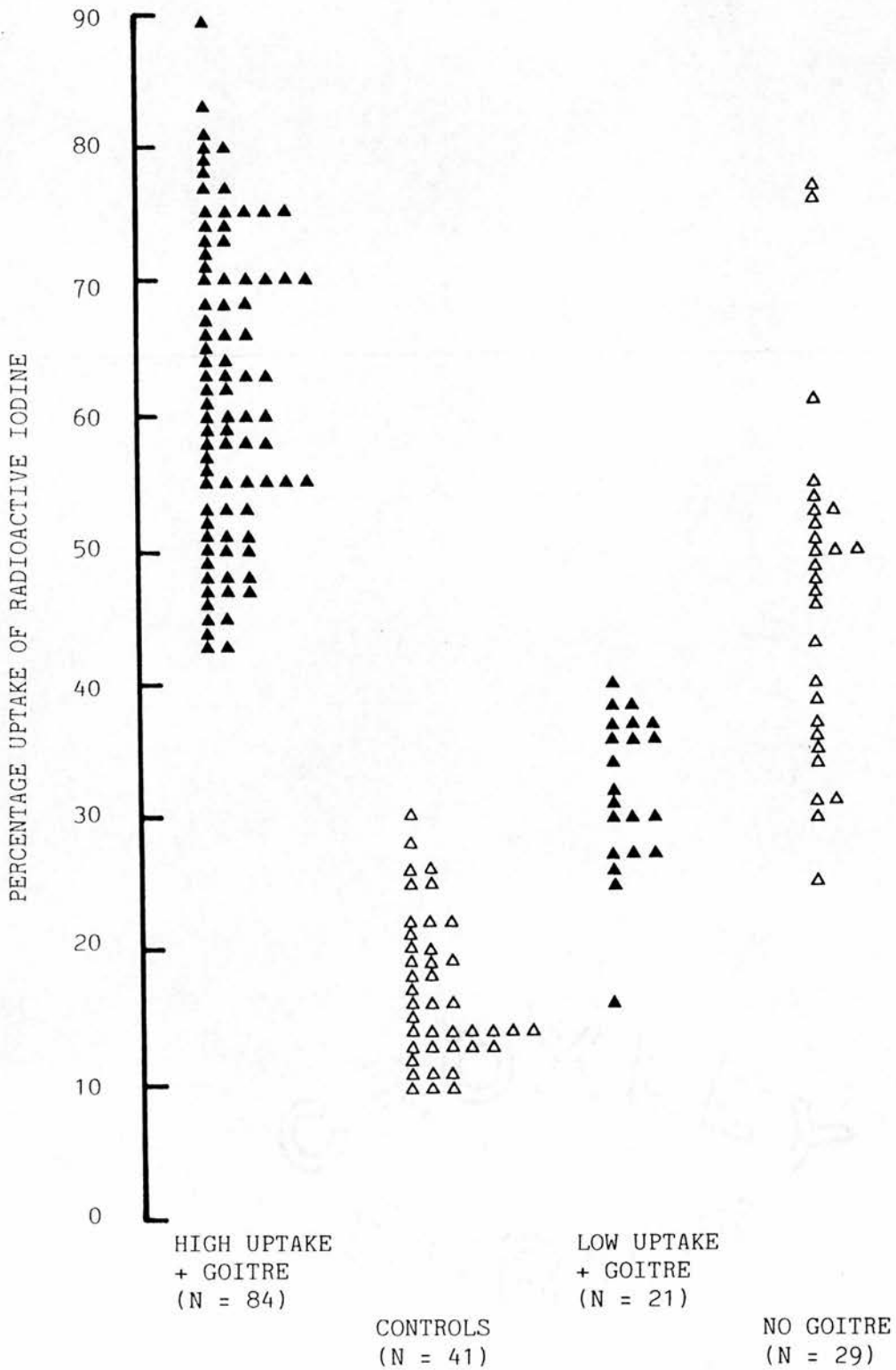
Group B - 21 patients (15%) with goitre but low radioactive iodine uptake (below 40%)

Group C - 29 patients (21%) with no detectable goitre

FOUR HOUR ^{131}I UPTAKE

Data for the four hour radioactive iodine uptake in the three groups is shown in FIGURE 4.1 along with results from 41 normal controls. The mean four hour uptake for the controls was 16.8% (SD 5.1, range 10 to 30). The mean ^{131}I uptake in the high and low uptake groups of toxic patients with goitre was 61.9% (SD 11.1, range 43 to 89) and

FIGURE 4.1 FOUR HOUR UPTAKE OF RADIOACTIVE IODINE IN NORMAL SUBJECTS AND IN THREE GROUPS OF PATIENTS WITH GRAVES' DISEASE



31.9% (SD 5.9, range 16 to 40) respectively. The mean uptake for the patients with no goitre (Group C) was 46.4% (SD 12.9, range 25 to 77) and this differed significantly from the means of normal controls and that of both groups of thyrotoxic patients with goitre (all $p < 0.001$).

AGE AT DIAGNOSIS

The mean age in group A at diagnosis was 40.9 years (SD 12.4) compared to 51.6 years (SD 11.6) in Group B ($p < 0.001$) and 52.6 years (SD 12.2) in Group C ($p < 0.001$) - see FIGURE 4.2. There was no significant difference between Groups B and C.

THYROID HORMONES

The values for T_3 are shown in FIGURE 4.3. Patients in Group A had a mean T_3 level of 6.6 nmol/l (SD 2.4) compared to 4.2 nmol/l SD 1.1, $p < 0.001$ for Group B and 5.4 nmol/l (SD 1.3, $p < 0.001$) for Group C. The mean T_4 in the three groups was 240.8 nmol/l (SD 56.3), 205.3 (SD 40.3) and 223.0 (SD 39.5) respectively for Groups A, B and C. The only significant difference was between Groups A and B ($p < 0.01$).

AUTOANTIBODIES

TSH receptor antibody levels are shown in FIGURE 4.4. The mean inhibition index for Group A was 24.8 as against 12.0 ($p < 0.001$) for Group B and 8.4 ($p < 0.001$) for group C. In group A 64/89 (72%) patients were positive compared to 9/21 (43%, $p < 0.05$) in Group B and 9/29 (31%, $p < 0.001$) in Group C - see TABLE 4.1.

FIGURE 4.2 AGE AT DIAGNOSIS IN THREE GROUPS OF PATIENTS WITH THYROTOXIC GRAVES' DISEASE

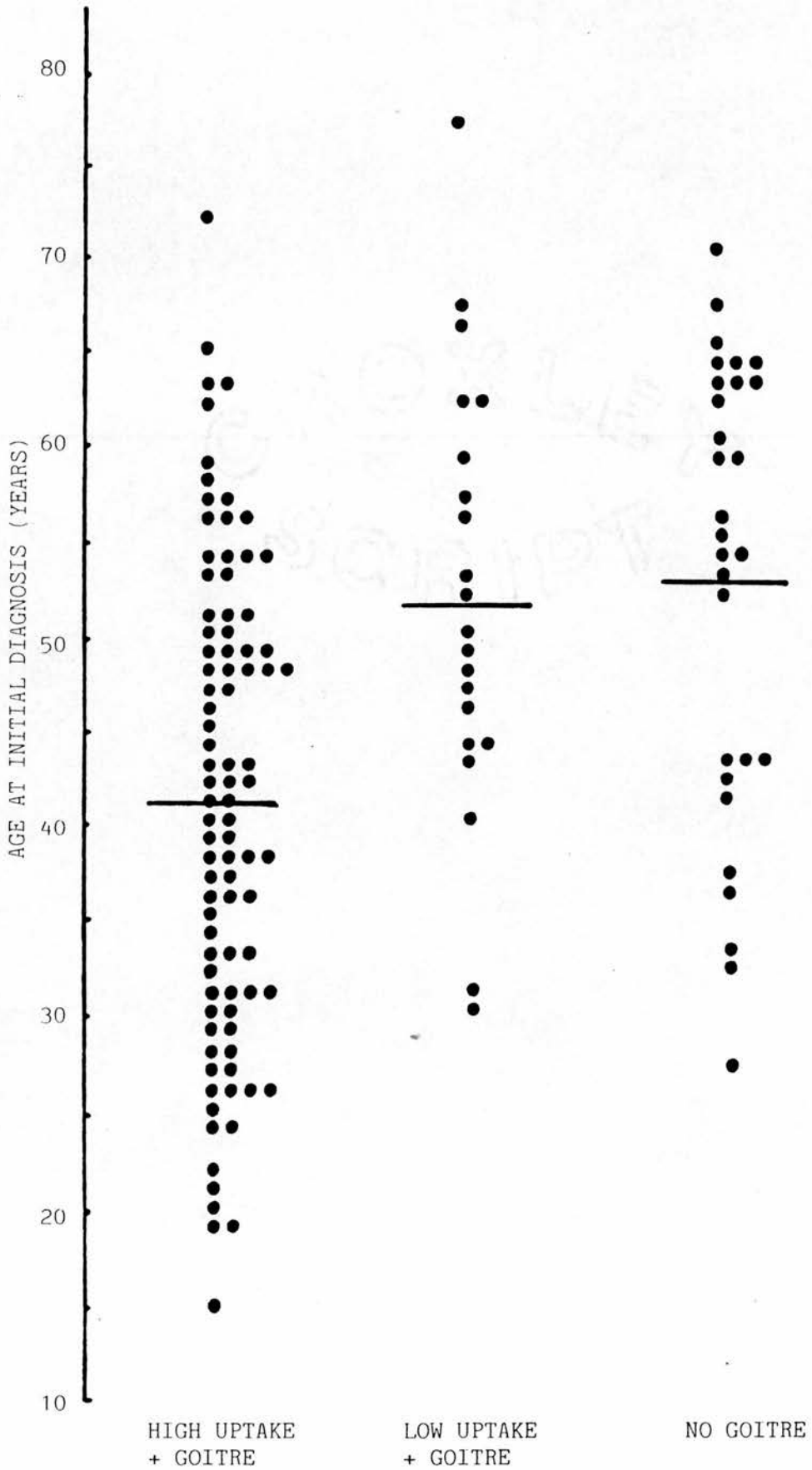


FIGURE 4.3 TRIIODOTHYRONINE LEVELS IN THREE GROUPS OF PATIENTS WITH GRAVES' DISEASE

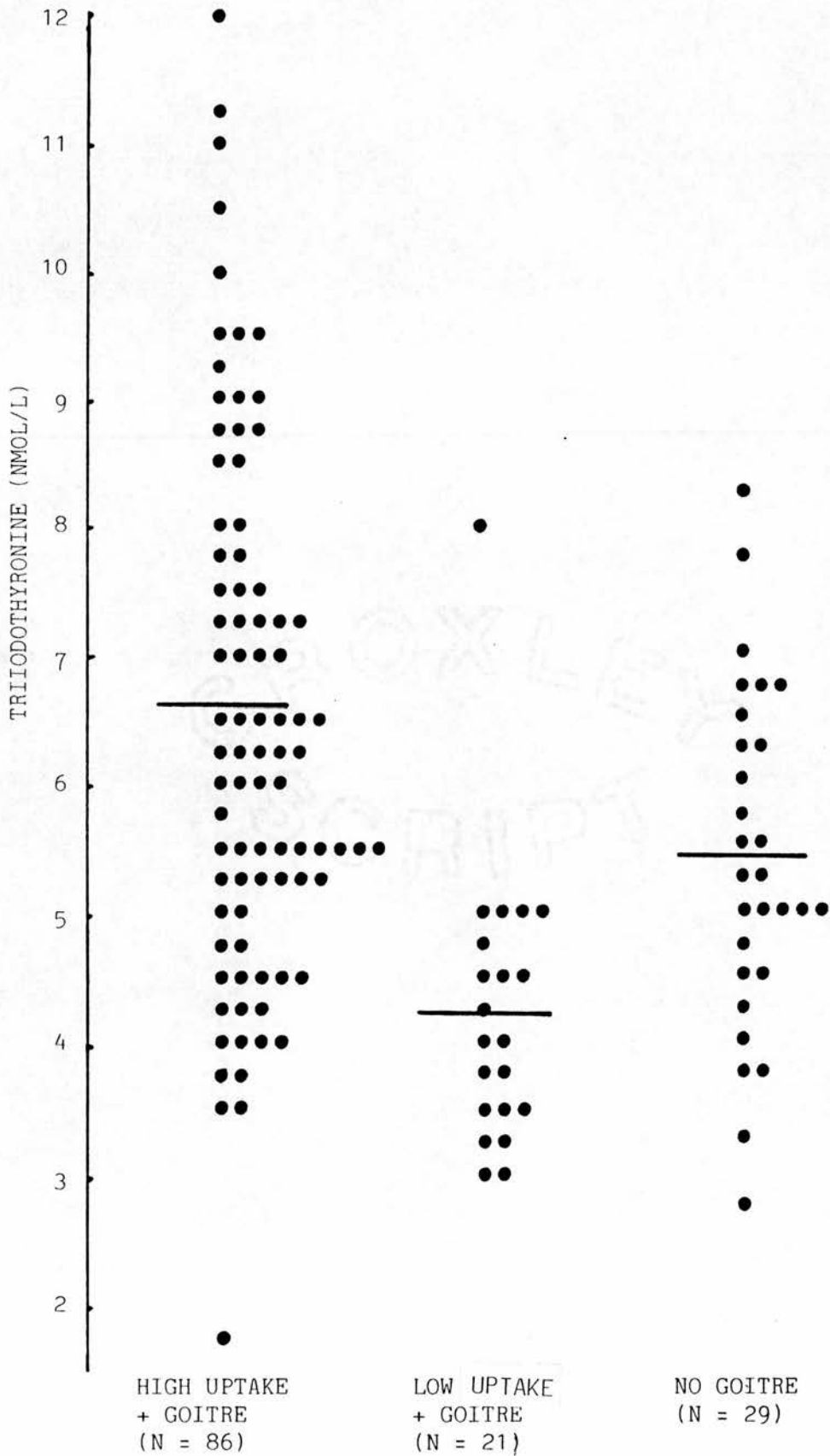


FIGURE 4.4 TSH RECEPTOR ANTIBODIES IN THREE GROUPS OF PATIENTS WITH GRAVES' DISEASE

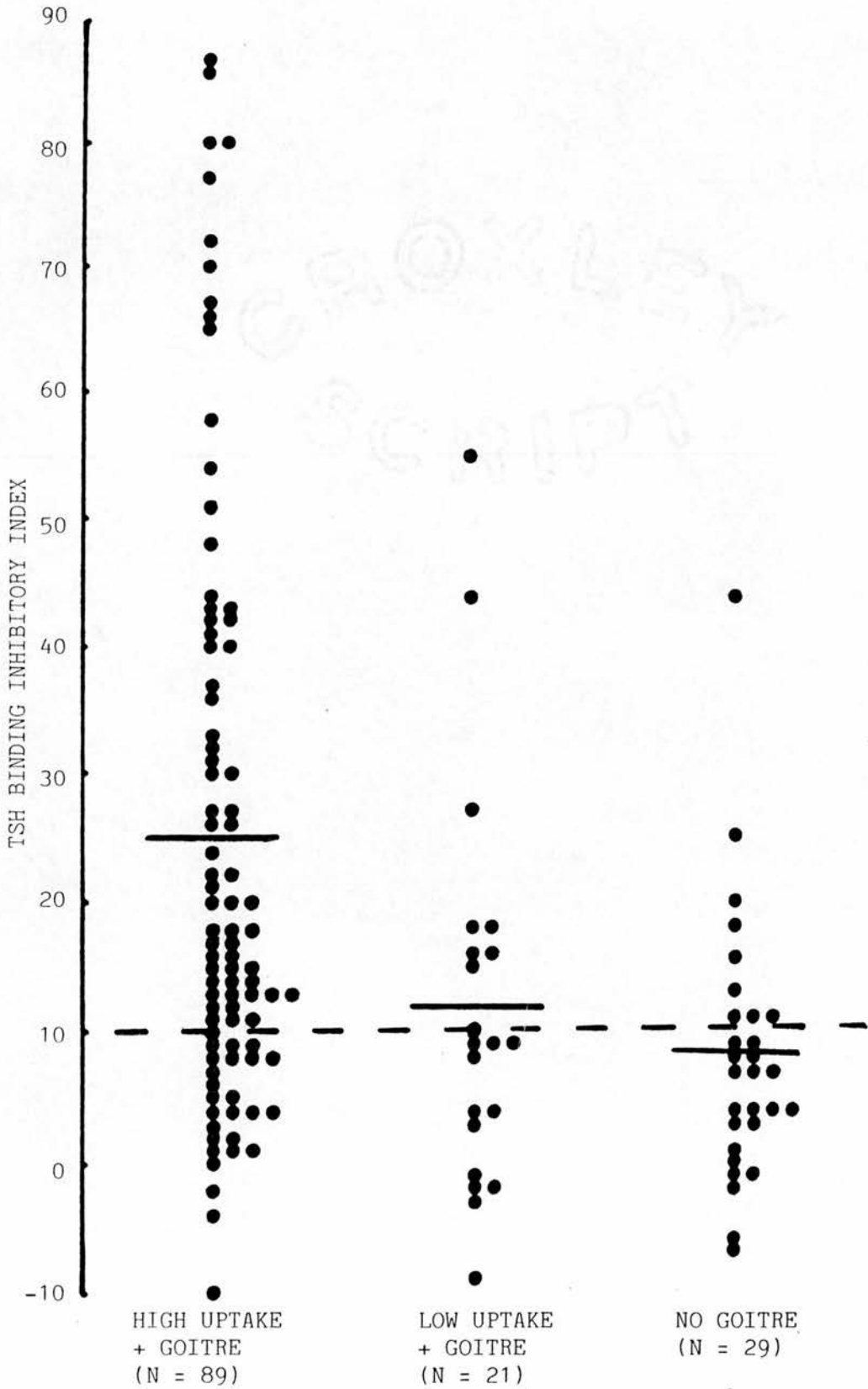


TABLE 4.1 AUTOANTIBODIES AGAINST THE THYROID AND OTHER TISSUES IN
THREE GROUPS OF PATIENTS WITH GRAVES'DISEASE

	GROUP A	GROUP B	GROUP C
	(n = 89)	(n = 21)	(n = 29)
	No. (%)	No. (%)	No. (%)
TBII POSITIVE	64 (72)	9 (43)*	9 (31)**
MICROSOMAL Ab.	43 (34)	10 (48)	8 (28)
THYROGLOBULIN Ab.	9 (10)	3 (14)	1 (3)
PARIETAL CELL	9 (10)	9 (43)**	2 (7)
ANTINUCEAR FACTOR	6 (7)	4 (19)	1 (3)
OTHER Abs.	Smooth Muscle (x1)	Mitochond. (x1)	-
PATIENTS WITH NON- THYROID ANTIBODIES	14 (16)	12 (57)**	3 (10)

* = $p < 0.05$ compared with Group A

** = $p < 0.001$ compared with Group A

Mitochond. = Mitochondrial antibody positive

Group A = High uptake + goitre

Group B = Low uptake + goitre

Group C = No goitre

The titres of other autoantibodies are also shown in TABLE 4.1. There was no significant difference in the numbers of patients in the three groups positive for anti-microsomal or anti-thyroglobulin antibodies. However, when the actual titres of thyroid microsomal antibodies were analysed using a rank correlation test, significantly lower values were found in the group of patients with no goitre when compared to the rest of the patients ($p < 0.01$).

There was an unusually high incidence of parietal cell antibodies in group B with nine out of 21 patients positive (43%) as opposed to 9/89 (10%) of Group A ($p < 0.001$) and 2/29 (7%) of Group C. The total number of patients in Group B with non-thyroidal autoantibodies was 12/21 (57%) compared to 14/89 (16%) of group A and 3/29 (10%) of group C.

DISCUSSION

Using the four hour radioactive iodine uptake and clinical observation on the presence or absence of a goitre, it has been possible to identify three putative subgroups of Graves' patients in a series of 139 patients. The first group (89 patients) had goitre and high ^{131}I uptake and may be regarded as having classical Graves' disease. The second group was of 21 patients with goitre but without grossly elevated ^{131}I uptake (less than 40% at four hours), while the third group was of 29 patients with no detectable goitre.

The patients with low radioactive iodine uptake and goitre (Group B) were older than the patients with high uptake and less severely thyrotoxic as judged by their thyroid hormone levels. They were studied as a separate group since minor degrees of elevation of the radioactive iodine uptake may occur in thyroiditis and multinodular goitre both of which may have some clinical and immunological overlap with Graves' disease. TSH receptor antibodies were detected in fewer of this group and the levels of TBII were lower than in the patients with goitre but with a high ^{131}I uptake. They also had a remarkably high incidence of non-thyroidal autoantibodies (mainly parietal cell) with 12/21 (57%) positive compared to 14/89 of the high uptake group. This group of patients may be analogous to the "Hashitoxicosis" patients in the study of Stenzky et al (1983). The association of Hashimoto's thyroiditis with HLA-DR5 (Farid et al, 1980) is of interest in the light of the high incidence of parietal cell antibody since pernicious anaemia is also associated with HLA-DR5. The coexistence of Graves' disease with other autoimmune diseases in this sort of patient was also noted by Fatourechhi et al (1971) who found five out of 24 patients with Graves' and histological thyroiditis to have diabetes mellitus and a further five to have close relatives with diabetes.

Patients with "Hashitoxicosis" may differ prognostically with a higher proportion becoming hypothyroid, either spontaneously or after destructive antithyroid therapy. Of the 57 patients studied in Chapter 3 after ^{131}I therapy, 31 had high uptake and goitre (Group A), 11 had low uptake and goitre (Group B) and 15 patients had no goitre (Group C). In all, six patients did not respond to their initial dose of radioactive iodine and required further therapy

(three in group A and three in Group B). Of the 28 patients who responded to a single dose in Group A, 15 patients were euthyroid after six months while 13 had become hypothyroid. In Group C, nine out of the fifteen patients, all of whom responded to a single dose, became hypothyroid. In Group B, there were eight responders and seven of them became hypothyroid in the six months following treatment. While no statistically significant differences were found, there is some indication that Group B patients are more likely to become hypothyroid after therapeutic doses of radioactive iodine.

Patients with no goitre are also older than patients in Group A and have less severe thyrotoxicosis. Since such patients may account for up to 20% of cases of thyrotoxicosis, it is clearly of importance to decide whether they are suffering from true Graves' disease or whether some other pathology is involved. The relatively high incidence of exophthalmos in patients with no goitre reported by Greenwood et al (1985) and the appreciable incidence of TSH receptor antibodies reported here support the hypothesis that some, at least, of these patients have true Graves' disease. In the diagnostic index for thyrotoxicosis devised by Crooks et al (1959) before sophisticated thyroid hormone measurements were available, the absence of goitre weighed heavily against the diagnosis. These authors did however, cite the case of a 63 year old man who had no goitre but did have a high diagnostic index supporting the diagnosis of thyrotoxicosis. Although the diagnosis of hyperthyroidism is now made much more readily, it is not always easy to substantiate a diagnosis of Graves' disease as the underlying cause since no single marker is always positive and it is possible for patients with early

disseminated thyroid autonomy to have a diffuse thyroid scan because individual thyroid follicles may begin to function autonomously before discrete nodules form (Studer et al, 1978).

Heterogeneity may therefore exist within Graves' disease and it may become^{possible} in time to devise a classification analagous to that for diabetes mellitus. Apparent heterogeneity may exist because of the variable expression of autoantibodies such growth promoting antibodies or cytotoxic antibodies or there may be an incomplete expression of some of the features of the disease in some patients due to changes in the immune system with advancing age.

Chapter Five

Immune Complexes in Autoimmune Thyroid Disease and Addison's Disease

SUMMARY

Immune complexes were studied using the C1q solid phase assay in the serum of patients with autoimmune thyroid disease and in patients with Addison's disease. Particular attention was paid to the relationship between circulating immune complexes (CICs) and the expression of organ specific autoantibodies. The Addison's patients were of special interest in this light because of the high frequency of associated autoimmune endocrine diseases found in this condition.

CICs were detected in 12 out of 55 (22%) patients with untreated Graves' disease. Only one of the patients with CICs was also positive for TSH receptor antibodies compared to 28 out of 42 (67%) patients with no CICs ($p < 0.001$). The mean value for TBII in the IC positive group was 5.4 as opposed to 22.2 in the IC negative group ($p < 0.02$). The presence of ICs at diagnosis may relate to the prognosis after radioactive iodine therapy. None of seven IC positive patients became hypothyroid within six months of treatment while seven of fifteen IC negative patients did so ($p = 0.056$, Fisher Exact Test). Four out of eleven (36%) patients with Graves' disease in remission after medical treatment had CICs as did four out of eleven patients with subclinical Graves' (flat TRH test, normal or slightly elevated thyroid hormones, diffuse thyroid scan and relatively few symptoms). In the patients in remission and in the subclinical group, there was an association between exophthalmos and IC positivity (all four exophthalmic patients positive) but none of the seven patients with untreated Graves' thyrotoxicosis who had documented exophthalmos was IC positive.

Of 40 patients with Hashimoto's thyroiditis, CICs were detected in ten out of 19 patients (53%) who were euthyroid and in 14 out of 21 (67%) who were hypothyroid. CIC positivity showed a biphasic distribution with respect to age at first diagnosis with the first peak corresponding mainly to euthyroid cases (mean age 32 years), followed by a trough in the incidence of IC positivity between the ages of 35 and 50 years and a further peak of patients presenting in later life and who were mainly hypothyroid (mean age 52 years). Thyroid microsomal antibodies were strongly positive in 15/19 (79%) euthyroid and 19/21 (90%) hypothyroid patients. Immune complex positivity and increasing titres of microsomal antibody were negatively associated in the euthyroid group but positively associated in the hypothyroid group. Anti-thyroglobulin antibodies were detected in five patients from each group. All five of the hypothyroid anti-thyroglobulin positive patients were also positive for CICs with a mean C1q-SP value of 7.7 while only one of the five corresponding euthyroid patients was IC positive and the mean C1q-SP value in this group was 5.4 ($p < 0.05$).

In primary atrophic hypothyroidism (PAH) CICs were detected in 69 out of 105 patients at the time of diagnosis. Once more there was a variation in IC positivity with age at diagnosis, but where there had been a trough incidence in middle life for the Hashimoto patients, the peak incidence of IC positivity in PAH was between 36 and 45 years (79% of patients positive). There was a significant ($p < 0.01$) trend towards IC positivity with increasing titre of thyroid microsomal antibody. CICs were found in seven out of eleven (64%) patients with subclinical hypothyroidism and in two patients with spontaneous hypothyroidism following Graves' thrototoxicosis.

Addison's disease is relatively uncommon and ICs have not been previously studied extensively. CICs were found in 122 out of 210 cases (58%) - 170 idiopathic, 38 tuberculous, one sarcoid and one post-septicaemia. Serum from the time of diagnosis, before replacement therapy was commenced, was available in 79 cases. Of the fourteen patients with tuberculous Addison's (nine male and five female), eight were IC positive. In this group, but not in the autoimmune group, there was a negative correlation between C1q-SP and age. In the 65 idiopathic cases, CICs were found in 41 (63%). There was no difference between the adrenal antibody negative cases (n = 31, 15 male and 16 female) and the adrenal antibody positive cases (n = 34, 9 male and 25 female) either in the incidence or level of CICs. However, in the antibody negative group, 11 out of 13 patients with non-adrenal autoantibodies or autoimmune diseases were positive compared to eight of the remaining 18 patients (p = 0.02).

Thirteen out of 24 (58%) patients with established tuberculous Addison's disease (one to 22 years from diagnosis) and 57 out of 105 (54%) with idiopathic disease (one to 30 years) were IC positive. While the proportion of patients with adrenal antibodies declined with time from diagnosis the IC positivity did not - but the incidence of other autoantibodies and other autoimmune diseases increased. Only 49% of patients with no extra-adrenal antibodies or autoimmune diseases were IC positive, compared to 58% of those with non-pathogenic antibodies, 67% of those with at least one other diagnosis and 79% of those patients with at least two other autoimmune diseases.

In conclusion, thyroid autoimmune diseases and Addison's disease (tuberculous and idiopathic) are associated with a high incidence of immune complexes. CICs may affect the detection of organ specific autoantibodies, both in untreated Graves' disease and in Addison's disease. In the latter condition, their presence on long term follow up is related to the incidence of other organ specific autoimmune diseases. In primary hypothyroidism, CICs are age related but show a different distribution with age in PAH and Hashimoto's. In Hashimoto's thyroiditis, the association of CICs with thyroid antibodies depends on thyroid status.

INTRODUCTION

Immune complexes are the result of non-covalent bonding between antigens, both endogenous and exogenous, and their specific antibodies. They form in the tissues and in the circulation of patients with a wide variety of infectious, malignant and autoimmune diseases (Lambert et al, 1978; Theofilopoulos, 1980; Poskitt & Poskitt, 1985). The nature and size of the complex depends on the antigen involved and the number of potential antibody binding sites as well as the type of antibody and its valency. The scope for variety is virtually unlimited although, in practice, there may be fairly simple rules governing the structure of immune complexes (ICs) which are actually formed and relatively small complexes tend to be found in the circulation (Steensgaard & Johansen, 1980). While the formation of ICs is the inevitable result of a humoral immune reaction, their detection in the circulation in disease states is

variable. This variability is partly due to differences in detection methods and particular methods or combinations of methods may be suitable for some disease states but not for others (Lambert et al, 1978; Migliorini et al, 1974). Also the clearance of ICs from the circulation depends, to a large extent on their size and composition (Mannick et al, 1974) with smaller complexes being cleared by the kidneys and larger ones by circulating lymphocytes, mononuclear and polymorphonuclear phagocytic cells and the fixed phagocytic cells of the reticuloendothelial system. In cutaneous vasculitic conditions, the clearance of ICs by the reticuloendothelial system is greatly accelerated (Dambuyant et al, 1984) and if this were to be generally applicable to immune complex-associated autoimmune diseases, then this might add to the difficulty experienced in detecting CICs. A possible role for red blood cells in clearing CICs has come to light recently: Inada et al (1982, 1983) have demonstrated defective erythrocyte C3b receptor function in SLE and have found that this relates to the presence of CICs and is associated with increased fragility of the red cells. Not only does the level of CICs decline with disease remission, but the C3b receptor function and the changes in osmotic fragility also return towards normal.

The variety of methods which have been developed to detect and quantify ICs is summarised in TABLE 5.1, a list which is by no means exhaustive. For reviews on methodology see - Lambert et al, 1978; Theofilopoulos, 1980; Williams, 1981; Ritzman & Daniels, 1982. Generally speaking, the physical methods have found limited usage: Analytical ultracentrifugation is relatively insensitive and is in any case too cumbersome for routine use. Polyethylene glycol precipitation may be a useful screening test but is subject to

TABLE 5.1 METHODS USED TO DETECT IMMUNE COMPLEXES

A. PHYSICAL METHODS

- Analytical Ultracentrifugation
- Polyethylene glycol Precipitation
- Laser Nephelometry

B. DETECTION IN TISSUES

- Immunofluorescence
- Immunoperoxidase

C. INTERACTION WITH ANTIGLOBULINS

- Polyclonal or Monoclonal Rheumatoid Factor Assay (fluid or solid phase)
- Latex Agglutination Inhibition

D. INTERACTION WITH COMPLEMENT FACTORS

- C1q Binding Assay (fluid phase)
- C1q Solid Phase Assay
- Inhibition of C1q Binding (by radioimmunoassay or by [cr] inhibition of latex agglutination)
- Conglutinin Solid Phase Assay

E. INTERACTION WITH CELLS

- 1) via complement receptors:
 - Raji Cell Radioimmunoassay
 - Inhibition of EAC-lymphocyte Rosetting
- 2) via Fc receptors
 - Platelet Aggregation Test
 - Inhibition of Antibody Dependent Cellular Cytotoxicity

F. BINDING TO STAPHYLOCOCCAL PROTEIN A

interference from a large number of factors including the presence of large amounts of monomeric IgG. Laser nephelometry measures the light scatter from a diluted serum sample, is highly sensitive but again strongly dependent on the physical state of the serum and many other variables. Results correlate poorly with those obtained using the C1q binding assay (Izzard et al, 1983). Assays based on antiglobulins rely on the inhibition of rheumatoid factor binding to aggregated or insolubilised IgG (bound to Sepharose or Latex particles) by immune complexes.

C1q is an 11S heat-labile protein which contains the immunoglobulin binding site of the first component of complement. It is widely used in the assay of immune complexes, either as a fluid phase assay (Zubler et al, 1976) where binding to labelled C1q is followed by polyethylene glycol precipitation, or as a solid phase assay where the C1q is immobilised on the surface of a plastic tube and bound complexes are detected using a labelled anti-IgG. The latter method is the one employed in this thesis. Conglutinin is a soluble protein found in the serum of bovine species which binds avidly to fixed complement components, particularly C3d (Lachman, 1967). It can be used in a solid phase assay analagous to that used with C1q and this assay proves to be both sensitive and reproducible (Casali et al, 1977).

Raji cells are a lymphoblastoid B cell line derived from a patient with Burkitt's lymphoma. They lack surface immunoglobulin and have few receptors for the Fc portion of IgG but have a large number of high affinity receptors for complement components including C1q. A radioimmunoassay for immune complexes bound to their surface is the

most reliable of the cell based techniques for measuring CICs. Fresh platelets will aggregate when cell surface receptors interact with immune complexes but a number of other agents including viruses and antiplatelet antibodies may also aggregate platelets. Antibody dependent cellular cytotoxicity is inhibited by ICs because they compete for the Fc receptors on effector cells. Staphylococcal protein A binds the Fc portion of IgG1, IgG2 and IgG4 and can be used directly as an assay for immune complexes or used as an anti-immunoglobulin in tests such as the C1q solid phase assay.

The precise role of immune complexes in the pathogenesis of autoimmune diseases is not clear. There are however a number of possible ways in which they might contribute to the autoimmune process. They can activate complement both by the classical and alternative pathways and thereby induce immune adherence via C3b receptors, chemotaxis of leukocytes via the C5a component or macrophage activation by the Bb fragment of factor B. They can interact with cellular complement or Fc receptors causing, for example, platelet aggregation with release of nucleotides or vasoactive amines, neutrophil degranulation with consequent release of proteolytic enzymes. Indeed ICs have the potential to interact and thus modulate the function of any cell with an Fc receptor. These include suppressor/cytotoxic T lymphocytes, K cells and activated T lymphocytes. Samurat and Revillard (1979) have shown that both IgG and IgM complexes will reversibly inhibit the formation of rosettes between peripheral blood lymphocytes and red cells sensitised with IgM. The inhibition with IgG complexes was not seen when the cell preparation was depleted of lymphocytes with receptors for IgG, indicating an immunoregulatory role for the latter cells. In

addition to an effect on immunoregulation, they may also block the antigen receptors on plasma cells and effector T cells. The effect of ICs on K cells is potentially twofold. In antibody excess, they may arm K cells against a specific antigen and ICs have been shown to stimulate lymphokine production by K cells (Neville & Lischner, 1981). On the other hand, they can cause irreversible loss of Fc-IgG receptors (Moretta et al, 1978) and this may account for the loss of K cell activity in some patients with systemic lupus erythematosus (Schneider et al, 1975), diabetes mellitus (Pozzilli et al, 1981) and Graves' disease (Endo et al, 1983). In the latter two studies, an inverse relationship between CICs and K cell activity was actually demonstrated. That immune complexes are detected in the circulation may be due to impaired phagocytic function in the autoimmune diseases. This has been documented in diabetics with severe microangiopathy (Iavicoli et al, 1982) and in Sjogren's syndrome with associated C4 deficiency (Hersey et al, 1983). It has been suggested by Lewis and Roberts (1980) that ICs could have a central role in the induction of autoimmune disease. Tissue inflammation, from any cause, will release into the circulation antigens such as DNA which, when cleaved into smaller fragments by circulating enzymes might form small ICs on binding with their appropriate antibodies. Such complexes, if they were of such a size to be cleared relatively slowly, could become deposited, for example on vascular endothelium, and hence initiate tissue damage.

Many of the diseases in which immune complexes are thought to be important have an HLA association and the expression of ICs may thus have a genetic basis. Probably the best evidence for a disease state being caused by ICs is in glomerulonephritis. Goodpasture's syndrome,

where nephritis is due to antibodies to the glomerular basement membrane is strongly associated with HLA-DR2 (Rees et al, 1978) and idiopathic membranous nephropathy has been linked to HLA-DR3 (Klouda et al, 1979). The renal lesions in strains of mice susceptible to autoimmune disease may be related to previous viral infection, particularly with retroviruses, and the formation of virus-antivirus immune complexes in (NZB x NZW) F1 hybrid mice is genetically determined (Maruyama et al, 1983) as is the formation of C1q binding complexes in mice persistently infected with the lymphocytic choriomeningitis virus (LCMV, Oldstone et al, 1983). Random terpolymers of L-glutamine, L-alanine and L-tyrosine have been used to characterise the immune response genes in guinea pigs (Bluestein et al, 1972) and, since these synthetic antigens are similar to many bacterial and virus coat antigens, the hypothesis has been advanced that the IC response to infection may be genetically determined and may, in part, underlie the association between HLA and autoimmune disease (Vickerman, 1979).

In terms of clinical application, immune complexes have been most extensively studied in the connective tissue diseases. They are found in the sera and synovial fluids of a high proportion of patients with rheumatoid arthritis (Halla et al, 1979a) and particularly in patients with circulating rheumatoid factor (Gupta et al, 1979). Both the studies of Halla and of Gupta employed three different methods to quantify CICs - one based on their binding to monoclonal rheumatoid factor, the C1q binding assay and the Raji cell radioimmunoassay. Both studies reported a poor correlation between the three methods in keeping with the findings of Reeback et al (1985) who used different methods but again found the results of them to correlate

poorly. The results in the C1q binding assay may correlate with some measures of disease activity (Halla et al, 1979b; McDougal et al, 1982) and relate to systemic features in juvenile rheumatoid disease (Possen et al, 1977) but methods based on polyethylene glycol precipitation and the C1q solid phase assay show no such correlations (Reeback et al, 1985).

CICs have been found in a small proportion of patients with progressive systemic sclerosis using assays based on C1q and conglutinin (Siminovitch et al, 1982) and their presence correlated with disseminated organ involvement, particularly lung disease. Certain serological markers correlate with the severity of the disease and with the extent of organ involvement. The centromere pattern of staining on immunofluorescence is found typically in the CREST syndrome (calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly and telangiectasia), a benign variant of the disorder, while the marker anti-Scl-70 is found particularly in those patients with pulmonary involvement (Cattogio et al, 1983). Recently, French et al (1985) have found immune complexes using the Raji cell radioimmunoassay in about a third of patients and they correlated strongly with the presence of anti-Scl-70 which is a highly specific marker for systemic sclerosis. CICs have also been correlated with disease activity in eosinophilic fasciitis, a disorder related to scleroderma where inflammation and thickening of the deep fascia is associated with hypergammaglobulinaemia and eosinophilia (Seibold et al, 1982).

In systemic lupus erythematosus (SLE), there are frequently CICs although there is some controversy surrounding their relationship to the activity and the extent of the disease, and hence their usefulness in monitoring therapy (for review, see Inman, 1982). Complexes detected by the C1q binding assay have been reported to correlate with disease activity, DNA binding capacity and the consumption of complement components (Nydegger et al, 1974; Zubler et al, 1976). By contrast, Abrass et al (1980) found this assay to be of little use but reported that complexes detected using the C1q solid phase assay correlated with disease activity and the presence of renal or articular manifestations. It has been suggested by Tung et al (1981) that the size of ICs may relate to the pathological changes they cause, with larger complexes (greater than 19S) being found in patients with membranous glomerulonephritis, small complexes (around 7S) in patients with cerebral involvement and a mixture of the two in patients with proliferative glomerulonephritis or no renal changes at all. As already discussed, the size or composition of CICs determines which assay is most appropriate for their detection. There is also evidence that ICs are frequently found and relate to disease activity in mixed connective tissue disease (Halla et al, 1979b) and in polymyalgia rheumatica with giant cell arteritis (Espinoza et al, 1982).

In diabetes mellitus, CICs may be detected in over half the patients tested at diagnosis depending on the method used and their presence correlates with that of islet cell antibodies (Irvine et al, 1977, 1980). Generally IC positivity decreases with time from diagnosis in parallel with islet cell antibody positivity and persistent antibodies are associated with persistent ICs (Irvine et al, 1980).

There is in fact a bimodal distribution of IC positivity with time from diagnosis (Irvine 1980) and this may in part relate to insulin-anti-insulin complexes which, it has been suggested, may be involved in the pathogenesis of microvascular complications (for review see Di Mario et al, 1984). Anderson (1976) did find serum insulin binding capacity to be slightly but not significantly increased in patients with complications and patients with very high titres of anti-insulin antibodies were especially likely to have complications. Generally, however, anti-insulin antibodies and complexes have been found to correlate poorly with microvascular disease (Botansky et al, 1982; Di Mario et al, 1983). There is an overall correlation between ICs and diabetic complications but these could clearly be secondary to vascular disease and their role in pathogenesis, if any, is not established (Andreani et al, 1982; Botansky et al, 1982).

Immune complexes have been demonstrated in tissue by immunofluorescence (Dixon et al, 1961) and can be found thus in a variety of IC-related diseases (Penner et al, 1982). They are demonstrable in the thyroid in animal models of thyroiditis - both in mice (Clagget et al, 1974) and in obese strain (OS) chickens (Katy et al, 1981). In both cases the deposition of ICs is age related, although it can be found as early as hatching in OS chickens, and precedes cellular infiltration (Kofler et al, 1983). Immune complexes have been found using electron microscopy in human patients with Hashimoto's thyroiditis or Graves' disease. They are seen in association with the follicular basement membrane and often occur near areas of lymphocytic or plasma cell infiltration (Werner et al, 1972; Kalderon et al, 1973; Kalderon & Bogaars, 1977).

Thyroglobulin-antithyroglobulin ICs have been shown to bind to extraocular muscle membrane where they may have a role in the production of Graves' exophthalmos (Konishi et al, 1974). A case of glomerulonephritis has been reported following radioactive iodine therapy where thyroglobulin was demonstrated in the IC deposits in the basement membrane of the kidney (Plath et al, 1978).

Immune complexes have been found in the circulation of patients with thyroid disorders by a number of authors. Using an anticomplementary assay, Calder et al (1974) found CIC in 59% of Hashimoto patients, 29% of patients with atrophic hypothyroidism and 17% of Graves' patients. McKenzie & Zakarija (1977) cited a study where 11 out of 24 (46%) Graves' patients were positive in an assay where the binding of C1q to sheep red blood cells was inhibited by the presence of CICs (the red cells having been previously sensitised with subagglutinating doses of rabbit anti-sheep haemolysin). Barkas et al (1976) used the inhibition of ADCC to quantify ICs, and found K cell activity to be reduced in the presence of sera from patients with Hashimoto's and from those with atrophic hypothyroidism as well as to a lesser extent with the sera from patients with thyrotoxicosis. The Raji cell radioimmunoassay was used by Al Khateeb et al (1978) to demonstrate CIC in 78% of Hashimoto's, 36% of atrophic hypothyroidism and 20% of thyrotoxic patients. CIC have been detected in a proportion of Graves' patients using the C1q fluid phase assay (van der Heide et al, 1980): 17/57 (30%) patients were positive and there was a negative correlation between CIC and TSH receptor antibodies. CIC appeared in many patients early in the course of drug treatment, just as TSH receptor antibodies were disappearing from the circulation. The possibility arises from this study that assays for

TSH receptor antibodies may not be positive in some Graves' patients because the antibody is complexed in the circulation with its specific antigen. Bogner et al (1979), however were unable to demonstrate thyroid antigens in Raji cell bound ICs when they were incubated with fluorescein conjugated antibodies against the TSH receptor, microsomal antigen or thyroglobulin.

The C1q solid phase assay is the one used in the studies reported in this dissertation and extensive studies of this method in autoimmune thyroid disease have not been previously reported. Endo et al (1983) found 34% of 41 Graves' patients to be positive but included in their study patients already on drug treatment. Brohee et al (1979) applied the assay to the serum of 171 patients with thyroid disease and found an overall positivity of 26%. They did, however, include patients with non-autoimmune thyroid disease and did not take into account the metabolic state of the patient or the time from diagnosis. C1q-SP was positive in 7/25 (28%) Graves' patients, 15/37 (40%) of those with asymptomatic thyroiditis and 5/29 (17%) with goitrous thyroiditis. CIC correlated with the presence but not with the titre of thyroid microsomal antibody and not with anti-thyroglobulin or the age or sex of the patient.

The aim of the work presented in this chapter is to investigate immune complexes as measured by the C1q solid phase assay in primary hypothyroidism, Graves' disease and Addison's disease. The latter condition was of particular interest, not only because of its relative rarity but also because of the high frequency of other organ specific autoantibodies and autoimmune disease found in association with the disease. Evidence was sought for the differential expression

of immune complexes in putative subgroups of the autoimmune endocrine diseases and for a possible reciprocal relationship between immune complex positivity and the expression of organ specific autoantibodies in the circulation which may prevent these antibodies being detected in standard tests.

NOTE: The limits of positivity for the C1q solid phase assays used here varies from one section to another. This is due to the use of different C1q preparations and to differences in the times for preincubation, the C1q binding being higher the longer the incubation period. Each assay is standardised using a bank of normal and abnormal serum and, for each set of experiments, the assay conditions were identical.

IMMUNE COMPLEXES IN GRAVES' DISEASE

I) Untreated Graves' disease

PATIENTS AND METHODS

The study included 55 patients (eight male, 47 female), a random selection from one of the series studied in Chapter 2. The mean age at diagnosis was 45.4 years (SD 14.3, range 15 to 70), the mean T₃ at diagnosis was 5.94 nmol/l (SD 2.31) and the mean T₄ was 231.5 nmol/l (SD 60.4). Thyroid microsomal and anti-thyroglobulin antibodies were assayed by passive haemagglutination using commercially available

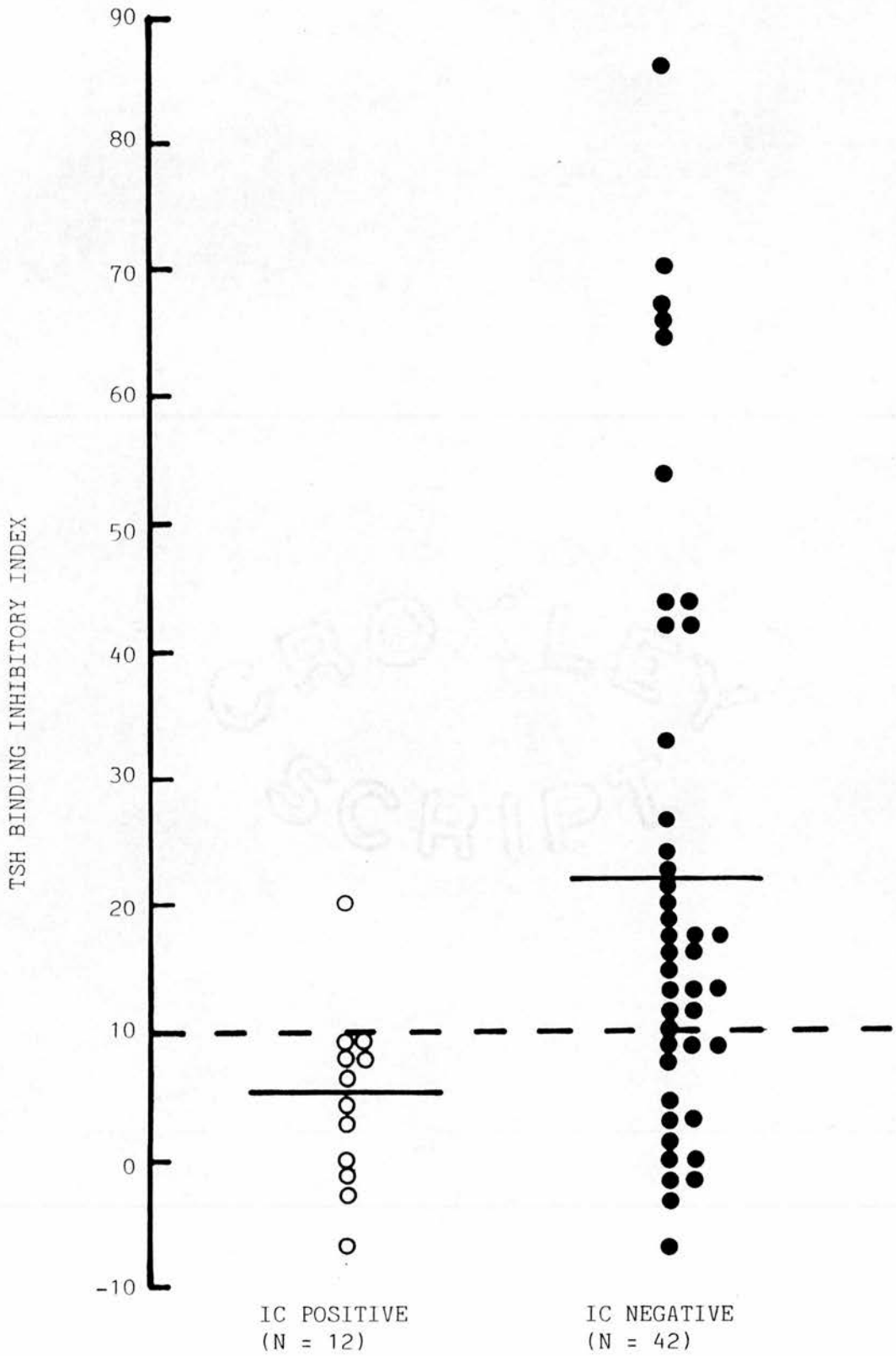
kits (Wellcome). Other autoantibodies were detected by indirect immunofluorescence and TSH receptor antibodies were assayed using immunoglobulin concentrates as described in APPENDIX 1. Immune complexes were measured by the C1q solid phase assay as detailed in APPENDIX 2.

RESULTS

Positive results in the C1q solid phase assay were obtained in 12 out of the 55 patients (22%). Thyroid microsomal antibodies were detected in 37 (67%) and anti-thyroglobulin in eight (15%). Parietal cell antibodies were found in nine (16%), antinuclear factor in four (7%) and smooth muscle antibodies in one patient. The value obtained in the C1q-SP assay did not correlate with the presence or titre of any of these antibodies, with any measure of thyroid function or with the sex of the patient. The mean age of IC positive patients was 55.5 years compared to 42.8 years for the IC negative patients ($p < 0.01$).

TSH receptor antibodies were found in 29 out of 54 (54%) patients. Only one of the twelve patients positive for immune complexes was also positive for TBII compared to 28 out of 42 (67%) patients with no immune complexes ($p < 0.001$, Chi Squared test). The mean value for TBII in the IC positive group was 5.4 as opposed to 22.2 in the IC negative group ($p < 0.02$, see FIGURE 5.1). Overall, there was a slight but non-significant negative correlation between C1q-SP and TBII.

FIGURE 5.1 TSH RECEPTOR ANTIBODIES IN IMMUNO COMPLEX POSITIVE AND IMMUNE COMPLEX NEGATIVE PATIENTS WITH UNTREATED GRAVES'



When the patients were divided, as in Chapter 4, into three groups - one with no goitre, one with goitre but low four hour ^{131}I uptake and one with goitre and high ^{131}I uptake, there was a slight association between the former two groups and immune complex positivity (see TABLE 5.2) although this did not reach statistical significance.

II) Graves' Disease in Remission

Of eleven patients tested while in remission after drug therapy for thyrotoxic Graves' disease, four (36%) were positive in the C1q solid phase assay. Two of the four IC positive patients had exophthalmos (C1q-SP = 8.3% and 9.1%), one had vitiligo (C1q-SP = 8.8%) and the other had rheumatic heart disease (C1q-SP = 11.1%). None of the seven IC negative patients had exophthalmos although one did have both Addison's disease and insulin dependent diabetes mellitus. Among the 55 patients tested for ICs prior to therapy, seven had documented exophthalmos, one had pernicious anaemia and another had both diabetes and vitiligo. None of these nine patients was IC positive using the C1q solid phase assay.

III) Subclinical Graves' Disease

Eleven patients were tested who were considered to have subclinical Graves' disease: The mean T_3 in this group was 2.3 nmol/l (SD 0.46, range 1.5 to 3.1) and the mean T_4 was 145.8 nmol/l (SD 26.0, range 112 to 193) and all had flat TRH tests. Four had palpable diffuse

goitres and exophthalmos was present in two cases. None had more than very mild symptoms of thyrotoxicosis. A thyroid scintigram showed a diffuse uptake of isotope in all cases and the mean four hour uptake of ^{131}I was 25.7% (SD 11.2). Thyroid microsomal antibodies were detected in the serum of four cases but none was positive for either anti-thyroglobulin or TSH receptor antibodies. Immune complexes were found in four of these eleven patients, and three of the four IC positive patients were also positive for microsomal antibodies. Both patients with exophthalmos were IC positive and one IC positive patient went on to develop florid thyrotoxic Graves' disease eleven months after her initial presentation and was subsequently treated with carbimazole.

IV) Immune Complexes and the Response to Radioactive Iodine

The outcome after therapeutic doses of radioactive iodine may relate in part to the presence of ICs in the circulation prior to treatment (see TABLE 5.3). Of 17 IC negative patients, treated with a mean dose of 6.4 mCi, seven were hypothyroid within six months. By contrast, none of seven IC positive patients treated with a mean dose of 7.8 mCi, developed hypothyroidism within six months of treatment ($p = 0.056$, Fisher Exact Test).

Immune complexes, TSH receptor antibodies and thyroid microsomal antibodies were tested sequentially in six patients following therapy. Immunological data from these patients is shown in TABLE 5.4. Patient details are given below:-

TABLE 5.2 IMMUNE COMPLEX POSITIVITY IN DIFFERENT GROUPS OF PATIENTS WITH UNTREATED GRAVES' DISEASE

	No. PATIENTS	IC POSITIVE	% POSITIVE
NO GOITRE	14	5	36%
GOITRE + LOW UPTAKE	7	3	43%
ALL PATIENTS WITH LOW U/T	15	6	40%
GOITRE + HIGH UPTAKE	34	4	12%

U/T = Four hour ¹³¹I uptake

TABLE 5.3 IMMUNE COMPLEXES AND THE RESPONSE TO RADIOACTIVE IODINE

	IMMUNE COMPLEX NEGATIVE (N = 17)	IMMUNE COMPLEX POSITIVE (N = 7)
NOT RESPONDING TO SINGLE DOSE	2	2
EUTHYROID	8	5
HYPOTHYROID	7	0

TABLE 5.4 IMMUNE COMPLEXES AND THYROID AUTOANTIBODIES FOLLOWING
RADIOACTIVE IODINE THERAPY FOR GRAVES' THYROTOXICOSIS

	MONTHS AFTER RADIOACTIVE IODINE THERAPY								
	0	1	2	3	4	5	6	7	8
CASE 1									
MICROSOMAL	20	N.T.	-	-	-	20	40	40	20
TBII	18.1	25.5	-	-	-	41.7	26.6	14.0	19.0
C1q-SP	9.7	8.5	-	-	-	8.4	8.9	8.8	8.9
CASE 2									
MICROSOMAL	80	40	-	-	160	160	80	-	80
TBII	57.6	54.1	-	-	44.6	73.5	54.1	-	73.5
C1q-SP	9.0	9.7	-	-	9.5	8.8	11.0	-	9.3
CASE 3									
MICROSOMAL	NEG	10	-	-	NEG	NEG	-	-	NEG
TBII	14.7	57.7	-	-	60.2	68.1	-	-	56.7
C1q-SP	8.8	8.6	-	-	9.2	9.5	-	-	8.7
CASE 4									
MICROSOMAL	40	80	80	-	-	-	N.T.	N.T.	N.T.
TBII	13.4	35.1	42.0	-	-	-	37.6	54.3	33.1
C1q-SP	8.4	9.5	8.3	-	-	-	8.3	8.5	11.6
CASE 5									
MICROSOMAL	NEG	-	-	20	80	80	80	40	40
TBII	17.0	-	-	58.5	64.7	58.9	66.2	78.2	80.4
C1q-SP	11.8	-	-	12.1	10.9	11.0	10.3	10.6	9.9
CASE 6									
MICROSOMAL	NEG	-	NEG	20	40	40	20	20	20
TBII	33.3	-	39.8	67.1	74.5	75.4	18.5	53.1	19.5
C1q-SP	10.1	-	10.2	9.6	10.2	10.5	9.2	8.7	9.0

MICROSOMAL = Titre of thyroid microsomal antibodies
 TBII = TSH receptor antibodies, Limit of positivity = 9.0
 C1q-SP = ICs by C1q-solid phase assay, positive > 10.9

PATIENT 1 44 year old female, T₃ 5.1 nmol/l and T₄ 183 nmol/l. No goitre and no eye signs. Diffuse scintigram, uptake 25% at four hours. Treated with 7 mCi and became hypothyroid at three months.

PATIENT 2 38 year old male with moderate goitre marked exophthalmos and severe myopathy. T₄ 320 nmol/l, diffuse thyroid scan, uptake 80%. HLA-DR 3+6. Treated with 7 mCi and still euthyroid at six months.

PATIENT 3 52 year old female, T₃ 6.8 and T₄ 157 nmol/l. No goitre or eye signs, diffuse thyroid scan and uptake 47%. HLA-DR 2. Treated with 5 mCi and hypothyroid at six months.

PATIENT 4 54 year old female, T₃ 7.0 nmol/l and T₄ 340 nmol/l. No goitre and no eye signs, diffuse scan and uptake 40%. Treated with 5 mCi and euthyroid at six months.

PATIENT 5 55 year old female, T₃ 6.3 nmol/l and T₄ 216 nmol/l. Small goitre but no exophthalmos, diffuse scan and uptake 50%. Treated with 5 mCi and hypothyroid at five months.

PATIENT 6 42 year old female, T₃ 5.1 nmol/l and T₄ 230 nmol/l. Small goitre and no eye signs. Diffuse scan, uptake 50%. HLA-DR 6+7. Treated with 6 mCi and hypothyroid at six months.

From the data shown in TABLE 5.4, immune complexes were found in only one patient of the six at diagnosis, they remained positive in this patient until five months after treatment but did not increase in parallel with TBII. Transient IC positivity in the months following therapy was seen in a further two patients but they were only

slightly positive and bore no relation to changes in other immunological tests. Both TSH receptor antibodies and thyroid microsomal antibodies showed significant increases after therapy in five out of the six patients.

IMMUNE COMPLEXES IN HASHIMOTO'S THYROIDITIS

The study included 19 patients who were biochemically and clinically euthyroid (18 female and one male) and 21 patients who were hypothyroid (all female). The mean age in the euthyroid group was 32.3 years (SD 14.7, range 14 to 63 years) while it was 51.6 years (SD 15.4, range 23 to 75 years) in the hypothyroid group. This difference was significant at the 0.001 level. In the euthyroid group, the mean T_4 at diagnosis was 78.6 nmol/l (SD 16.8, range 49 to 105, normal range 70 - 150) and the mean basal TSH was 15.7 mU/l (SD 15.4, range 3.8 to 68.6, normal less than 5.7 mU/l). The ten patients with low or borderline TSH (between 3.8 and 12.2 mU/l) all had exaggerated response to 0.2 mg of TRH intravenously with a mean basal TSH of 7.38 mU/l rising to a mean of 50.4 mU/l twenty minutes after injection of TRH. The mean T_4 in the hypothyroid patients was 43.3 nmol/l (SD 14.3, range 20 to 64) and the basal TSH was 65.4 mU/l (SD 53.5, range 6.5 to 233).

Of the 19 euthyroid patients, ten (52.6%) were positive for immune complexes with a mean value of 6.54% of the maximal radioactivity bound in the C1q solid phase assay (SD 2.23, limit of positivity 5.9%). Fourteen of the 21 (67%) hypothyroid patients were positive

for immune complexes with a mean value of 6.39% (SD 1.43). FIGURE 5.2 shows the values for the C1q solid phase assay in all 40 patients with Hashimoto's thyroiditis plotted against age at first diagnosis. The figure illustrates the younger age of diagnosis in the euthyroid patients, but also suggests an overall biphasic distribution of immune complex positivity with respect to age at diagnosis with a peak for those patients diagnosed before the age of 35 years of age, a trough between 35 and 55 years and a further peak for those patients (mainly hypothyroid) diagnosed in later life. This data is also shown in TABLE 5.5. There are two very high values in patients diagnosed between the ages of 35 and 55 years. The first was in a 38 year old euthyroid lady with a strong family history of autoimmune disease, her mother having both pernicious anaemia and primary hypothyroidism and her sister having been treated for Graves' thyrotoxicosis (C1q-SP = 15.3). The second was a 44 year old hypothyroid lady whose serum was also strongly positive for antinuclear factor and anti-thyroglobulin.

ANTITHYROID ANTIBODIES AND OTHER AUTOANTIBODIES

Thyroid microsomal antibodies were detected at a titre of 40² or above in 15 out of 19 (79%) euthyroid patients and in 19 out of 21 (90%) hypothyroid patients. The modal value for both groups was 80². There was no significant correlation in either group between the value obtained in the C1q-SP assay and the titre of thyroid microsomal antibodies. However the distribution of immune complex positivity with respect to microsomal antibody titre differed in the two groups - see FIGURE 5.3. In the euthyroid group, all four patients with low or undetectable microsomal antibodies were IC positive, and, with increasing titre of antibodies, there was a

FIGURE 5.2 RELATIONSHIP BETWEEN AGE AT DIAGNOSIS AND IMMUNE COMPLEXES
 IN EUTHYROID AND HYPOTHYROID HASHIMOTO'S THYROIDITIS

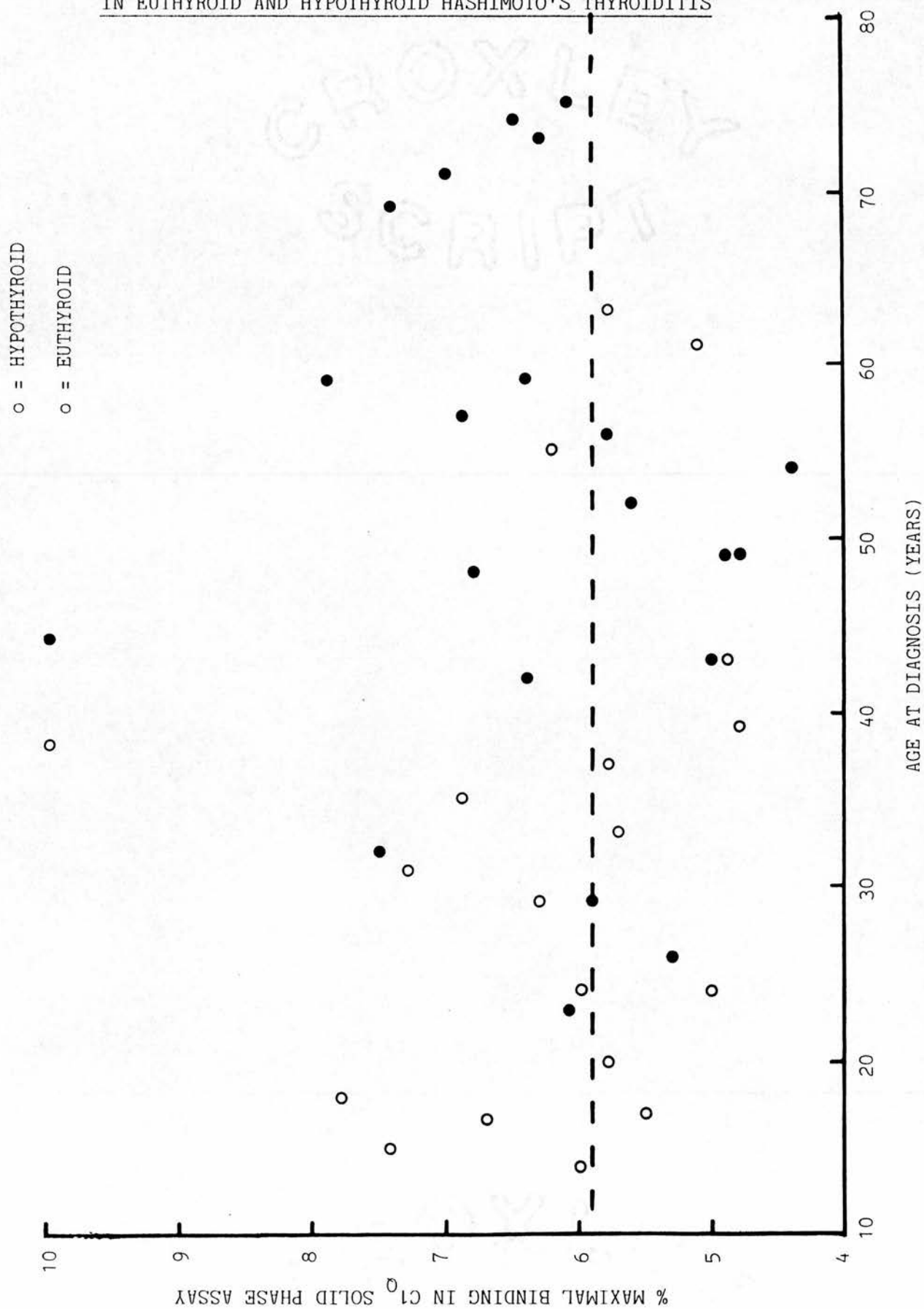
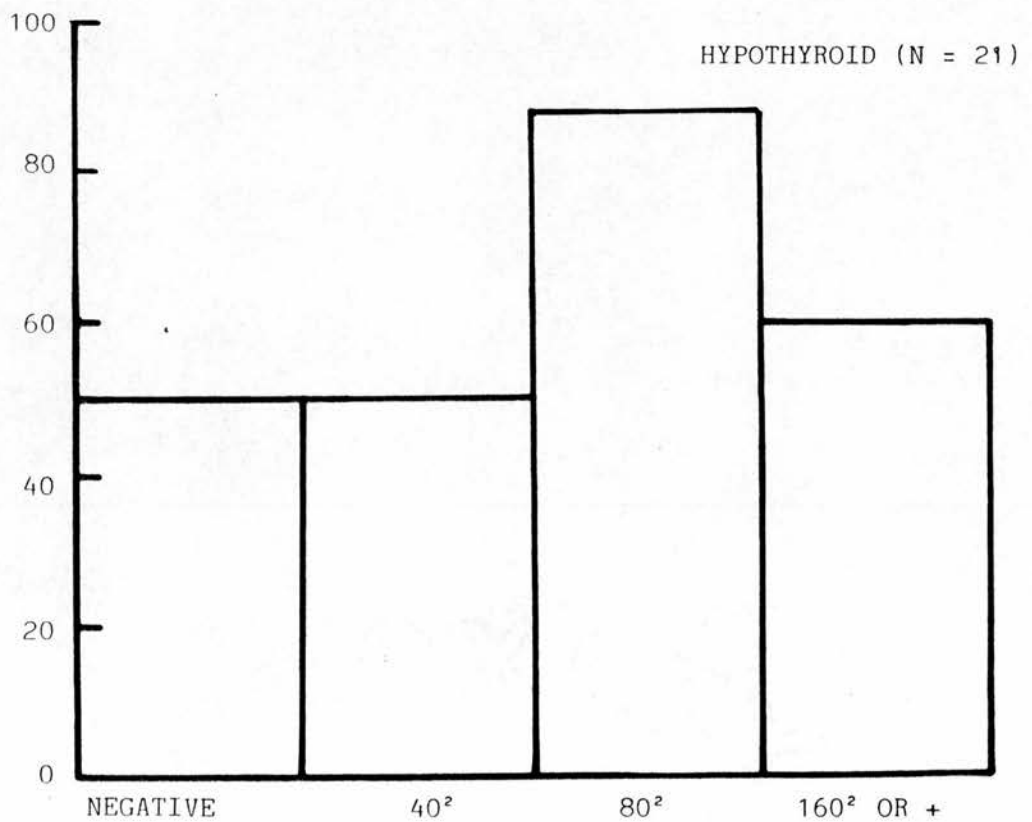


FIGURE 5.3 RELATIONSHIP BETWEEN IMMUNE COMPLEX POSITIVITY AND THYROID MICROSOMAL ANTIBODY TITRE IN EUTHYROID AND HYPOTHYROID HASHIMOTO'S



decreasing proportion of patients with immune complexes. By contrast, hypothyroid patients with high antibody titres were more likely to be positive for immune complexes.

Anti-thyroglobulin antibodies were detected in five (26%) of the euthyroid patients only one of whom was also positive for immune complexes. Five of the hypothyroid patients (24%) had circulating anti-thyroglobulin antibodies and all were positive for immune complexes. The mean value in the C1q-SP assay for the euthyroid patients with anti-thyroglobulin was 5.4% compared to 7.7% for the five hypothyroid patients with anti-thyroglobulin ($p < 0.05$). In the hypothyroid group, the mean C1q-SP binding was 6.07% for the patients with no anti-thyroglobulin which was significantly different to that of the patients with antibodies to thyroglobulin ($p < 0.05$, see FIGURE 5.4).

Parietal cell antibodies were detected in two of the 19 euthyroid Hashimoto's (11%) and in five of the 21 (24%) hypothyroid cases. The mean C1q binding in the seven parietal cell antibody positive cases was 5.85 compared to 6.6% for the antibody negative cases (not significant). Antinuclear factor was found in five cases (two hypothyroid and three euthyroid) but once more there was no significant association with C1q-SP.

FIGURE 5.4 IMMUNE COMPLEXES AND ANTITHYROGLOBULIN ANTIBODIES IN EUTHYROID AND HYPOTHYROID HASHIMOTO'S

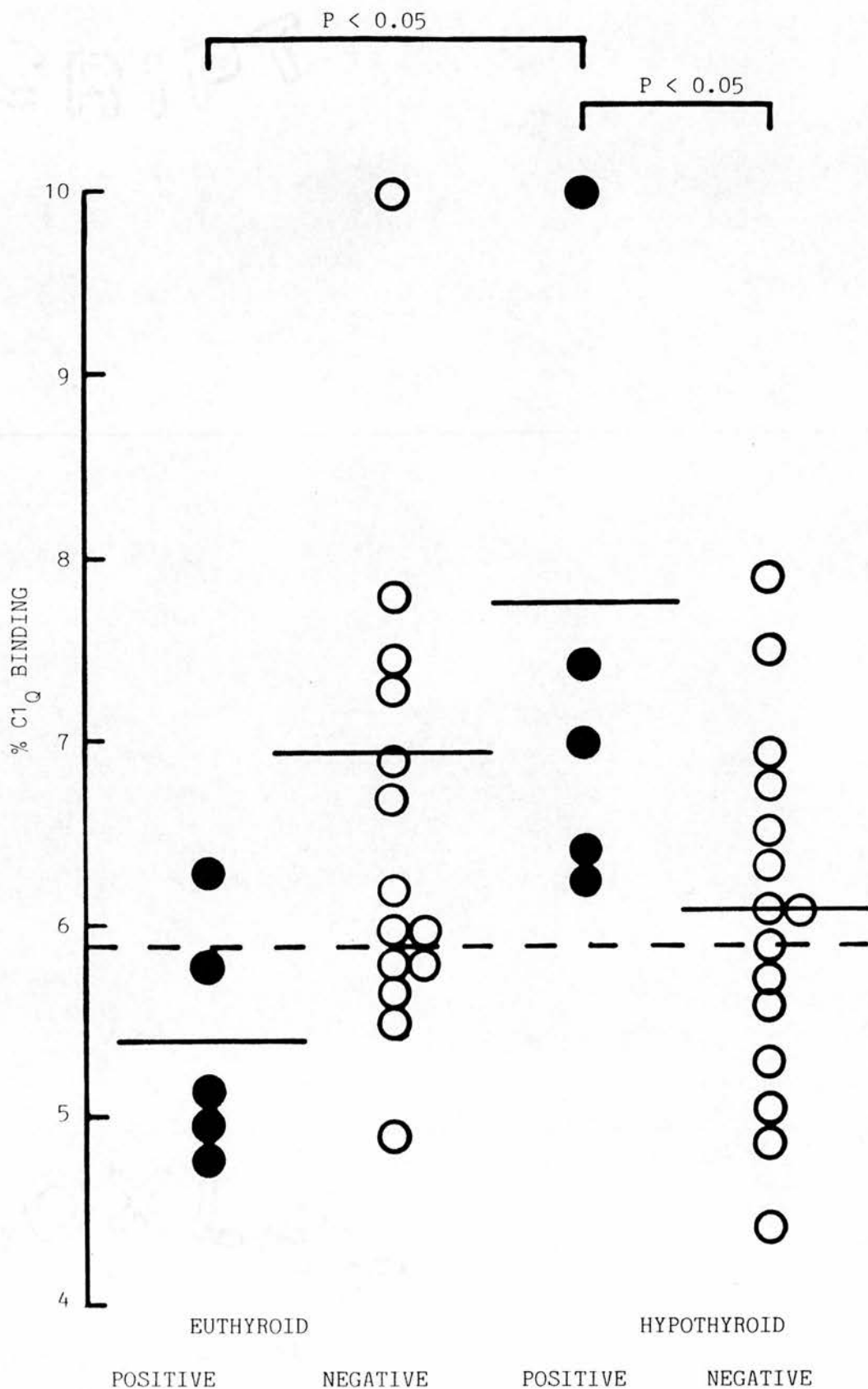


TABLE 5.5 RELATIONSHIP BETWEEN IMMUNE COMPLEX POSITIVITY AND AGE AT DIAGNOSIS IN HASHIMOTO'S THYROIDITIS

	AGE AT DIAGNOSIS (YEARS)					
	UP TO 25	26-35	36-45	46-55	56-65	OVER 65
No. PATIENTS	9	7	7	6	7	5
No. POSITIVE	6	5	3	2	4	5
% POSITIVE	67%	71%	43%	33%	57%	100%

TABLE 5.6 AGE AT DIAGNOSIS AND IMMUNE COMPLEX POSITIVITY IN PRIMARY ATROPHIC HYPOTHYROIDISM

	AGE AT DIAGNOSIS (YEARS)					
	UP TO 25	26-35	36-45	46-55	56-65	OVER 65
No. PATIENTS	5	13	14	29	21	23
No. POSITIVE	3	8	11	18	14	16
% POSITIVE	60%	62%	79%	62%	67%	70%

PRIMARY ATROPHIC HYPOTHYROIDISM

In this study, 105 patients (eleven male and 94 female) were included and the mean age at diagnosis was 52 years (SD 15.2, range 14 to 85 years). The mean T_4 at diagnosis was 36.0 nmol/l (SD 16.8, range 12 to 77) and the mean TSH was 59.1 mU/l (SD 47.2, range 7.3 to 330). Immune complexes were detected in 69 (66%) patients with a mean C1q-SP value of 6.5% (SD 1.26, range 4.8 to 10.8, normal less than 5.9%). There was no significant difference between male and female patients.

There was no significant overall correlation between age at diagnosis and C1q-SP values but, as with Hashimoto patients, there was a variation in the proportion of patients positive with age (see TABLE 5.6). Immune complexes were most commonly found in patients aged 36 to 45 years at the time of diagnosis.

THYROID MICROSOMAL ANTIBODIES AND OTHER AUTOANTIBODIES

Microsomal antibodies were detected by haemagglutination in 90 of the 98 (92%) patients tested and by indirect immunofluorescence in 89 of the 105 patients (85%). Immune complexes were found in five of the eight (63%) patients negative by haemagglutination and in nine of the 15 (60%) negative by immunofluorescence. The mean C1q SP in patients with no or very low levels of microsomal antibody was 6.38%, in patients with a titre of 40^2 it was 6.26% compared to 6.61% in patients with a titre of 80^2 and 7.00% in patients with a titre of 160^2 or above. This trend did not reach statistical significance but there was a significant trend ($p < 0.01$) towards increasing numbers of patients positive in the C1q solid phase assay with increasing

titres of microsomal antibody (see TABLE 5.7). This trend was even more marked when patients with antibodies against the stomach were excluded (the presence of other autoantibodies may affect the relationship between ICs and thyroid antibodies).

Anti-thyroglobulin antibodies were detected by passive haemagglutination in 20 out of 101 patients tested. Sixteen of these patients (80%) were immune complex positive and the mean C1q-SP value was 6.92%. Of the 81 patients negative for anti-thyroglobulin, 50 (62%) had detectable ICs and the mean C1q-SP was 6.42% (no significant difference). Of 22 patients positive for parietal cell antibodies, 12 (55%) were also positive for ICs and the mean C1q-SP binding was 6.42%.

SUBCLINICAL HYPOTHYROIDISM

Eleven patients, all female were studied. The mean age at diagnosis was 51.5 years (SD 15.2, range 28 to 76), the mean T_4 was 81 nmol/l (SD 16.1, range 63 to 116) and the mean TSH was 7.2 (range 3.4 to 30.2). All had an exaggerated TSH response to injected TRH. None had a palpable goitre or marked symptoms of hypothyroidism.

Microsomal antibodies were detected in ten of the eleven patients (91%), anti-thyroglobulin in one (10%) and parietal cell antibodies in five (45%). Immune complexes were found in seven cases (64%) and

TABLE 5.7 CORRELATION BETWEEN POSITIVITY IN THE C1q-SP ASSAY AND TITRE OF MICROSOMAL ANTIBODY IN PRIMARY ATROPHIC HYPOTHYROIDISM

		MICROSOMAL ANTIBODY TITRE			
		20 ²	40 ²	80 ²	160 ²
ALL PATIENTS (N = 98)	No. PATIENTS	32	27	22	17
	No. POSITIVE	20	18	15	13
	% POSITIVE	63%	67%	68%	76%
EXCLUDE PC POSITIVE (N = 83)	No. PATIENTS	31	21	18	13
	No. POSITIVE	19	14	14	11
	% POSITIVE	61%	67%	78%	85%
EXCLUDE ALL STOMACH Abs. (N = 81)*	No. PATIENTS	31	21	17	12
	No. POSITIVE	19	14	14	11
	% POSITIVE	61%	67%	82%	92%

PC = Parietal cell antibodies.

* = This group excludes all patients with either parietal cell or anti-smooth muscle antibodies (both detected using rat stomach as tissue substrate)

the mean C1q-SP value was 6.48%. No correlation was found between the results of the C1q-SP assay and the presence of thyroid or non-thyroid antibodies.

SPONTANEOUS HYPOTHYROIDISM IN GRAVES' DISEASE

Two patients were found, both strongly positive for immune complexes, who had been previously treated for thyrotoxic Graves' disease with antithyroid drugs many years before the diagnosis of hypothyroidism. Neither had recieved destructive antithyroid therapy:

CASE 1

A 62 year old lady presented with mild hypothyroidism (T_4 = 70 nmol/l, TSH = 9.6 mU/l). She had been treated with a full course of antithyroid drugs over twenty years previously. There was no goitre or eye signs when she developed hypothyroidism. Microsomal antibodies were positive by passive haemagglutination at 40^2 and also positive by immunofluorescence. Parietal cell antibody was also found and the C1q-SP binding was strongly positive at 8.9%.

CASE 2

A 54 year old lady developed symptoms of hypothyroidism developed symptoms of hypothyroidism (T_4 56 nmol/l, TSH 28.2 mU/l). She had been treated with antithyroid drugs in her late teens for a single episode of Graves' thyrotoxicosis. She had no goitre or eye signs at her second presentation. Microsomal antibodies were weakly positive

at 20² (negative by immunofluorescence). Parietal cell antibodies were found in the circulation and C1q-SP was strongly positive at 7.2%.

IMMUNE COMPLEXES IN ADDISON'S DISEASE

PATIENTS AND METHODS

Serum was available for study from 210 patients. In 79 cases this serum was from the time of diagnosis, before steroid replacement therapy was commenced. 14 of these cases were of tuberculous origin and 65 idiopathic. Serum from follow up was available from 24 tuberculous cases (one to 22 years from diagnosis) and 105 idiopathic cases (one to 30 years) as well as one case where sarcoidosis was thought to have caused adrenal failure and another case following septicaemia. Where tuberculous Addison's disease was diagnosed, there had been evidence of disseminated tubercle and most cases had adrenal calcification on a plain abdominal X ray.

The diagnosis of Addison's was made on the basis of symptoms and signs (pigmentation and hypotension) and supported by steroid measurements and ACTH stimulation tests although the latter were clearly not available from those patients followed up long after the diagnosis had been made. Associated autoimmune diseases were also diagnosed on the basis of clinical histories and were confirmed by appropriate biochemical tests in all cases.

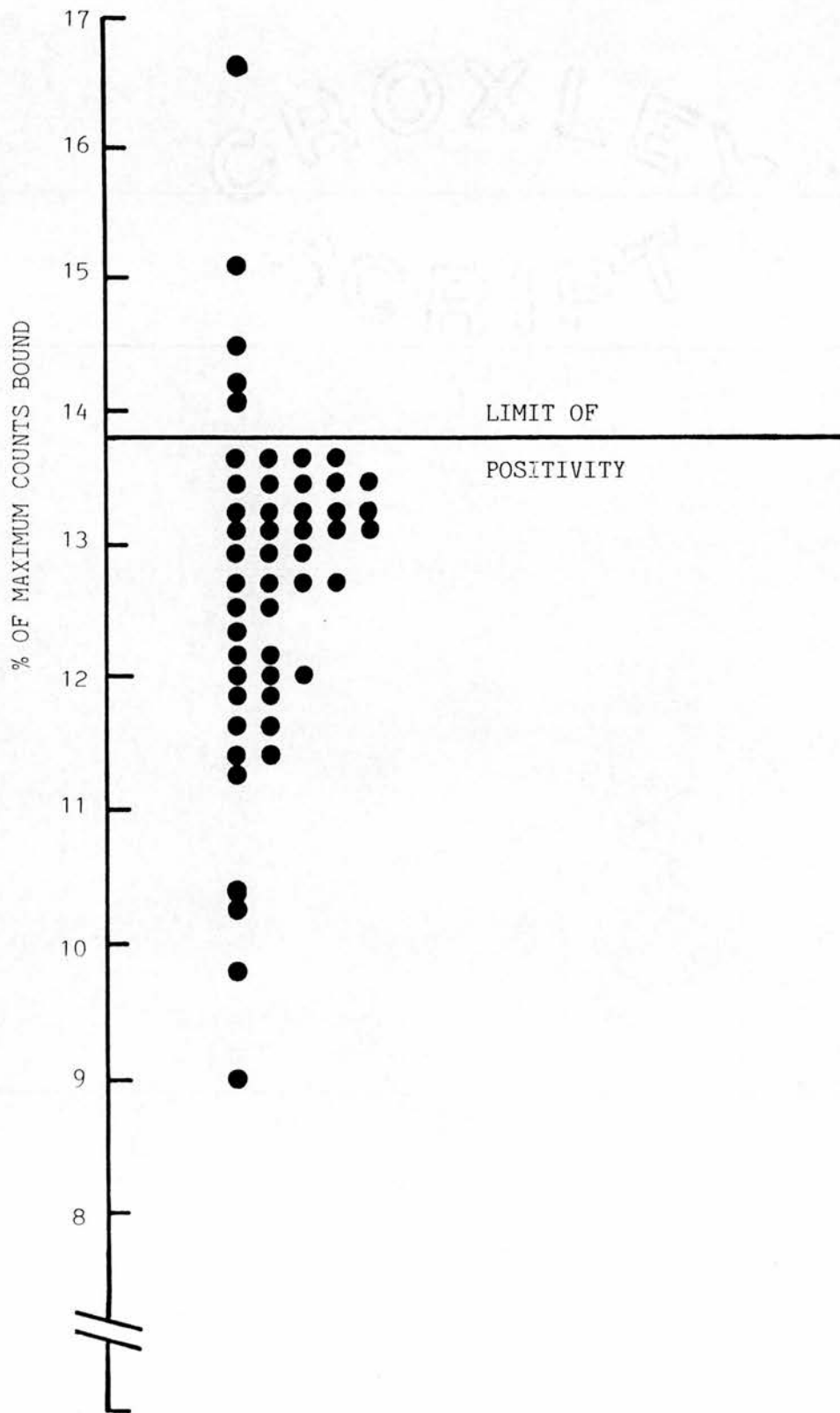
All sera were stored in aliquots, continuously frozen at -20°C and only thawed prior to assay for immune complexes. The C1q assay was performed as detailed in APPENDIX 2. The limit of positivity for this assay was 13.8% of the maximal counts bound and was set using sera from 50 normal blood donors. The use of Addison's sera which had been stored long term was validated by testing 40 control sera which had been stored with the Addison's sera for comparable periods of time see FIGURE 5.5. None of these storage controls had evidence of endocrine or autoimmune disease. The 90th centile for the C1q assay in these 40 controls was 13.7% i.e. virtually identical to that for the normal blood donors used to set the limit of positivity. Autoantibodies were measured by indirect immunofluorescence on five micrometer sections of adrenal, thyroid, pancreas, ovary and stomach. Statistical analyses were performed using linear regression, Student's t test and the Wilcoxon Rank Sum Test as appropriate.

A: ADDISON'S DISEASE AT DIAGNOSIS

Fourteen tuberculous cases were studied (nine male and five female, mean age 42.4 years). Immune complexes were detected in eight of these cases (57%) with a mean C1q-SP value of 15.4% but none of these patients had adrenal autoantibodies.

Of the 65 idiopathic cases studied at diagnosis, adrenal autoantibodies were found in 34 (25 female, nine male, mean age 31.8 years). Immune complexes were found in 22 out of these 34 cases (65%) with a mean C1q-SP of 16.9% for the whole group. Adrenal autoantibodies were not detected in 31 of the 65 idiopathic patients

FIGURE 5.5 STORAGE CONTROLS FOR ADDISON'S EXPERIMENTS



(17 male, 14 female, mean age 40.2 years) and 19 of these cases (61%) were immune complex positive. The mean C1q-SP for this group was 17.9%. The results of the C1q solid phase assay in the different groups of Addison's disease at diagnosis are shown in FIGURE 5.6. Neither the incidence nor the C1q-SP level differed significantly between the adrenal antibody positive and negative idiopathic cases.

In tuberculous Addison's disease, there was a negative correlation between age at diagnosis and the results obtained in the C1q-SP assay (see FIGURE 5.7, $r = -0.67$, $p < 0.01$). No such relationship was found for the idiopathic cases.

In the idiopathic group with no adrenal autoantibodies (31 patients), 18 had no autoantibodies of any sort while the remaining 13 had autoantibodies to tissues other than the adrenal. Of the former group of 18 patients, eight (44%) were positive for immune complexes and the mean C1q-SP value for these patients was 15.6%. Eleven of the 13 patients with non adrenal autoantibodies were IC positive and the mean C1q binding for this group was 21.1% ($p = 0.02$, see FIGURE 5.8).

B: ADDISON'S DISEASE ON FOLLOW-UP

Thirteen out of 24 (54%) tuberculous patients were positive for immune complexes but none had adrenal autantibodies. ICs were not found in the patient with sarcoidosis (male, 32 years old at diagnosis and tested two years later) nor in the patient with Addison's following septicaemia (33 year old female tested three years after diagnosis).

FIGURE 5.6 IMMUNE COMPLEXES AT DIAGNOSIS IN ADDISON'S DISEASE

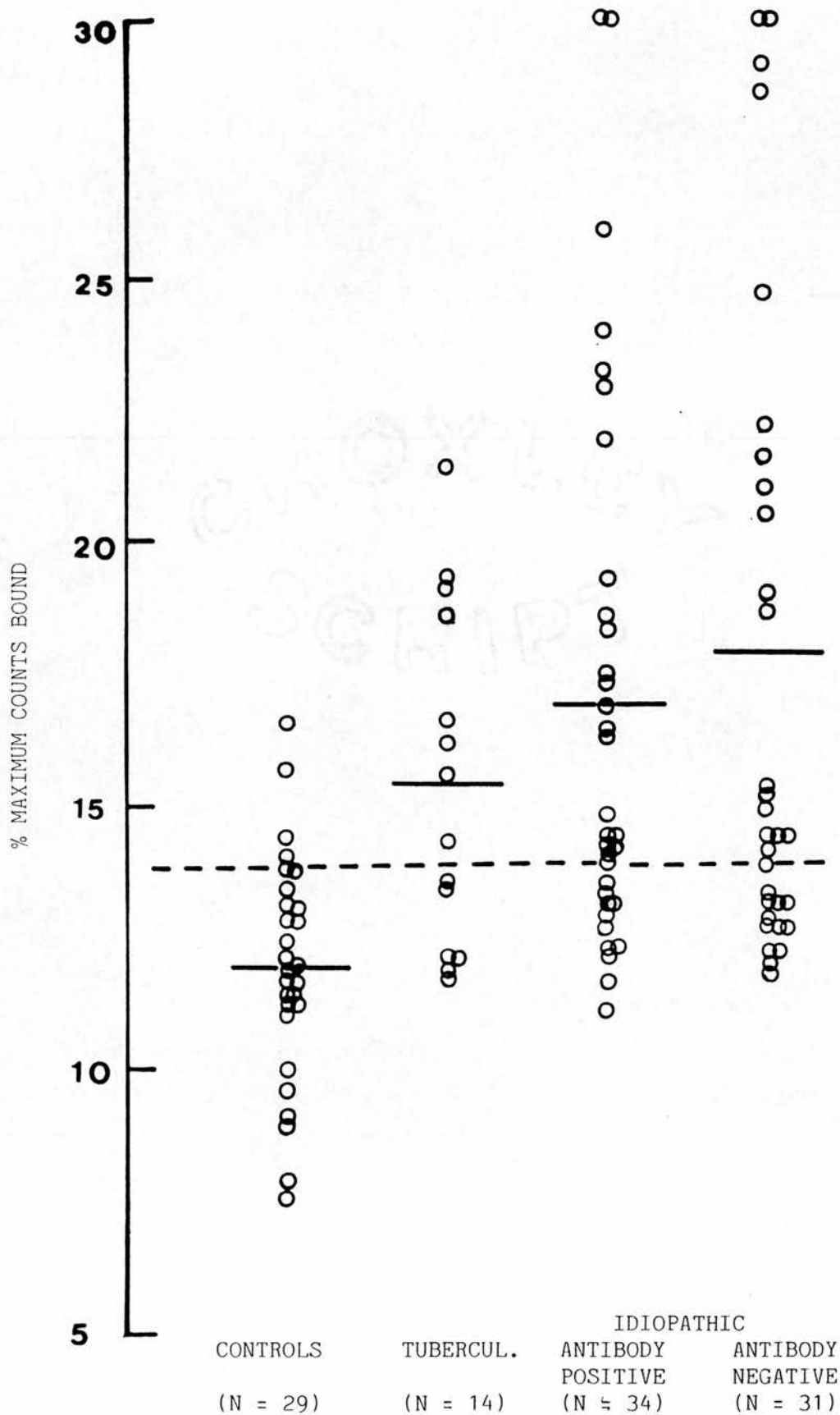


FIGURE 5.7 NEGATIVE CORRELATION BETWEEN AGE AND IMMUNE COMPLEXES
IN TUBERCULOUS ADDISON'S DISEASE

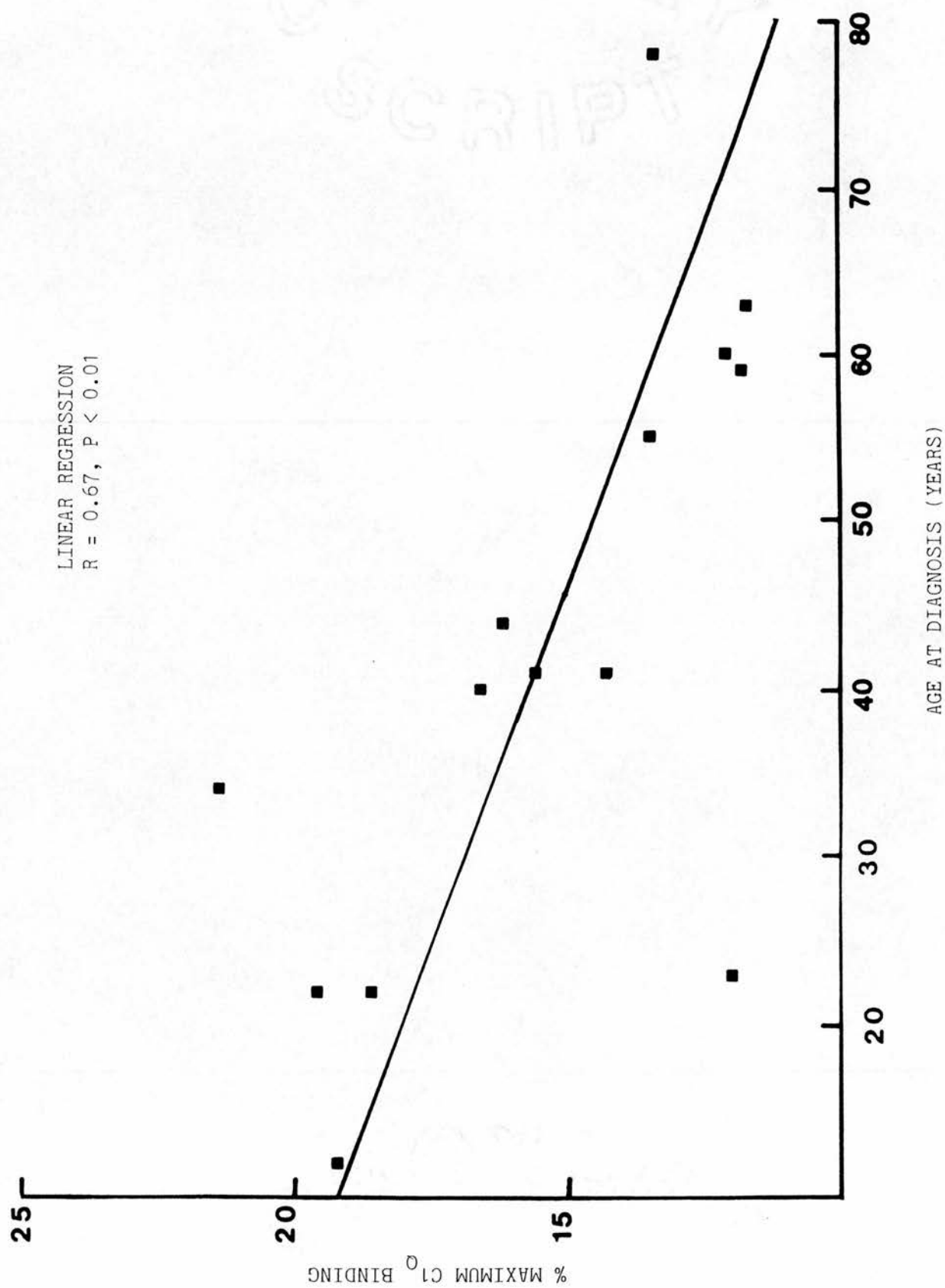
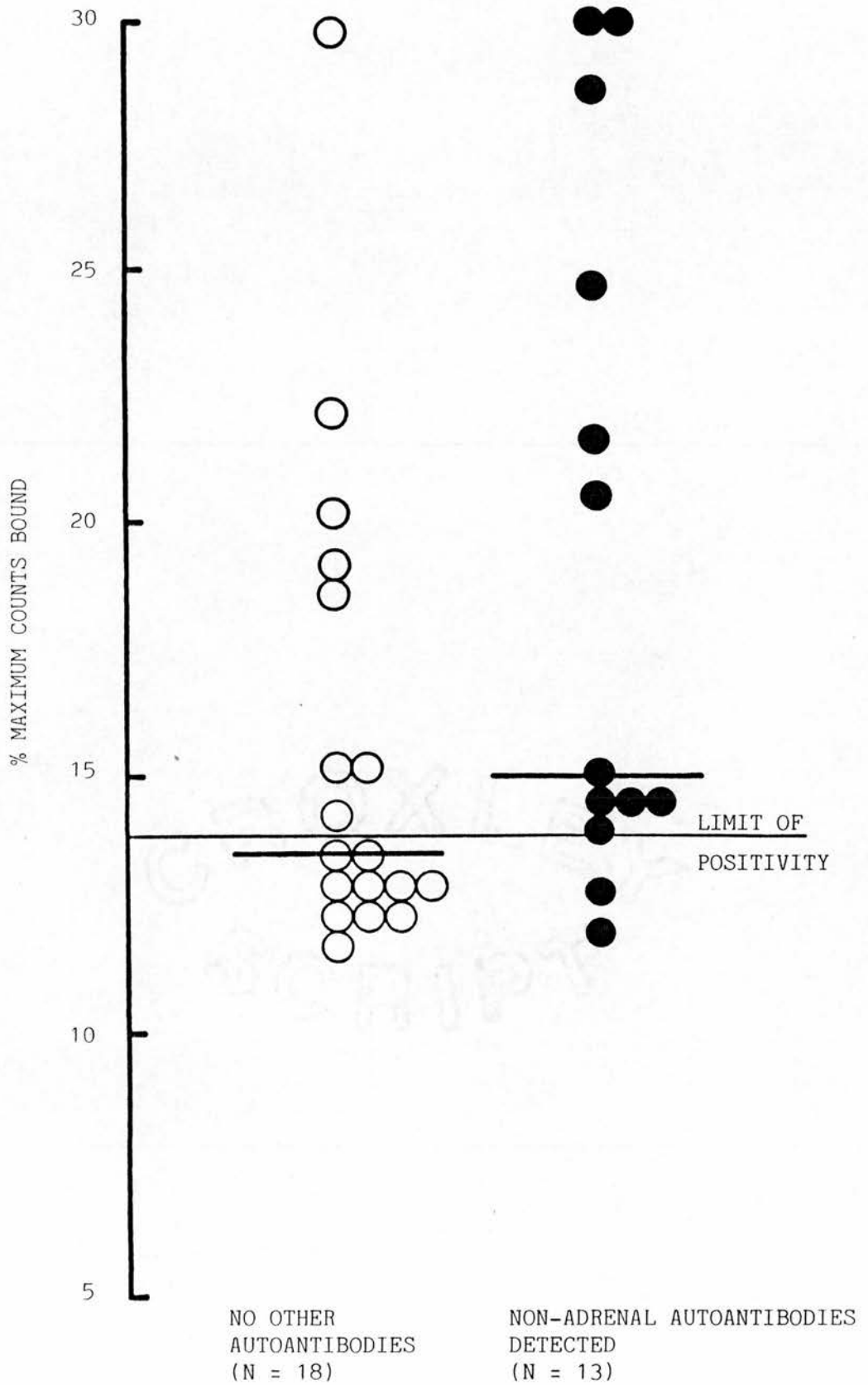


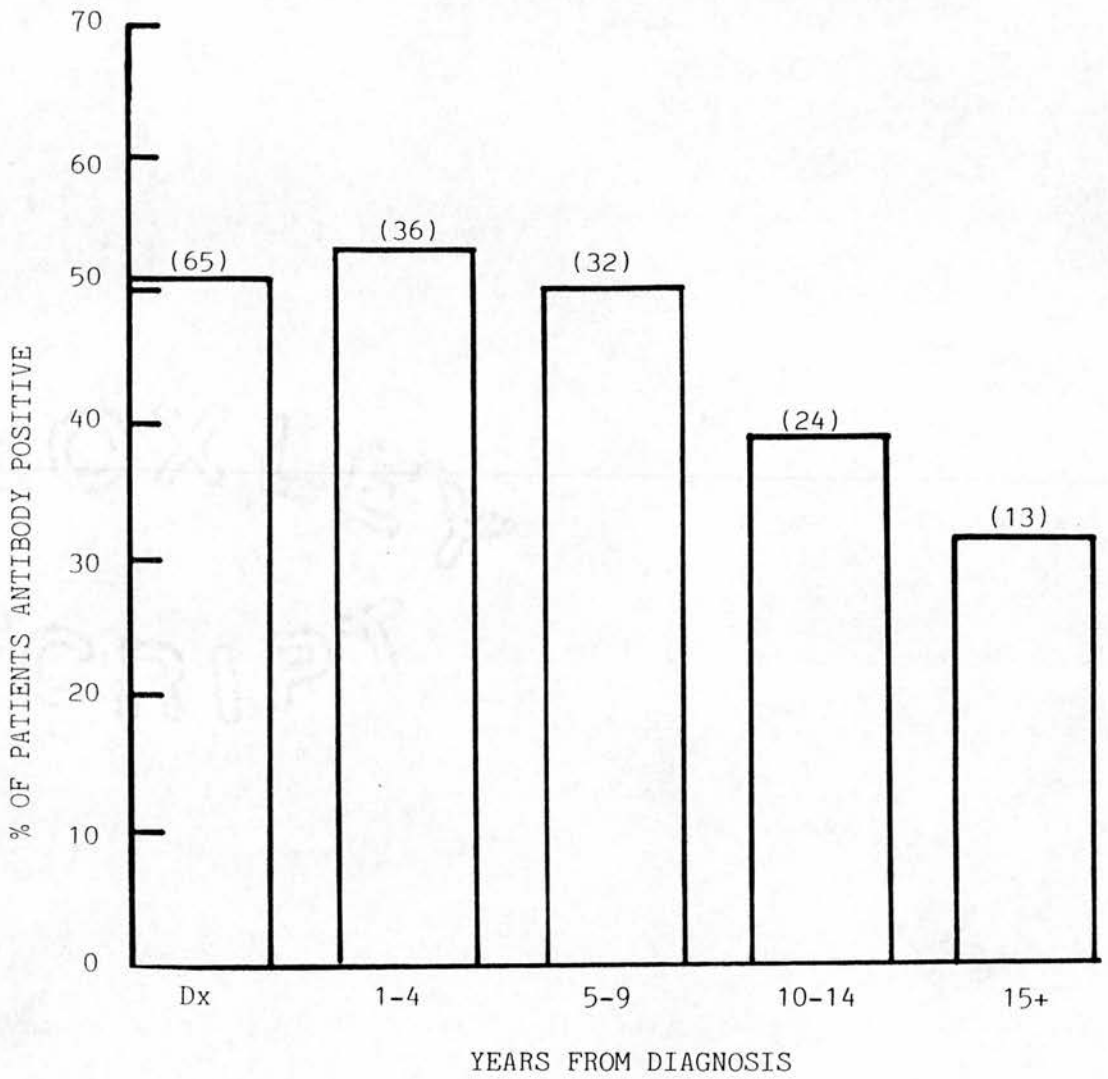
FIGURE 5.8 IMMUNE COMPLEXES IN ADRENAL ANTIBODY NEGATIVE
 IDIOPATHIC ADDISON'S DISEASE



In idiopathic Addison's disease, there was a progressive decline in adrenal antibody positivity with time from diagnosis (see FIGURE 5.9): 51% of 65 patients at diagnosis were positive, 53% of 36 patients tested at one to four years from diagnosis had antibodies, 50% of 32 patients five to nine years from diagnosis, 39% of 24 patients ten to 14 years from diagnosis and 32% of 13 patients at 15 years or more from the time of diagnosis. This decline in adrenal antibody positivity was not however paralleled by a decrease in IC positivity (see FIGURE 5.10) - 54% of patients were still IC positive 15 years from diagnosis. There was a progressive increase from the time of diagnosis of Addison's in the incidence of other autoantibodies or autoimmune diseases (see FIGURE 5.11). 55% of patients at diagnosis of Addison's had evidence of other autoimmune disease, either clinical or subclinical. This was 56% at one to four years, 69% at five to nine years, 67% at ten to 14 years and 85% at 15 years or more from the time of diagnosis of Addison's.

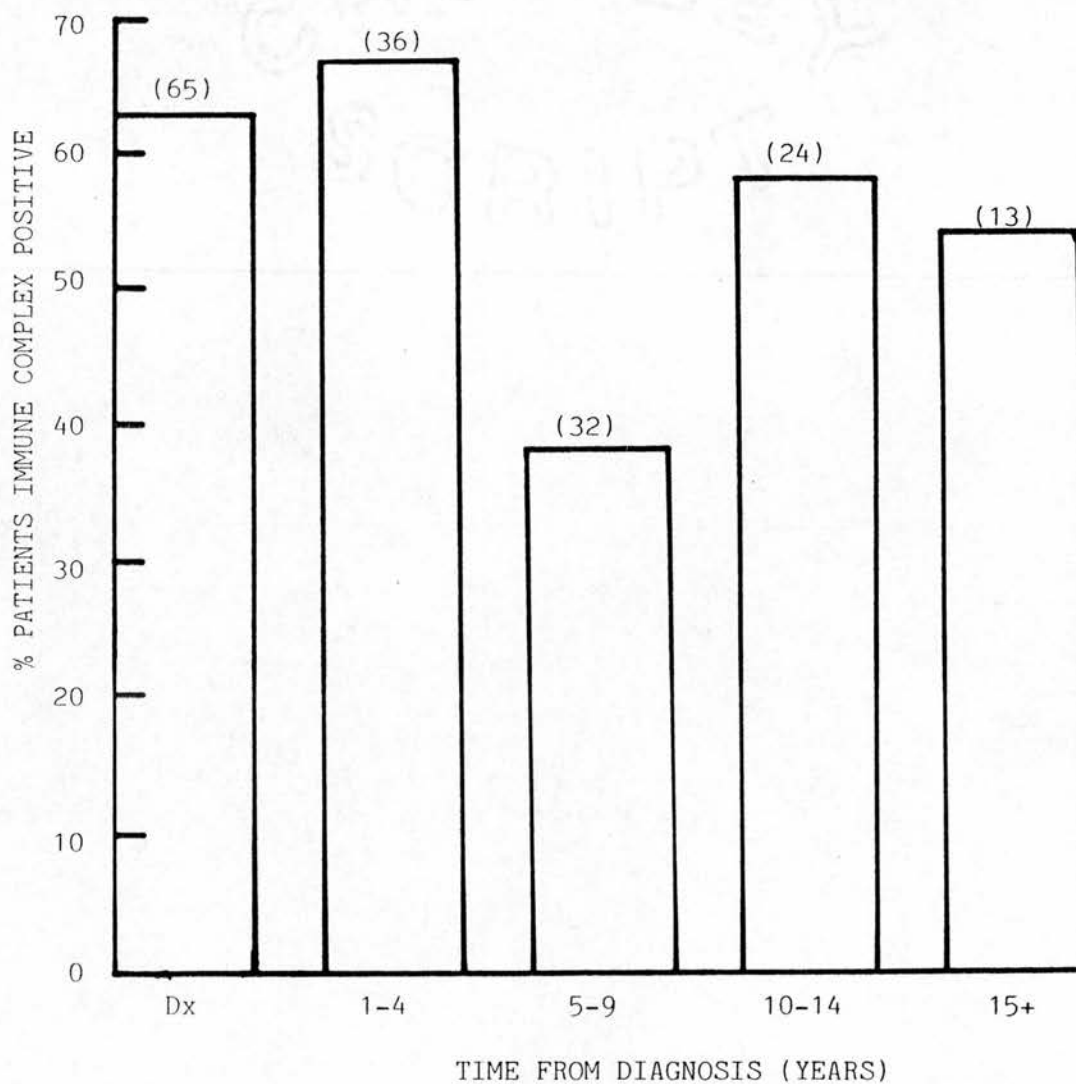
The incidence of non-adrenal autoimmune diseases in this series of idiopathic Addison's is shown in TABLE 5.8 along with the immune complex positivity associated with each disease. Evidence that the increase in the incidence of autoimmune diseases with time from diagnosis of Addison's is related to increasing immune complex positivity is shown in FIGURE 5.12. Of 66 patients with no other autoantibody or autoimmune disease, 32 (48%) were immune complex positive. 43 patients had subclinical diseases (autoantibodies with no clinical features of the associated disease) and 25 of them (58%) were IC positive. Of 61 patients with at least one other autoimmune disease, 41 (67%) were IC positive compared to 19 out of 24 (79%) patients with at least two other diseases.

FIGURE 5.9 DECLINE IN ADRENAL ANTIBODY POSITIVITY WITH TIME FROM DIAGNOSIS IN IDIOPATHIC ADDISON'S DISEASE



NUMBERS AT TOP OF COLUMNS REPRESENT THE TOTAL NUMBERS OF PATIENTS IN EACH GROUP

FIGURE 5.10 IMMUNE COMPLEX POSITIVITY WITH TIME FROM DIAGNOSIS IN IDIOPATHIC ADDISON'S DISEASE



NUMBERS AT THE TOP OF EACH COLUMN REPRESENT THE TOTAL PATIENTS IN EACH GROUP

FIGURE 5.11 INCREASE IN THE INCIDENCE OF NON-PATHOGENIC AUTO-ANTIBODIES AND AUTOIMMUNE DISEASES WITH TIME FROM DIAGNOSIS IN ADDISON'S DISEASE

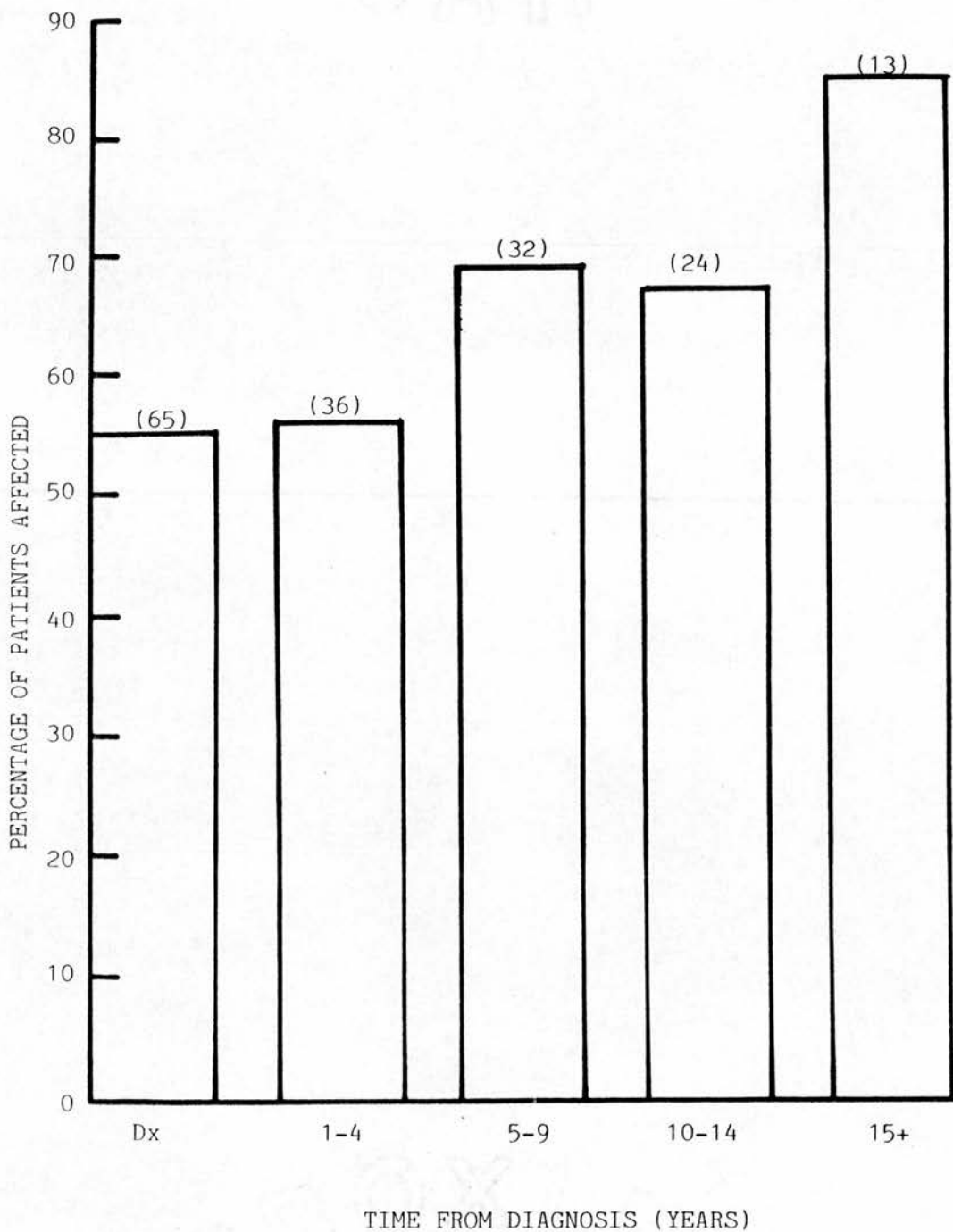
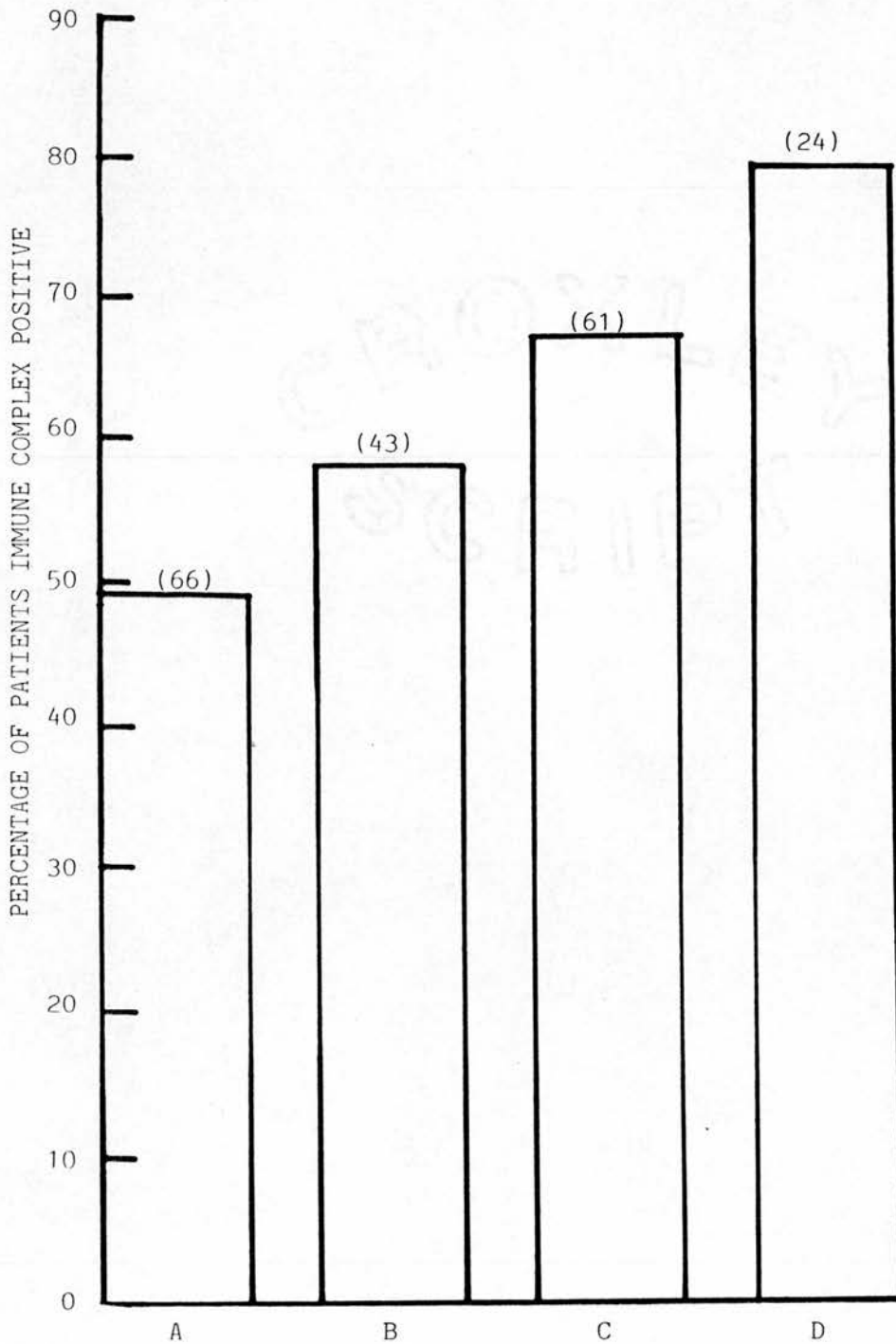


TABLE 5.8 THE INCIDENCE OF IMMUNE COMPLEXES IN AUTOIMMUNE
DISEASES ASSOCIATED WITH ADDISON'S DISEASE

<u>DISEASE</u>	<u>No. of PATIENTS</u>	<u>IC POSITIVE (%)</u>
PERNICIOUS ANAEMIA	8	4 (50%)
DIABETES MELLITUS	13	8 (62%)
HASHIMOTO'S THYROIDITIS	8	5 (62%)
GRAVES' DISEASE	16	10 (62%)
OVARIAN FAILURE	21	13 (62%)
ATROPHIC HYPOTHYROIDISM	14	11 (79%)
HYPOPARATHYROIDISM	5	4 (80%)
VITILIGO	6	5 (83%)

FIGURE 5.12 INCIDENCE OF IMMUNE COMPLEXES IN IDIOPATHIC ADDISON'S ASSOCIATED WITH OTHER AUTOIMMUNE DISEASES AND AUTOANTIBODIES



- A NO OTHER AUTOANTIBODY OR AUTOIMMUNE DISEASE
- B NON-PATHOGENIC AUTOANTIBODIES BUT NO OTHER AUTOIMMUNE DISEASE
- C AT LEAST ONE OTHER AUTOIMMUNE DISEASE
- D AT LEAST TWO OTHER AUTOIMMUNE DISEASES

DISCUSSION

Immune complexes have been sought using the C1q solid phase assay in large series of patients with Graves' disease, Hashimoto's thyroiditis, primary atrophic hypothyroidism and Addison's disease. Immune complexes (ICs) were found in 12/55 (22%) patients with untreated Graves' disease, 10/29 (53%) patients with euthyroid Hashimoto's, 14/21 (67%) with hypothyroid Hashimoto's, 69/105 (66%) with primary atrophic hypothyroidism at diagnosis and in 41/65 (63%) patients with idiopathic Addison's disease at diagnosis. The finding of a small, but significant, percentage positivity in Graves' disease is in keeping with the findings of Brohee et al (1979) and Endo et al (1983) while ICs have been reported in primary hypothyroidism by a number of authors (Calder et al, 1974; Al Khateeb et al, 1978; Brohee et al, 1979). Addison's disease has not been extensively studied previously.

The relationship of ICs to the expression of autoantibodies was explored because of the variable positivity of organ-specific antibodies in the various autoimmune endocrine diseases. For example, in large series of patients with Graves' disease, TSH receptor antibodies are only detectable by the radioreceptor assay in up to 80% of patients (Biro et al, 1982; see also results presented in Chapter 2), while only about a half of patients with idiopathic Addison's disease are positive for adrenal autoantibodies (Blizzard et al, 1967). Although this may in part reflect a relative insensitivity of the assay methods, clearly the autoantibodies might not be detected if they were complexed with antigen in the

circulation. In Graves' disease, a negative correlation was found between TSH receptor antibodies and ICs, in agreement with the results of van der Heide et al (1980), although ICs did not correlate with other thyroid autoantibodies. Only 1/12 IC positive patients was also positive for TSH receptor antibodies compared to 28/42 (67%) IC negative patients ($p < 0.001$). A possible role for ICs in modifying autoantibody expression was also apparent in those idiopathic Addison's patients negative for adrenal antibodies. Eight out of eighteen (44%) patients with no non-adrenal antibodies were positive for ICs compared to eleven out of thirteen (85%) patients with autoantibodies to organs other than the adrenal. The existence of associated autoimmune disease in the latter group makes it virtually certain that their Addison's is autoimmune in origin despite the lack of antibodies to the adrenal.

In their study of 171 patients with various thyroid diseases, Brohee et al (1979) found a positive correlation between IC positivity and thyroid microsomal antibodies. In the patients with newly diagnosed Graves' disease studied here, there was no such relationship, nor did ICs correlate with anti-thyroglobulin antibodies, parietal cell antibodies or antinuclear factor. The relationship between thyroid microsomal antibodies and ICs in Hashimoto's depended on thyroid status. All four patients who were euthyroid and had low microsomal antibody titres were IC positive, with increasing titre of antibodies there was a decreasing proportion of patients with ICs. In hypothyroid Hashimoto's, on the other hand, patients with high antibody titres were most likely to be IC positive. It is possible that all patients with euthyroid Hashimoto's produce comparable amounts of microsomal antibody but that these are not expressed in

some patients because they form immune complexes. The positive correlation in hypothyroid Hashimoto's may relate to the degree of tissue destruction, the amount of residual tissue or the degree of lymphocytic infiltration. A similar relationship existed between anti-thyroglobulin antibodies and ICs: Significant levels of anti-thyroglobulin were found in five euthyroid patients, only one of which was also positive for ICs, while all five anti-thyroglobulin positive hypothyroid patients were also IC positive. In primary atrophic hypothyroidism, there was a significant ($p < 0.01$) trend towards increasing numbers of patients positive in the C1q solid phase assay with increasing microsomal antibody titre. The presence of ICs may thus mask the expression of organ specific autoantibodies in Graves' disease, idiopathic Addison's disease and in euthyroid Hashimoto's but in primary hypothyroidism, whether atrophic or goitrous, ICs correlate with indices of thyroid destruction.

In the normal population, the prevalence of immune complexes increases with age (di Mario et al, 1981). A relationship between IC positivity and age at diagnosis was seen in some of the autoimmune diseases studied here. The mean age of 12 untreated Graves' patients with immune complexes was 55.5 years compared to 42.8 years for the 43 IC negative patients ($p < 0.01$). This may be of relevance to the groups of patients with Graves' disease identified in the previous chapter - patients without a palpable goitre were older and had lower levels of TSH receptor antibodies while patients with a low ^{131}I uptake were also older and had less severe thyrotoxicosis but showed a remarkable incidence of non-thyroid autoantibodies. Of the 14 patients in this study with no goitre, 5 (36%) were IC positive and three out of seven patients (43%) with goitre but low radioactive

iodine uptake were positive. By contrast, only four out of 34 (12%) patients with goitre and high ^{131}I uptake were positive for ICs. Although these results do not reach statistical significance, they would tend to support the hypothesis that immune complexes have a role in the age-related change in the presentation of Graves' disease.

In Hashimoto's thyroiditis, the distribution of IC positivity with age at diagnosis was a biphasic one. Eleven out of 16 (69%) patients presenting at 35 years or younger were IC positive and most of these patients were euthyroid at the time of presentation. By contrast, only 5/13 (38%) of patients presenting in middle life (36 to 55 years) were IC positive, but 9/12 (75%) of cases above the age of 55 at diagnosis had circulating ICs and most of these patients were hypothyroid at presentation. This distribution was not seen with primary atrophic hypothyroidism and, if anything, IC positivity was most common in middle life with 11/14 (79%) of patients presenting between the ages of 36 and 45 being IC positive. No relation between age at diagnosis and IC positivity was seen in idiopathic Addison's disease.

The increase in thyroid autoantibodies following ^{131}I therapy for thyrotoxicosis in Graves' disease was discussed in Chapter 2. There was, however, no indication of a parallel increase in IC positivity in the six patients tested sequentially here. The C1q assay measures complexes with a relative antigen excess and the presence of greatly increased levels of autoantibodies may suggest that a method such as the Raji cell radioimmunoassay, which detects complexes with a relative antibody excess could be more appropriate. IC positivity

prior to radioiodine treatment appeared to influence the prognosis: 7/17 IC negative patients were hypothyroid six months after treatment but none of seven IC positive patients became hypothyroid ($p = 0.056$, Fisher Exact Test). One possibility is that ICs prevent the expression of blocking antibodies to the TSH receptor since, although TSH receptor antibodies rise after ^{131}I therapy, these antibodies are not stimulatory.

The incidence of ICs in patients with subclinical Graves' disease or Graves' disease in remission was comparable to that of thyrotoxic patients. All four exophthalmic patients in these two groups (two in each group) were IC positive but none of the seven patients in the group with frank thyrotoxicosis who had documented exophthalmos was IC positive. It may be that IC positivity is associated with multiple autoimmune diseases (infiltrative exophthalmos and Graves' thyroid disease) but, as thyrotoxicosis develops, the release of antigen including thyroglobulin changes the characteristics of ICs in the circulation, preventing them being detected by the C1q solid phase assay. Both the patients with spontaneous hypothyroidism in Graves' disease were strongly IC positive.

The study of patients with Addison's disease is of particular interest, not only because of the presence of organ-specific autoantibodies to the adrenal (Anderson et al, 1957; Goudie et al, 1966; Irvine & Barnes, 1972) which occur particularly in female patients (Irvine et al, 1967) but also because of the high frequency of other organ-specific autoantibodies for example, to steroid secreting cells in the gonads (Anderson et al, 1968) and to the thyroid (Blizzard et al, 1968). There is also an increased incidence

of organ specific antibodies in first degree relatives of these patients (Wuepper et al, 1969). The association between idiopathic Addison's disease and other autoimmune diseases is well documented (Morse et al, 1961; Kenny & Holliday, 1964) and this has led to the description of polyendocrine deficiency syndromes (Loriaux, 1985). In the patients tested in this study, the incidence of adrenal antibody positivity declined with time from diagnosis but this was not paralleled by a decrease in IC positivity. This may be due to an increase with time from diagnosis of the prevalence of other autoimmune diseases. The high incidence of associated autoantibodies and autoimmune diseases in patients with idiopathic Addison's disease was confirmed in this study and there was direct evidence for increasing IC positivity with increasing organ involvement.

In conclusion, ICs are detectable by the C1q solid phase method in a large proportion of patients with Hashimoto's thyroiditis, primary atrophic hypothyroidism and idiopathic Addison's disease but in a lesser proportion of patients with Graves' disease. Their presence may prevent circulating organ-specific autoantibodies being detected and they may be particularly common in multiple disease states. The information available from IC studies is at present limited until more information is available about the antigens involved. Only in the case of thyroglobulin have antigen-specific ICs been studied (Takeda & Kriss, 1977). However, the microsomal antigen and the TSH receptor have both been solubilised and the combination of physical methods of separation and the use of monoclonal antibodies (Kohn et al, 1983; Weetman et al, 1985a) may yield further information.

Chapter Six

HLA-DR Typing in Graves' Disease

SUMMARY

HLA-DR typing was performed by a standard B lymphocyte microcytotoxicity test on 82 patients with thyrotoxic Graves' disease. 53 of the patients (65%) were positive for the DR-3 allele in keeping with data from the published literature. There was no significant difference between DR-3 positive and DR-3 negative patients in terms of age at first diagnosis, thyroid hormones or four hour ^{131}I uptake.

Thyroid microsomal antibodies were found particularly in patients who were DR-3 homozygotes or those who had the phenotype DR-3+4 (such patients may be specially susceptible to autoimmune endocrine disease): Ten out of eleven (91%) of these patients were positive for thyroid microsomal antibodies compared to 23 (55%) of the remaining 42 patients tested ($p = 0.05$).

TSH receptor antibodies (TBII) were found in 44/53 (83%) patients tested. The median value for TBII in DR-2 positive patients was 25.2 compared to 17.5 in non DR-2 positive patients ($p < 0.05$). The median TBII in seven DR-2 homozygotes was 36.2 compared to 19.9 in the 15 other DR-2 positive patients ($p < 0.01$).

Of 24 patients followed up after starting carbimazole, eight were DR-2 positive. The median TBII was not only higher at diagnosis in these patients but but it also took six months to return to normal after starting treatment despite adequate biochemical control of their thyrotoxicosis. By contrast, in sixteen other patients studied TBII was normal in two months. 26 DR typed patients were tested for

TBII at least 18 months after ^{131}I therapy. DR-3 was found in 12/15 (80%) patients with persisting TBII compared to 5/11 (45%) with no TBII.

These results suggest that autoimmune thyroid stimulation and thyroid destruction might be under separate genetic control and that HLA-DR2 may be a marker for the former. HLA-DR3, although strongly associated with Graves' disease, does not appear to be a useful marker for any particular aspect of the disease.

INTRODUCTION

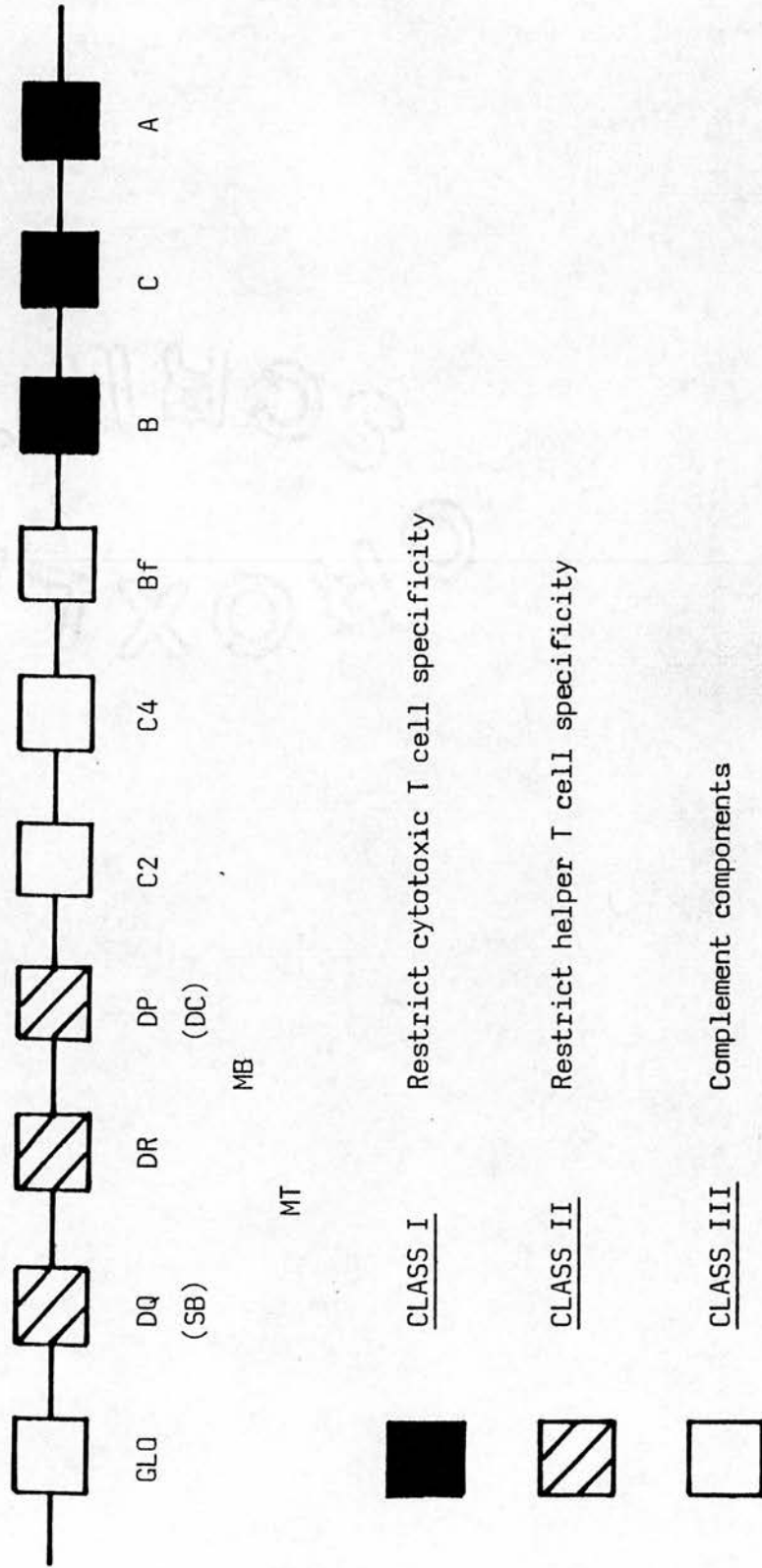
The Human Leukocyte Antigen (HLA) system is a complex of some 1000 genes on the short arm of chromosome six. Its discovery arose from the observation of antibodies in the sera of parous women or multiply transfused patients which would agglutinate white blood cells (Dausset, 1954; Payne, 1957). Very quickly, a polymorphism became apparent in the system whereby the serum from a particular patient would agglutinate the cells of some patients but not of others. This polymorphism became relatively easy to study after Terasaki and McClelland (1964) introduced a microcytotoxicity assay based on complement-mediated lysis of leukocytes in microwell plates. The HLA system is now known to code for glycoproteins on the surface of all nucleated cells in the body and comprises four loci (A, B, C and D). One haplotype is inherited from each parent and the alleles at each

locus are expressed in a codominant fashion. Because the gene complex is relatively small, recombination is quite uncommon and the haplotypes are generally inherited intact.

The structure of the human Major Histocompatibility Complex (MHC) is shown in FIGURE 6.1. Products of the A, B and C loci are known as Class I antigens and are expressed on the surface of all nucleated cells and blood platelets. Class II antigens include the ten (eighth histocompatibility workshop) recognised alleles of the DR locus and are expressed on the surface of B lymphocytes, accessory cells of the immune system (monocytes, macrophages and Langerhans cells), activated T lymphocytes and some endothelial cells. Until recently typing for alleles of the D locus could only be performed by a mixed lymphocyte reaction whereby stimulator cells treated either by irradiation or with mitomycin would induce transformation in HLA non-identical responder cells. A microcytotoxicity assay on B cells, similar to that used for alleles of the A, B, and C loci, is now widely employed and specificities thus identified are known as DR (D-related). Other Class II antigens are coded for in the DQ and DP loci (formerly SB and DC respectively) and, in addition, two systems - MB and MT - are recognised each with three alleles in linkage disequilibrium with those of the DR locus. The complex also codes for three complement components - C2, C4 and properdin factor B (Bf) of the alternative pathway, as well as two enzyme systems -the red cell glyoxylase (Glo) and the adrenal 21-hydroxylase.

The gene product of the HLA -A, B, and C loci is a 43,000 molecular weight glycoprotein found at the cell surface in association with a 10,000 Dalton beta₂ microglobulin (coded for on chromosome 15). Class

FIGURE 6.1 THE HUMAN MAJOR HISTOCOMPATIBILITY COMPLEX



I antigens are thought to restrict the action of cytotoxic T cells i.e. antigen bearing cells need to be similar in their HLA complement to that of the cytotoxic T cells for the latter to be active (McMicheal et al, 1977; Dickmeiss et al, 1977). Class II (DR) antigens differ in structure: They consist of two similar chains, an alpha chain of 32,000 Daltons and a beta chain of 28,000 Daltons, both of which are coded for at the DR locus. These antigens are thought to restrict helper cell function and are therefore important in the relationship between immunoregulatory T cell subsets and effector cells. For example, Thorsby et al (1983), in studying the T cell response to purified protein derivative of tuberculin and herpes simplex virus, showed that the T cell response to these antigens was reduced by about a half if the effector cells and antigen presenting cells shared only one DR determinant, while it was very poor indeed if they were completely non identical for Class II antigens. The function of Class II antigens is discussed in detail in Chapter 8.

The importance of HLA typing in clinical practice lies in the association between certain HLA alleles and disease states, particularly those with an autoimmune aetiology. The association between histocompatibility antigens and disease was first appreciated in the mid 1960s when susceptibility to virus-induced leukaemia in the mouse was shown to be related to the major histocompatibility complex (Lilly et al, 1964). The susceptibility to a disease is quantified in terms of relative risk (RR) which is defined as the ratio of the particular antigen frequency in patients with the disease to the allele frequency in control subjects (Svejgaard et al, 1974). In the autoimmune diseases, early reports of associations with the HLA-A, B and C alleles have largely been superceded by

associations with the DR locus which are generally more marked. The recognised associations between HLA-DR loci and autoimmune diseases are summarised in TABLE 6.1.

The mechanism underlying these HLA associations with autoimmune diseases is not clear. One of the problems is the incomplete association between particular disease states and HLA alleles although this may become less of a problem as new specificities become characterised. For example, coeliac disease has recently been shown to be more closely linked to the DC3 phenotype than it is to HLA-DR3 (Tosi et al, 1983). Hypotheses that the HLA system restricts the range of antigens to which the individual is capable of responding or that the glycoprotein gene products act as receptors for viruses or chemically modified antigens remain largely unproven. The main research thrust has been towards identifying genes in the major histocompatibility complex which control the immune response such as those described in the mouse or the guinea pig (McDevitt and Chinitz, 1969; Benacerraf and McDevitt, 1972). Unfortunately, the experimental evidence for a direct role of the histocompatibility complex in determining individual differences in immune response in Man is sparse. Sasazuki et al (1978) have shown in normal Japanese subjects, that the response to tetanus toxoid is under the influence of HLA-B5, and to a greater extent of an HLA-D determinant. A similar effect with the response to influenza A and vaccinia has been reported (for review see Sasazuki et al, 1983). These authors have further studied the antigen-specific T cell response to streptococcal cell wall antigen in normal families which were HLA typed. The correlation between the level of response in HLA identical sibs was very high, but was less marked in sibs sharing only one haplotype and

TABLE 6.1 ASSOCIATIONS BETWEEN HLA-DR AND AUTOIMMUNE DISEASES

<u>DISEASE</u>	<u>PHENOTYPE</u>	<u>RELATIVE RISK</u>
Goodpasture's syndrome	DR2	15.9
Multiple sclerosis	DR2	4.1
Dermatitis herpetiformis	DR3	15.4
Coeliac disease	DR3	10.8
Systemic lupus erythematosus	DR3	5.8
Sicca syndrome	DR3	9.7
Idiopathic membranous nephropathy	DR3	12.0
Myasthenia gravis	DR3	2.5
Addison's disease (idiopathic)	DR3	6.3
Graves' disease	DR3	3.7
Type I diabetes	DR3	3.3
Rheumatoid arthritis	DR4	4.2
Hydralazine-induced SLE	DR4	5.6
IgA nephropathy	DR4	4.0
Type I diabetes	DR4	6.4
Juvenile rheumatoid (pauciarticular)	DR5	5.2
Pernicious anaemia	DR5	5.4
Hashimoto's thyroiditis	DR5	3.2

* Data from Svejgaard, Platz and Ryder (1983).

very low in HLA non-identical sibs. The response to the antigen in vitro could be abolished by the addition of monoclonal anti-DR indicating that the reaction between antigen presenting cells and T cells was restricted by Class II antigens. The mechanism for the HLA-mediated control of response to streptococcal antigen was thought to be the generation of Leu-2a positive suppressor cells and a linkage between this response and the MT1 allele was proposed. Some direct evidence that HLA phenotype might affect lymphocyte subsets has recently accumulated: Lawley et al (1981) have shown decreased numbers of cells with Fc receptors for IgG in HLA B8/DR3 positive patients with dermatitis herpetiformis and also in normal subjects with this phenotype. This subset of cells contains those lymphocytes with suppressor cell activity (see Chapter 7). In the study of Caruso et al (1984), the DR1 phenotype was associated with increases of both T-gamma and T-mu cells, while both subsets were decreased, although not significantly, in DR3 positive normal subjects. These authors (Caruso et al, 1985) have recently extended their study to include the use of monoclonal antibodies to define T cell subsets. Both OKT4 and OKT8 positive cells were increased in association with DR1 while, once more there was a non-significant decrease in DR3 positive subjects.

The association between HLA-B8 and Graves' disease was first recognised in 1973 by Grumet et al and has been confirmed in many subsequent studies (Farid et al, 1975; Thorsby et al, 1975; Whittingham et al, 1975; Balazs et al, 1978; Mather et al, 1980). In a further report by Grumet et al (1974), the association with HLA-B8 was confirmed but was not found in patients with Graves' disease following external neck irradiation. In the study by Farid et al

(1976), not only was the B8 allele frequency increased but so too was the incidence of homozygosity compared to the general population. An association with HLA-DR3 is now recognised and this is more marked than that with B8 (Bech et al, 1977; Farid et al, 1979; Allanic et al, 1980; Farid et al, 1980a; Dahlberg et al, 1981; McKenna et al, 1982). HLA-B8 and DR3 are in linkage disequilibrium and the latter is more closely linked to the gene or genes which confer susceptibility to Graves' disease while the association of the former may only be due to the linkage disequilibrium with DR3. These associations only apply to caucasians with Graves' disease: For example, Grumet et al (1975) found no association with HLA-B8 in Japanese patients while, more recently, an increase of HLA-Bw35 has been reported (Kawa et al, 1977; Nakao et al, 1978). A recent study in a Chinese population (Hawkins et al, 1985) found an increase in HLA-Bw46 in early onset Graves', and of B5 in late onset disease. A younger age of onset in DR3 positive caucasian patients was reported by Farid et al (1980a) but this finding has not been a universal one (Balazs et al, 1978).

There is therefore no doubt that, in caucasian populations, the B8/DR3 axis relates to the development of Graves' disease, but does it relate to the clinical features of the disease? An association between HLA-B8 and thyroglobulin antibodies and in vitro lymphocyte transformation was found by Balazs et al (1978) but, generally, no correlation between HLA alleles and thyroid microsomal antibodies or anti-thyroglobulin has been found (Whittingham et al, 1975; Bech et al, 1977; Mather et al, 1980; Dahlberg et al, 1981). Similarly, the B8/DR3 alleles do not correlate particularly with the presence or titre of TSH receptor antibodies (McGregor et al, 1980a; Mather et al, 1980; Schernthaner et al, 1980). In a family with multiple cases

of Hashimoto's disease, no correlation was found between HLA haplotype and the expression of antithyroid antibodies (Gordin et al, 1979). By contrast, in systemic lupus erythematosus, Ahearn et al (1982) have found the DR2 and DR3 phenotypes to correlate with circulating autoantibodies. Briones-Urbina et al (1982) have reported high levels of immunoglobulins in patients with Graves' disease and that the IgG level was higher in DR3 positive patients than in those without this phenotype. Exophthalmos has been associated with HLA-B8 (Balazs et al, 1978) and with HLA-DR3 (Farid et al, 1980b) although Bech et al (1977) found no such association.

In patients euthyroid after antithyroid drugs, Irvine et al (1977) found the persistence of thyroid microsomal antibodies to correlate with HLA-B8, a relationship which was confirmed by Schernthaner et al (1979) although the latter study found no correlation with the presence of TSH receptor antibodies. Indeed, after drug therapy, McGregor et al (1980a) found TSH receptor antibodies to be higher in patients negative for DR3. When Stenszky et al (1983) attempted to divide Graves' patients into subgroups, those patients with eye disease, high levels of autoantibodies, strong family history and a tendency to relapse after medical therapy had a particularly high incidence of HLA-B8. Thyroid abnormalities are common in the first degree relatives of patients with Graves' disease (for example, thyroid antibodies and abnormal responses in the TRH test) but these do not necessarily correlate with HLA (Chopra et al, 1977). It seems therefore, that HLA B8/DR3 may relate to susceptibility to disease and to the persistence of autoantibodies but not to the clinical features of the disease.

HLA-B8 has also been associated with primary atrophic hypothyroidism but not with Hashimoto's thyroiditis (Irvine et al, 1978; Moens et al, 1979). Similarly, in polyglandular autoimmune disease, the incidence of B8 is increased in diabetic patients with Graves' but not in patients with Hashimoto's and diabetes (Farid et al, 1980). When an early report of increased HLA-DR3 in Hashimoto's was re-examined the apparent increase was found to be due to patients with atrophic hypothyroidism (Moens & Farid, 1978; Farid et al, 1981). Goitrous thyroiditis is now known to be associated with HLA-DR5 (Farid et al, 1980c; Weissel et al, 1980; Farid et al, 1981). The overlap between Graves' disease and Hashimoto's thyroiditis and the possibility of a DR5 related subgroup of Graves' was discussed in Chapter 4 - Schleusener et al (1983) have identified a group of thyrotoxic patients with a diffuse goitre but no ophthalmopathy or TSH receptor antibodies where the incidence of DR5 was increased. The syndrome of painless thyroiditis with transient thyrotoxicosis (PTTT) has only recently been recognised and an association with HLA-DR3 documented, although cases which occur in the post partum period also have an increased incidence of DR5 (Farid et al, 1983). TSH receptor antibodies have been detected in patients with Hashimoto's thyroiditis by Bliddal et al (1982b): Positivity in the radioreceptor assay was associated with DR5 while, in an assay based on the stimulation of human thyroid adenylate cyclase, it was the negative patients who tended to be DR5 positive. These two subpopulations of antibodies may thus be under independent genetic control.

The presence of genetic influences other than DR3 is also shown by family studies. For example, Farid et al (1980d) looked at two families with multiple cases of Graves' disease and showed that disease susceptibility was not necessarily related to B8/DR3. In another family reported by these authors (Farid et al, 1979), Graves' disease was strongly associated with a maternal haplotype containing HLA-DR2. Reviewing 14 pairs of sibs with Graves' disease, they (Farid et al, 1980) found nine of them to be HLA identical suggesting that a contribution from both parental haplotypes might be important. These cases did not show an increase in B8, although this allele was frequently found in the parental haplotype which did not relate to disease susceptibility.

The value of HLA typing in predicting the response to therapy in Graves' disease is controversial. Early reports suggested that B8 and DR3 were markers for relapsing disease (Irvine et al, 1977b; Bech et al, 1977). By combining the measurement of TSH receptor antibodies after drug therapy with DR typing, McGregor et al (1980a) were able to successfully predict the ultimate outcome in 95% of cases. HLA-DR3 has also been reported to relate to the outcome after radioactive iodine (Davies et al, 1982) - in this study, there was a high incidence of the allele in TSH receptor antibody negative patients who did not respond to therapy. The consensus from recent follow up studies after drug therapy is that HLA-B8 or -DR3 is not an accurate predictor of relapsing disease (Scherthaner et al, 1980; Dahlberg et al, 1981; McKenna et al, 1982; Young et al, 1985). The benefit of DR typing in clinical practice is, at best, marginal and since it is so technically demanding, it probably has no place in routine patient management.

The aim of the work presented in this chapter was firstly to confirm the high incidence of HLA-DR3 in thyrotoxic Graves' disease which is reported in the literature and thus to validate the inclusion of patients studied in other studies described in this dissertation. Secondly, to seek correlations between HLA markers and clinical or immunological features of the disease and evidence to support the hypothesis of disease heterogeneity put forward in Chapter 4. The role of HLA-DR typing in predicting the response to antithyroid drugs and to radioactive iodine therapy was re-examined in limited clinical studies.

PATIENTS AND METHODS

HLA-DR typing was performed by a lymphocyte microcytotoxicity test as described in APPENDIX 3 on 82 unselected patients with thyrotoxic Graves' disease. The study comprised 14 male and 68 female patients with a mean age at diagnosis of 38.3 years (SD = 13.1, range 10 to 69 years). The diagnosis was made, as before, on the basis of symptoms and signs, raised T₃ and/or T₄, flat TRH test and diffuse goitre and/or scintiscan. Also typed were three patients with pure ophthalmic Graves' disease (two men aged 48 and 49, one woman aged 61 at diagnosis). All three of these patients had a flat TRH test but none was clinically or biochemically thyrotoxic. It was not possible in three patients to obtain a definite tissue type. Only one antiserum was available for HLA-DR1 and therefore results for this allele may not reflect the true incidence in Graves' disease.

Thyroid hormone assays were performed by in-house radioimmunoassay (Department of Clinical Chemistry, Royal Infirmary, Edinburgh). Anti-thyroglobulin and anti-microsomal antibodies were detected by passive haemagglutination using commercially available kits (Wellcome) and TSH receptor antibodies were assayed using neat serum as described in APPENDIX 1.

STATISTICS

Thyroid hormone and age data were compared using Student's t Test and TSH receptor antibody values were analysed with the Mann-Witney U Test. Other data were subjected to the Fisher Exact Test.

RESULTS

The DR phenotypes of 82 patients with thyrotoxic Graves' disease are shown in TABLE 6.2 and the frequency of the individual alleles compared to that in the normal United Kingdom population is shown in FIGURE 6.2. The only allele showing a significant difference is DR-3 with 65% of Graves' patients positive compared to 27% of the normal population.

AGE AT DIAGNOSIS

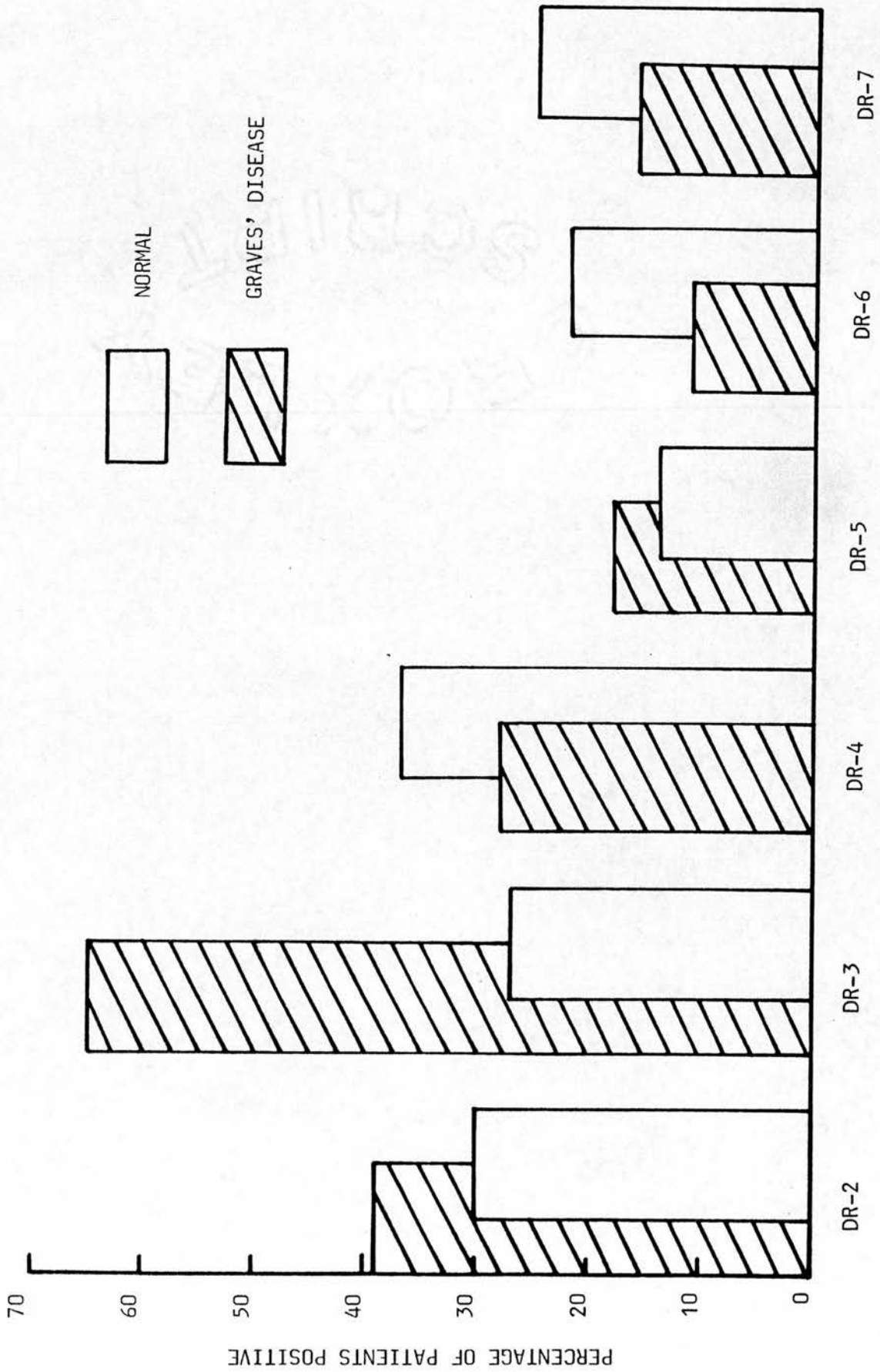
The mean age of the 82 patients at diagnosis was 38 years and no group of patients with any one allele differed significantly from this. The mean age for DR-3 homozygotes (n = 7) was 35.2 years compared to 37.6 years for other DR-3 positive patients (n = 46) and 40.3 years for non DR-3 positive patients (n = 29). The mean age at diagnosis for all DR-5 positive patients (n = 15) was 43.9 years

TABLE 6.2 DISTRIBUTION OF HLA-DR TYPES IN PATIENTS WITH THYROTOXIC GRAVES' DISEASE

	<u>DR-2</u>	<u>DR-3</u>			
<u>DR-2</u>	9		<u>DR-3</u>		
<u>DR-3</u>	10	7		<u>DR-4</u>	
<u>DR-4</u>	8	12	1		<u>DR-5</u>
<u>DR-5</u>	4	7	1	1	<u>DR-6</u>
<u>DR-6</u>	0	7	0	1	0
<u>DR-7</u>	1	10	0	1	1

Figures given refer to the number of patients with each phenotype (total number = 81, one patient who was DR-1+4 is not included in this table).

FIGURE 6.2 THE FREQUENCY OF HLA-DR ALLELES IN THYROTOXIC GRAVES' DISEASE AND IN THE NORMAL POPULATION



compared to 36.4 for DR-3 positive patients (excluding those who were also DR-3 positive, $n = 46$, $p = 0.07$, Fisher Exact Test).

THYROID HORMONES AND RADIOACTIVE IODINE UPTAKE

There was no difference in triiodothyronine, thyroxine or four hour radioactive iodine uptake between groups of patients with different HLA-DR phenotypes.

THYROID MICROSOMAL AND ANTI-THYROGLOBULIN ANTIBODIES

Microsomal antibodies were detected at a titre of 20^2 or above in 33 out of 53 (62%) patients at diagnosis. Of eleven patients who had DR-3 only or DR-3+4, ten were positive (91%) compared to 23 of the 42 (55%) other patients ($p = 0.05$, Fisher Exact Test). Anti-thyroglobulin antibodies were found in six out of 52 (12%) patients at diagnosis and showed no association with HLA DR type.

TSH RECEPTOR ANTIBODIES

In the 53 patients tested at diagnosis, there was no difference in the incidence or level of TSH receptor antibodies between DR-3 positive ($n = 32$) and DR-3 negative ($n = 21$) patients. The twelve patients with DR-3 only or DR-3+4 did not differ significantly from other patients. HLA DR-2 was, however, associated with increased TBII with 20 out of 22 DR-2 positive patients (91%) having TBII compared to 24 out of 31 (77%) other patients and the median TBII in the two groups was 25.2 and 17.5 respectively ($p < 0.05$, see FIGURE 6.3). The median TBII in seven DR-2 homozygotes was 36.2 compared to 19.9 in the 15 other DR-2 positive patients ($p < 0.01$, see FIGURE 6.4).

FIGURE 6.3 TSH RECEPTOR ANTIBODIES IN DR-2 POSITIVE AND DR-2
NEGATIVE GRAVES' PATIENTS

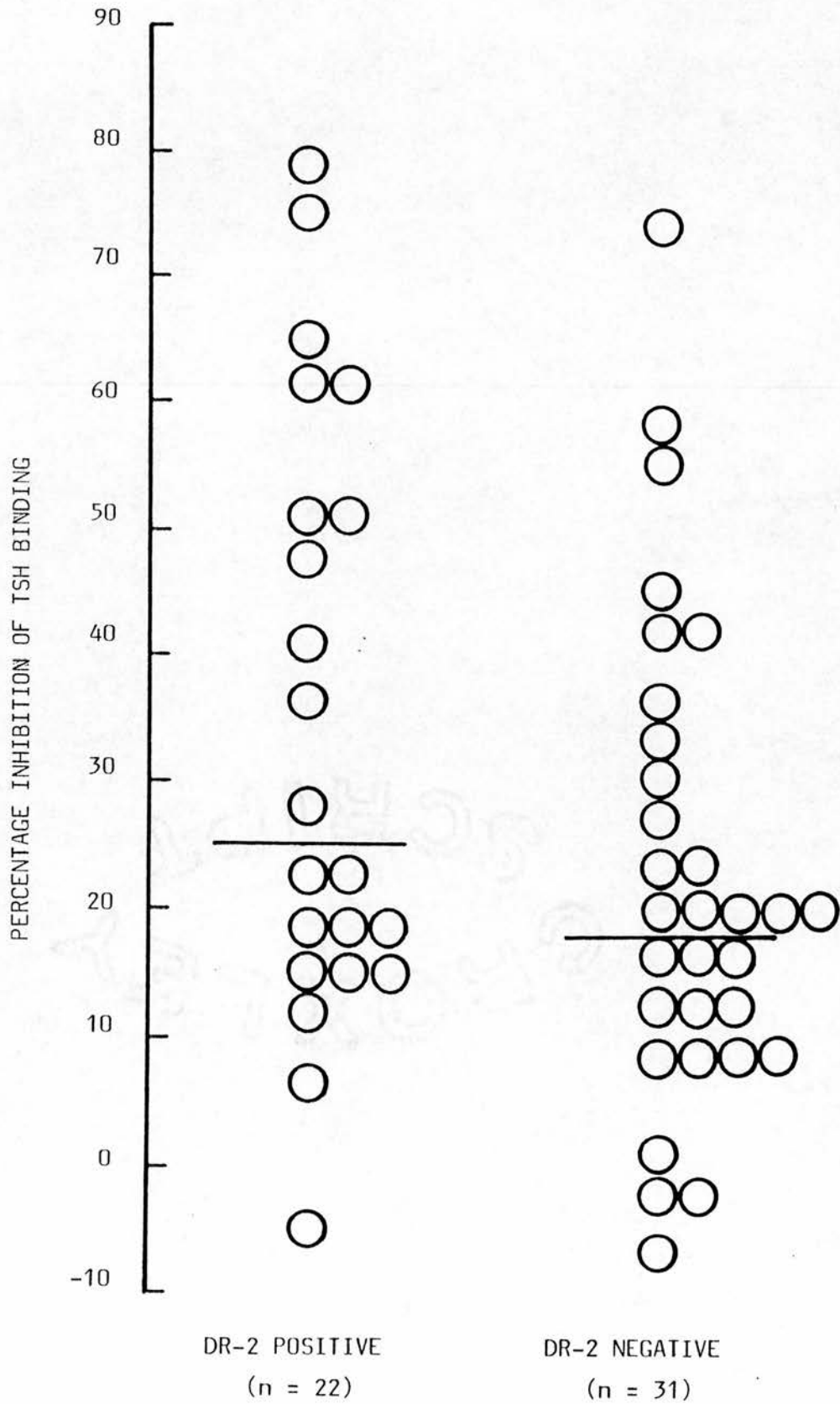
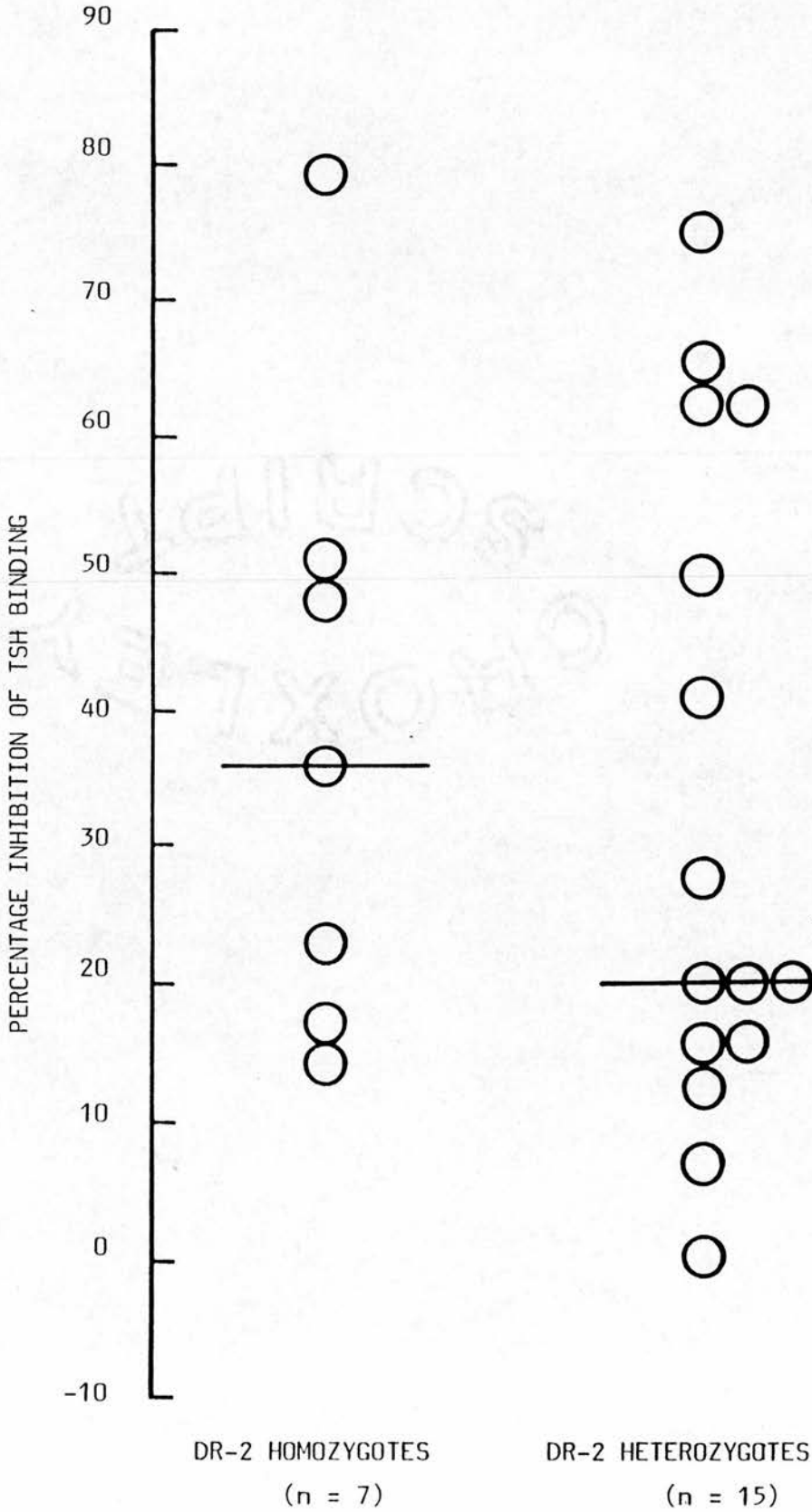


FIGURE 6.4 TSH RECEPTOR ANTIBODIES IN DR-2 HOMOZYGOTES COMPARED TO DR-2 HETEROZYGOTES



RESPONSE TO CARBIMAZOLE

The change in TBII in the six months after starting carbimazole treatment was studied in eight DR-2 positive patients and compared to that in sixteen other patients. The DR-2 positive patients were five females and three males with a mean age at diagnosis of 35.6 years (SD 16.8, range 16 to 69 years). The other patients, ten of whom were HLA typed, consisted of 13 females and three males with a mean age at diagnosis of 29.7 years (SD 7.1, range 15 to 42 years). The response of TBII during carbimazole treatment is shown in FIGURE 6.5. The median TBII at diagnosis in the DR-2 positive patients was 48.8 compared to 19.3 in the others ($p < 0.05$). TBII had reached normal levels in the latter group by two months after therapy was initiated but took six months to do so in the DR-2 positive group.

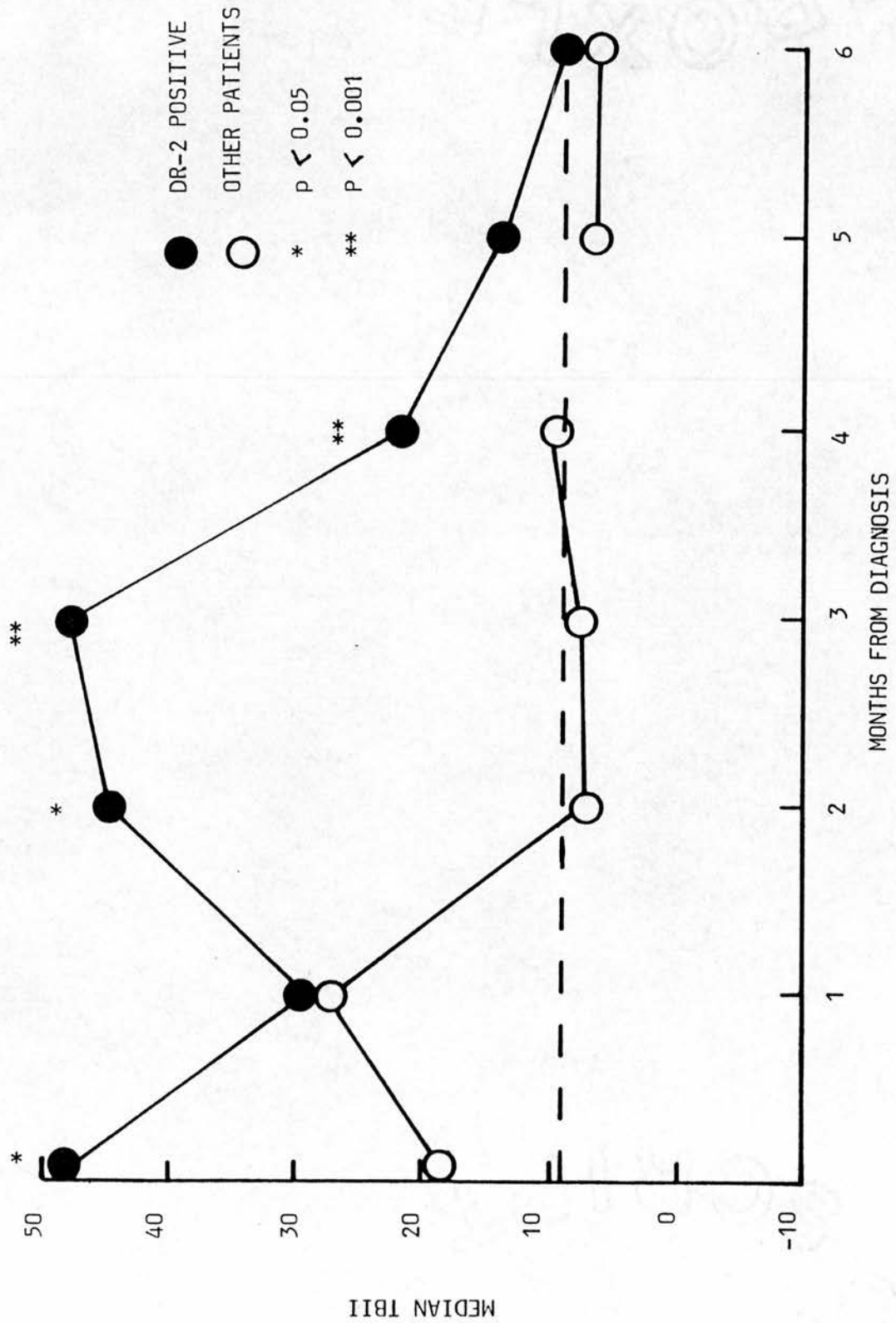
HLA DR-2 AND RESISTANCE TO THERAPY IN GRAVES' DISEASE

Consistent with the above observations, several patients were encountered where the DR-2 phenotype was associated with a resistance to medical therapy. Three such examples are reported below:-

CASE HISTORY 1

A 24 year old female (DR-2+3) presented with symptoms and signs of thyrotoxicosis in August 1982. She had a moderate diffuse goitre but no ophthalmopathy. T_3 at diagnosis was 6.6 nmol/l, T_4 was 230 nmol/l and the TRH test was flat. TBII were strongly positive at 75.2 and thyroid microsomal antibodies were detected weakly at 10^2 . After four months of carbimazole therapy with which she complied well, she was still requiring 30 milligrams to keep her clinically euthyroid and even then here T_3 and T_4 were mildly elevated at 3.0 and 158 nmol/l

FIGURE 6.5 RESPONSE OF TSH RECEPTOR ANTIBODIES TO CARBIMAZOLE IN DR-2 POSITIVE AND OTHER PATIENTS



respectively. She was therefore submitted to subtotal thyroidectomy after which she became biochemically euthyroid.

CASE HISTORY 2

An 18 year old girl (DR-2+4) was treated for thyrotoxicosis in late 1975 with carbimazole. She had a large diffuse goitre and severe bilateral exophthalmos. Because of poor response to drugs, she underwent subtotal thyroidectomy in March 1976. She remained euthyroid until April 1979 when she again developed symptoms of hyperthyroidism (T_3 5.3 nmol/l, T_4 196 nmol/l). She was recommenced on carbimazole but her thyroid hormones remained marginally elevated. In July 1981, she therefore recieved 15 milliCuries of ^{131}I and four months later became hypothyroid. In October 1984, TBII was still elevated at 14.5.

CASE HISTORY 3

A 20 year old lady (DR-2) was referred in January 1983 having been taking 30 milligrams of carbimazole per day intermittently since September 1982. She was eight weeks pregnant at presentation and therapy was reinstituted with atenolol 100 mg. per day in addition to carbimazole 30 mg. The response of thyroid hormones was poor and in March 1983, when T_3 was 5.2 nmol/l and T_4 was 162 nmol/l underwent subtotal thyroidectomy. TBII prior to operation was 24.4. Throughout her pregnancy, the thyroid hormones remained towards the upper limit of normal and the TBII was consistently raised. On the 18th July 1983, she gave birth to a normal female child by Caesarean section. Two months later TBII had risen from the pre-delivery value of 29.4 to 44.4 and T_3 was borderline high at 2.8 nmol/l with a normal T_4 of 136 nmol/l. By June 1984, these values had risen to 5.4 and 299

nmol/l respectively and she restarted carbimazole, following which she became euthyroid.

OPHTHALMOPATHY

The three patients with isolated Graves' eye disease had HLA types DR-3+4 (two cases) and DR-2+3. In the 82 thyrotoxic patients, ophthalmopathy was found in 35 of which 22 (63%) were DR-3 positive. Neither this nor any other allele differed significantly in frequency between patients with and without exophthalmos. In four of the above cases, the exophthalmos only occurred after treatment for thyrotoxicosis and three of these patients were DR-2 positive.

RADIOACTIVE IODINE THERAPY

Of ten consecutive patients rendered hypothyroid in the six months after therapy, three were DR-3 positive compared to eight out of ten patients who were euthyroid at the end of six months (not significant). HLA DR-3 was found in 12 out of 15 (80%) patients with TBII persisting long after radioactive iodine therapy (mean 42 months, range 24 to 71) compared to five out of eleven (45%) patients with normal TBII (mean period of follow up 58 months, range 18 to 147). Microsomal antibodies were detected in eight out of 14 (57%) patients with persistent TBII and in four out of eleven (36%) of those with no TBII on long term follow up.

DISCUSSION

The HLA-DR3 allele has been found in 53 out of 82 (65%) patients with thyrotoxicosis and a diffuse goitre and or scintiscan confirming the incidence which is already recognised in Graves' disease (Bech et al, 1977; Farid et al, 1977; Allanic et al, 1980; Dahlberg et al, 1981; McKenna et al; 1982). In keeping with these studies, there was no difference in the thyroid hormone values or in the radioactive iodine uptake of the thyroid gland between DR-3 positive and DR-3 negative patients. The age of the patient at diagnosis similarly showed no significant correlation with HLA although a trend was seen with the mean age for apparent DR-3 homozygotes of 35.2 years, 37.6 years for other DR-3 positive patients and 40.3 for DR-3 negative patients. Farid et al (1980a) did find a younger age of onset for DR-3 positive patients although this was not seen in the study by Balazs et al (1978).

No correlation was found between any DR allele and circulating anti-microsomal or anti-thyroglobulin antibodies and this is in agreement with the reports of Whittingham et al (1975), Bech et al (1977), Mather et al (1980) and Dahlberg et al (1981). HLA-B8 has been reported to be associated with higher levels of anti-thyroglobulin and lymphocyte transformation in response to thyroid antigen by Balazs et al (1978). DR-3 homozygotes and those with DR-3+4 might be expected to have a particular tendency towards autoimmune endocrine destruction: The DR-3 phenotype is associated not only with Graves' disease but also with primary atrophic hypothyroidism, Addison's disease and diabetes mellitus while over 95% of Type I diabetics possess either this allele or DR-4 and

DR-3/DR-4 heterozygosity has been associated with a higher than anticipated risk of diabetes (Thomsen et al, 1979). Of eleven patients who were either DR-3 only or DR-3+4, ten (91%) were positive for anti-microsomal antibody compared to 23 out of 42 (55%) other patients ($p = 0.05$).

HLA-DR3 is not generally found to correlate with the presence or titre of TSH receptor antibodies within a group of patients with Graves' disease (McGregor et al, 1980a; Mather et al, 1980; Schernthaner et al, 1980) and this has been confirmed by the present study. A surprising correlation between DR-2 and TBII was, however noted. The median TBII in 22 DR-2 positive patients was 25.2 compared to 17.5 for 31 other patients ($p < 0.05$). In seven apparent DR-2 homozygotes the value was 36.2 as opposed to 19.9 for the other 15 DR-2 positive patients ($p < 0.01$). This observation clearly needs to be confirmed with a larger series of patients but it does fit with our present knowledge that TSH receptor antibody secretion is not directly under the control of DR-3. Genetic influences other than DR-3 have previously been indicated from family studies (Farid et al, 1980d) and indeed one family has been described where the susceptibility to Graves' disease was linked to DR-2 (Farid et al, 1979) It is noteworthy that two of the DR-2 positive patients in the present study were sisters, both with the phenotype DR-2+3.

The response to carbimazole was also different in DR-2 positive patients: After starting the drug, the median TBII for eight DR-2 positive patients took six months to return to normal despite adequate biochemical control of their thyrotoxicosis while, in sixteen other patients, the TBII was normal in two months. In the

study by McGregor et al (1980a), TBII were higher after therapy in DR-3 negative patients than they were in DR-3 positive patients.

The response to radioactive iodine therapy may relate to HLA status: Three out of ten patients hypothyroid at six months were DR-3 positive compared to eight out of ten euthyroid patients. If hypothyroidism, euthyroidism and persisting toxicosis are regarded as part of a continuum in the response to therapy, these results are in agreement with those of Davies et al (1982) who showed that poor response to therapy at three months related to DR-3 positivity. ^{131}I therapy is not only of interest because it is frequently used in the treatment of thyrotoxicosis but also because of the autoimmune reaction it initiates which makes it a potentially useful model.

The production of non-stimulatory TSH receptor antibodies after ^{131}I therapy (Bech et al, 1982) and their persistence long after treatment was discussed in Chapter 3. This persistence may relate to HLA with 12 out of 15 (80%) patients with persistent antibodies being DR-3 positive compared to five out of eleven (45%) of those in whom antibodies did not persist. A similar trend was noted with anti-microsomal antibodies.

For the study described in Chapter 4, a large series of patients with thyrotoxic Graves' disease was divided into three groups - one with goitre and high ^{131}I uptake, the second with goitre but low ^{131}I uptake and the third group with no goitre. Patients in the latter two groups were not only older but had less severe thyrotoxicosis while those in the second group had a very high incidence of autoantibodies including parietal cell antibodies. Since pernicious anaemia is

associated with DR-5 and patients with high thyroid antibody titres may have coexisting Hashimoto's thyroiditis which is also DR-5 associated, it was of interest to examine this apparent disease heterogeneity with respect to DR typing. However the twelve patients with low uptake and goitre and the 21 patients with no goitre did not show any deviation from the incidence of HLA alleles seen in the majority of Graves' patients. Nor was there any difference in patients with ophthalmopathy although Balazs et al (1978) have previously reported an association with HLA-B8 and Farid et al (1980b) showed DR-3 to be increased in patients with eye disease.

In conclusion, no correlation was found between HLA-DR3 and any clinical or biochemical feature of the disease, as expected. TBII were higher in DR-2 positive patients and in DR-2 homozygotes compared to heterozygotes. These patients were also less susceptible to the specific immunosuppressive effects of carbimazole. Microsomal antibody secretion and the persistence of TBII after 131 I therapy -both measures of thyroid autoaggression - may be under the influence of DR-3. There may therefore be separate genetic control of thyroid stimulation and destruction by the immune system and this could in part underlie the heterogeneity which exist in the expression of immunological markers.

Chapter Seven

Helper and Suppressor

Lymphocyte Subsets

in Graves' Disease

SUMMARY

Lymphocyte subsets were studied in Graves' disease using monoclonal antibodies. There was an increase in the absolute lymphocyte count in untreated Graves' ($2.33 \times 10^9/1$) compared to controls ($1.80 \times 10^9/1$, $p < 0.05$). Total T lymphocytes, as measured by the monoclonal antibody OKT3, did not differ significantly. There was a reduction in the percentage of OKT8 positive cells in untreated Graves' (17.2% vs. 22.4% in controls, $p < 0.001$), which was not present in patients rendered euthyroid after treatment. The OKT4/OKT8 ratio in untreated Graves' was 2.53 compared to 1.95 in controls ($p < 0.001$). The absolute count of OKT8 positive cells did not, however, differ in the two groups and, indeed, there was a moderate increase in the absolute OKT4 positive cell count in the Graves' patients ($9.82 \times 10^8/1$ vs. $7.62 \times 10^8/1$ in controls, $p = 0.05$).

The decrease in the percentage of suppressor cells was also demonstrated with the antibody UCHT4. Overall, the correlation between OKT8 and UCHT4 was good ($r = 0.763$, $p < 0.001$) but this was largely due to the nine controls ($r = 0.933$, $p < 0.001$), while the correlation between the results with the two antibodies was poor in the Graves' patients ($r = 0.096$, not significant).

In normal female subjects, the proportion of OKT8 positive cells in peripheral blood declined with age ($r = 0.641$, $p < 0.001$) with an increase in the OKT4/OKT8 ratio ($r = 0.696$, $p < 0.001$). The absolute OKT8 count did not change significantly with age.

In conclusion, data on lymphocyte subsets needs to be interpreted with caution since changes in the proportion of a subset might not reflect changes in absolute numbers of cells. Measurements using different monoclonal antibodies may not be wholly comparable in disease states. The influence of age means that controls for studies should be carefully selected.

INTRODUCTION

A: PERIPHERAL BLOOD T AND B LYMPHOCYTE SUBSETS IN GRAVES' DISEASE

Lymphocytosis is a well recognised feature of Graves' thyrotoxicosis but it is by no means clear whether this is due to an overall increase in lymphocytes or only to an increase in particular subsets. Non-thymus derived, B lymphocytes are responsible for antibody secretion and constitute up to 15% of circulating lymphocytes. they may be identified by their surface immunoglobulin or by receptors on their surface including the CR2 receptor which forms the basis for the EAC rosetting test. Increased numbers of B lymphocytes have been reported in untreated Graves' disease using this method (Hsu et al, 1976; Mori et al, 1980). Lundell et al (1976), however, found no alterations in B cells in Graves' patients, in keeping with the findings of Mulaisho et al (1975) who also found no alterations in goitrous or non goitrous hypothyroidism. Urbaniak et al (1974) point out that differences in separation procedures for lymphocytes may give rise to variations in

the yield of T lymphocytes and therefore produce apparent changes in the ratios of T to B cells. Using two different methods, however, they did not find alterations of either subset in Graves' or Hashimoto's diseases compared with normal controls. An increase in cells staining with the monoclonal antibody OKIa (anti-DR) has been attributed to an elevated B cell count (Wall et al, 1983), although such cells may equally well represent activated T cells (Jackson et al, 1983, see Chapter 8). The published literature is thus rather ambiguous with respect to changes in B cells in Graves' disease.

Cell mediated immunity is much harder to study than is humoral immunity but there is now good evidence that it is disturbed in conditions such as systemic lupus erythematosus, diabetes mellitus and Graves' disease. Much of the recent work in autoimmunity has been concerned with subsets of thymus derived lymphocytes. T cells can be distinguished by their proliferative response to certain soluble mitogens and are classically enumerated by a rosetting technique with sheep red blood cells. There are conflicting reports on T lymphocyte numbers in Graves' disease: Early studies suggested that they may be increased (Farid et al, 1973; Aoki et al, 1973) presumably reflecting a state of activation of the cell mediated immune system. It was even suggested by Farid et al (1974) that the E rosette test may be useful in predicting the response to antithyroid drug therapy since it was shown in 13 subjects to correlate well with T₃ suppression of radioactive iodine uptake. Later work did not, however confirm this and other workers have found the proportion of T cells to be normal in Graves' disease (Mulaisho et al, 1975; Calder et al, 1976; Hsu et al, 1976; Lundell et al, 1976). In one study, Marciel et al (1976) showed the

percentage of T cells to be normal but the absolute number of T cells was elevated.

These studies all used the red cell rosetting technique and their findings have been confirmed recently by Okabe et al (1983) but most recent work suggests that, if anything, T cells are reduced in active Graves' disease (Wall et al, 1977; Grinblat et al, 1979; Mori et al, 1980). The monoclonal antibody OKT3 has been used by Wall et al (1983) and these authors found about 46% of peripheral blood mononuclear cells to be OKT3 positive compared to 52% in controls. A similar finding using another monoclonal antibody has been reported by Iwatani et al (1983). Not all studies with monoclonal antibodies show reduced T cells - Sridama et al (1982) and Ludgate et al (1984) found them to be normal.

There is uncertainty therefore, concerning changes in T and B lymphocyte numbers in untreated Graves' disease but clearly gross changes in cell numbers are not a prerequisite for an autoimmune disease. Experimental differences, including lymphocyte separation methods may underlie part of this controversy but even where the method is fairly standard as with the use of monoclonal antibodies, the results have proved to be conflicting.

B: HELPER AND SUPPRESSOR SUBSETS OF T LYMPHOCYTES

Two subpopulations of lymphocytes with major immunoregulatory roles are now recognised - helpers (T_h) and suppressors (T_s). The former are important in promoting the immune response by cooperating with B lymphocytes and other subsets of T lymphocytes with effector

functions. The latter limit the immune response and may be important in the prevention of autoimmune disease, which may be achieved as much by the presence of an active immunoregulatory process as by the absence of "self-reactive" clones of cells. Thus, a relative deficiency of T_S may permit both humoral and cell-mediated immune attack on organs such as the thyroid. These cell subsets have been studied both in animals (for a review of T_H and T_S in animal models of thyroiditis, see Rose et al, 1981) and in a variety of autoimmune disease states affecting Man.

Several approaches have been used to study these lymphocyte subsets: Firstly, using in vitro systems, attempts have been made to reverse changes in humoral and cell mediated immunity by the addition of normal lymphocytes which are presumed to contain the suppressors deficient in the patient's cells. Secondly, they may be estimated qualitatively by using certain properties of T_S in culture - they are rapidly activated by the mitogen concanavalin A and they are selectively depleted on long term incubation of the culture. Thirdly, Fc receptors on the surface of T lymphocytes allow helpers and suppressors to be distinguished - T_S mainly have receptors for IgG while the receptors on T_H are mainly for IgM. Finally, monoclonal antibodies have proved to be powerful tools in the investigation of lymphocyte subsets. They give no direct information on the activity of the cells nor on their specificity but the assays are reproducible and readily applied to large numbers of samples.

Early evidence linking autoimmune thyroid disease to changes in cell-mediated immunity came from studies on the migration inhibition factor (MIF, Lamki et al, 1973): The normal migration of peripheral

blood mononuclear cells in a culture chamber is inhibited when they are exposed to an antigen to which they have been previously sensitized. Kovalczyk and Zembala (1978) have shown that the migrating cells are mainly T lymphocytes and have refined the test by using a T cell enriched fraction as both the indicator cells and MIF-producing cells. Using this test, Okita et al (1981) have provided further evidence of T lymphocyte sensitization in Graves' disease and Hashimoto's thyroiditis. They have gone on to show that the migration inhibiting activity of these two patient groups could be abolished by the addition of normal T lymphocytes and this was thought to be due to their suppressor cell content (Okita et al, 1981). Furthermore, the T_S defect may be organ specific - the same group (Topliss et al, 1983) have demonstrated that the migration inhibition by lymphocytes from Graves' and diabetic patients is specific for thyroid and pancreatic antigens respectively. Migration inhibition by diabetic lymphocytes could be reversed by the addition of Graves' lymphocytes and vice versa. While it has been possible in the MIF test to reverse the abnormality in patients cells by the addition of normal cells, this has proved difficult in systems involving humoral immunity. Beall (1972) has cultured B lymphocytes from patients with autoimmune thyroiditis and found them to produce anti-thyroglobulin antibody. Antibody production was not inhibited by the addition of normal lymphocytes. Similarly, McLachlan et al (1980) have shown that neither mitogen- nor antigen-stimulated antibody production by lymphocytes from thyroiditis patients was inhibited by normal lymphocytes. A recent report by Shinomiya et al (1984) tells of the inhibition of acetylcholine receptor antibody formation by the addition of culture supernatant of normal lymphocytes to B cells from patients with myasthenia gravis.

A subset of lymphocytes which is preferentially activated by concanavalin A has been shown to have suppressor cell activity (Sakane & Green, 1977). They will suppress the response of B cells to mitogen and antigen as well as antibody-dependent cellular cytotoxicity and natural killer activity (Hallgren et al, 1977; Nair et al, 1981) and they are present in the peripheral blood of normal subjects (Shou et al, 1976). The activity of such cells is selectively lost after 24 hours of incubation (Dutton et al, 1972). These two properties have been used to qualitatively assess T_S in autoimmune disease. Thus, a decrease in suppressor activity has been found in newly diagnosed diabetics (Buschard et al, 1980), returning to normal with treatment. Horowitz et al (1977) and Lederman et al (1981) reported similar findings, although Slater et al (1980) found no difference in diabetics of long standing. A decrease in T_S measured by this method has also been demonstrated in SLE (Bresnihan & Jasin, 1977) and in active multiple sclerosis (Gonzalez et al, 1979). Balazs et al (1979) have found a reduced con A-activated and short lived suppressor cell function in patients with untreated Graves' disease. These increased with treatment but not to normal levels. Aoki et al (1979) reported similar results but there was no change in Hashimoto's thyroiditis or in thyroid cancer. A more recent study by Jones et al (1982) showed no change in con A-activated suppressor cell activity in untreated Graves' disease. Although T_S activity has generally been found to be reduced in autoimmune disease when studied by this method, it may not correlate well with suppressor cell numbers as measured by monoclonal antibodies: Schandene et al (1983), in a series of patients with various immunological disorders found a poor correlation between con

A-activated suppressor activity and percentage OKT8 positive cells although a significant correlation with the OKT4/OKT8 ratio was noted.

Distinctive cell surface markers allow us to enumerate subsets of lymphocytes. T cells bearing Fc receptors for IgG (T_g cells) and for IgM (T_m cells) differ in function - T_g cells possessing suppressor activity and T_m cells having helper activity (Moretta et al, 1977). T_g cells are reduced in active SLE (Moretta et al, 1979) but normal levels have been reported in Hashimoto's thyroiditis (Canonica et al, 1982). It may well be, however, that the diagnosis of Hashimoto's is made long after the autoimmune process is initiated. Increased T_g cells are described in subacute thyroiditis (Wall et al, 1981) and reduced levels have been found in Graves' disease in association with increased numbers of activated lymphocytes (Canonica et al, 1983). T_g cells returned to normal following radioactive iodine therapy and were not elevated in patients with toxic adenoma. By contrast, Okabe et al (1983) found no change in active Graves' disease using this method.

The availability of monoclonal antibodies to cell surface antigens has transformed research into lymphocyte subsets and is the method reported in this thesis. A variety of autoimmune diseases has been associated with a decrease in suppressor cells with a consequent increase in the ratio of helper to suppressor cells. Thus, Buschard et al (1983) have investigated patients with Type I diabetes mellitus with the antibodies OKT3 (pan T cell), OKT4 (helper/inducer cells) and OKT8 (suppressor/cytotoxic cells). At diagnosis patients showed no change in absolute lymphocyte numbers or total T cells but had

increased OKT4⁺ as well as decreased OKT8⁺ cells. Diabetics of long standing also had reduced OKT8⁺ cells but this was not as marked as in the new patients. Morimoto et al (1980) have reported reduced OKT8⁺ cells in active SLE and similar changes have been found in multiple sclerosis - particularly in the acute phase and in progressive forms (Bach et al, 1980). Pernicious anaemia has recently been added to the list of conditions associated with a reduced percentage of OKT8⁺ cells (Imamura et al, 1984).

A reduced proportion of suppressor cells in thyroid diseases has been shown in a number of studies: The reduction in total T lymphocytes found by Thielemnas et al (1981) in 25 patients with Graves', atrophic hypothyroidism and Hashimoto's was attributed to a decrease in OKT8⁺ cells. These authors found no correlation between thyroid hormone status and suppressor cell numbers. Sridama et al (1982) also report a decrease in OKT8⁺ cells in untreated Graves' and Hashimoto's thyroiditis although treated Graves' patients did not differ from controls. Lymphocyte subsets were investigated in a spectrum of thyroid disorders by Bonnyns et al (1983) who found a negative correlation between OKT8⁺ cells and thyroid hormone levels. Thus, Graves' patients had a low OKT8% but a high free thyroxine and the converse was true of Hashimoto's. A direct influence of thyroid hormones on the immune system was suggested. A different set of monoclonal antibodies was used by Iwatani et al (1983): Leu2⁺ (cytotoxic/suppressor) and Leu3⁺ cells (helper/inducer) were present normal numbers in Graves' but there was a qualitative difference in that the fluorescence peak on the cell sorter was shifted in the Graves' patients. One recent study has shown normal levels of helper and suppressor cells using the OKT series (Wall et al, 1983). Ludgate

et al (1984) showed not only that OKT8⁺ cells were reduced in active Graves' and that these returned to normal with treatment, but also that they remained low in patients whose thyrotoxicosis relapsed after antithyroid drug therapy.

There is good evidence, therefore, for reduced suppressor cell activity in untreated Graves' disease although this has not been confirmed in all published studies. The work presented here was aimed at finding out whether the reduction in the proportion of OKT8⁺ cells was the result of a decrease in the absolute number of these cells or whether it may be secondary to an increase in the levels of another cell subset. Two separate monoclonal antibodies defining the suppressor cell subset are compared and further support for the hypothesis that age-related changes in the immune system might be important in the genesis of autoimmune disease is sought.

PATIENTS AND METHODS

PATIENTS The study included 21 consecutive cases of untreated Graves' disease. The diagnosis was made on the basis of an elevated T₃ and/or T₄, flat TRH test and a diffuse goitre and/or scintigram. TSH receptor antibodies were detected in neat serum as already described and were found in 18 of the 21 new cases. The other three all had exophthalmos. Seven cases were studied while clinically and biochemically euthyroid six to nine months after starting carbimazole (maintenance dose 5 to 15 mg.). A further nine cases were in remission and were receiving no antithyroid therapy (five had previously been treated with carbimazole and the others had been

treated with radioactive iodine). Subclinical Graves' disease was diagnosed in seven cases: Two were TSH receptor antibody positive, the total T_3 ranged from 2.6 to 3.8 nmol/l (normal upper limit = 2.8) and the T_4 from 121 to 162 nmol/l (normal upper limit = 150). All seven had flat TRH tests and all had a diffuse scintigram but none had severe symptoms or signs of thyrotoxicosis.

Controls were selected from staff of the Royal Infirmary, Edinburgh and were matched approximately to the age and sex of the patients. None had a history of autoimmune disease or any other intercurrent illness.

METHODS Venous blood was collected in preservative-free heparin at the same time each day (9 to 11 A.M.) since lymphocyte subsets may show a diurnal variation (Bertouch et al, 1983; Ritchie et al, 1983). At least one control was processed with each patient sample. Absolute lymphocyte counts were supplied by the Department of Haematology, Royal Infirmary, Edinburgh using a Coulter counter followed by a 200 cell manual differential count. TSH receptor antibodies were analysed on neat serum as described in APPENDIX 1.

Statistical analysis involved the use of Student's t test and linear regression throughout.

RESULTS

The study included 24 controls (20 female, four male), 21 patients with thyrotoxic Graves' disease prior to starting carbimazole (16 female and five male), seven patients who were taking carbimazole but who were euthyroid, nine patients in remission following therapy and seven patients with subclinical Graves' disease. The following monoclonal antibodies were used to enumerate lymphocyte subsets:-

OKT3	Total T lymphocytes
OKT4	Helper/Inducer cells
OKT8	Suppressor/Cytotoxic cells
UCHL4	Suppressor/Cytotoxic cells

OKT8 POSITIVE CELLS There was a significant reduction in the percentage of OKT8 positive cells in untreated Graves' disease (mean OKT8 = 17.2%) compared to controls (mean = 22.4%, $p < 0.001$) - see FIGURE 7.1. The mean OKT8 in the seven patients euthyroid on carbimazole was 20.3% with a value of 21.7% for the patients in remission and 21.9% for the patients with subclinical disease. None of these values differed from controls.

OKT4 POSITIVE CELLS These values are illustrated in FIGURE 7.2 The mean percentage of OKT4 positive cells in controls was 42.6% compared to 42.4% in untreated Graves', 42.8% in those euthyroid on carbimazole, 40.6% in the group in remission and 42.9% in the subclinical group. There was no significant difference between the various groups.

FIGURE 7.1 PERCENTAGE OKT8 POSITIVE CELLS IN TREATED AND UNTREATED

GRAVES' DISEASE

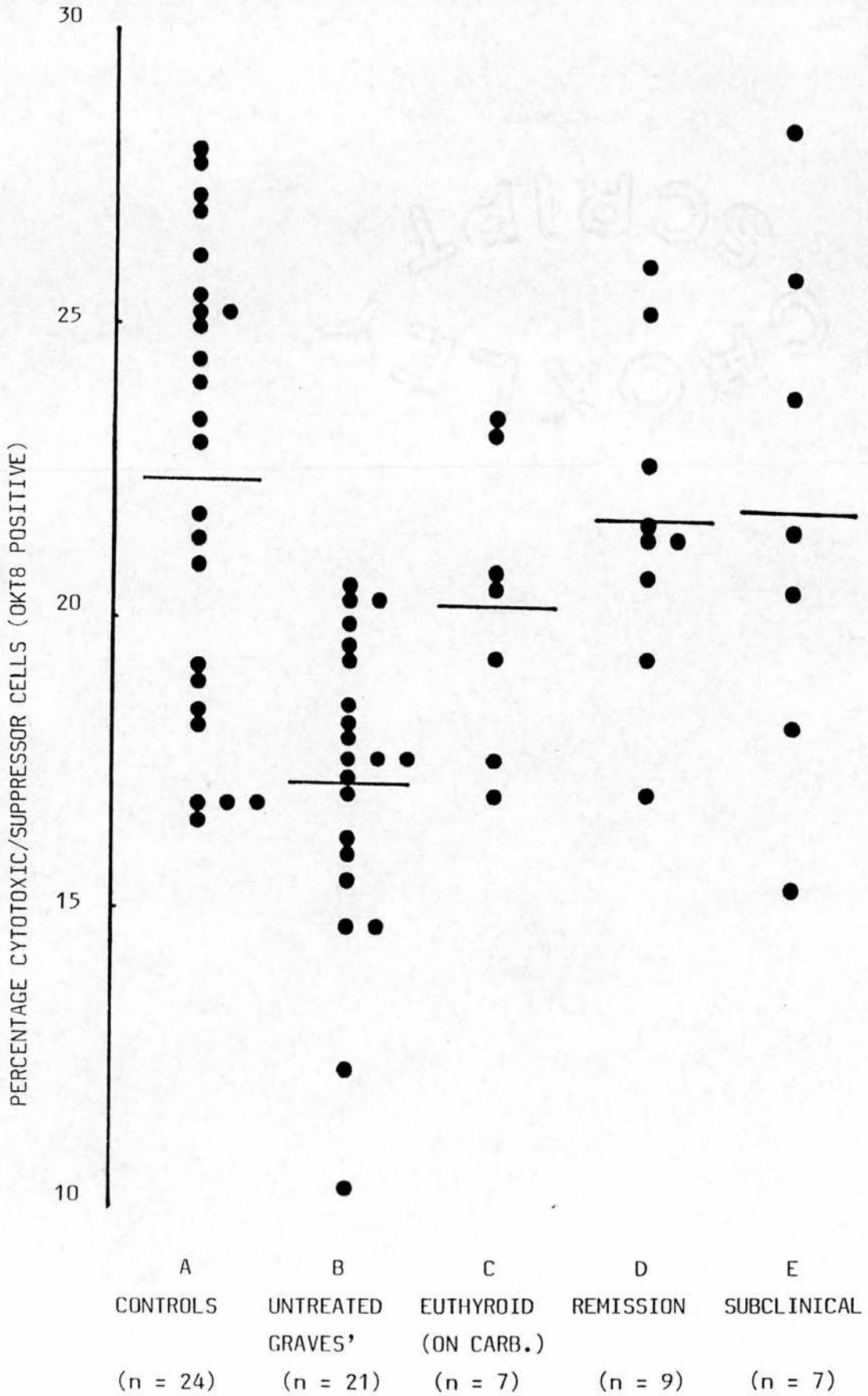
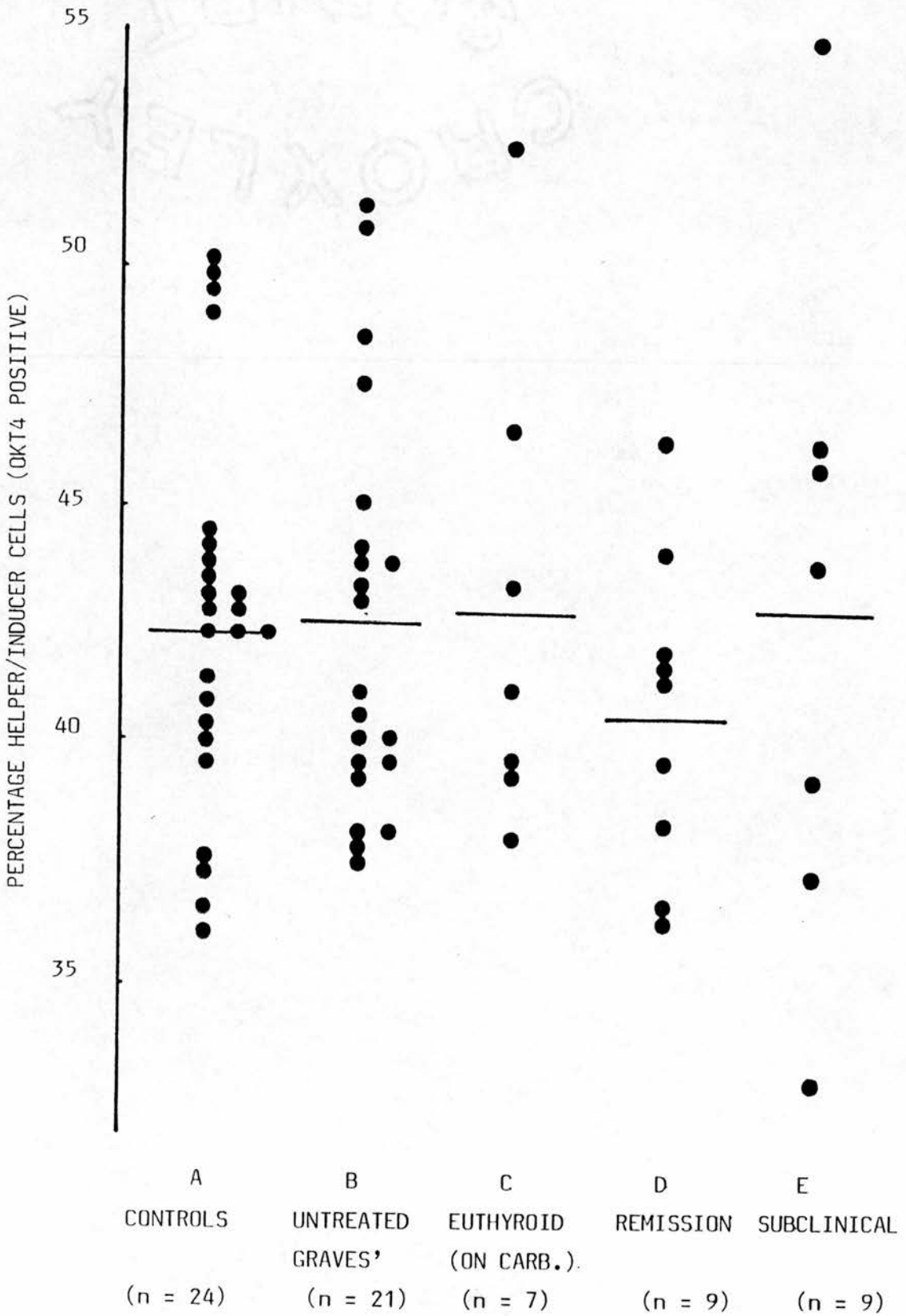


FIGURE 7.2 PERCENTAGE OKT4 POSITIVE CELLS IN TREATED AND UNTREATED

GRAVES' DISEASE



OKT4/OKT8 RATIO See FIGURE 7.3. The mean ratio in controls was 1.95 while that in untreated Graves' was 2.53 ($p < 0.001$). Patients euthyroid on carbimazole had a mean ratio of 2.14, those in remission after treatment 1.90 and the subclinical cases 2.09 - none of which differed from controls.

OKT3 POSITIVE CELLS In 29 controls, the mean percentage of T cells was 58.4% (SD = 6.52) compared to 57.3% in 20 patients with untreated Graves' disease (SD = 5.55, not significant).

COMPARISON OF THE MONOCLONAL ANTIBODIES OKT8 AND UCHT4

Suppressor/cytotoxic cells were measured with these two antibodies in nine controls and in nine patients with untreated Graves' disease. The correlation between the two is shown in FIGURE 7.4. The overall correlation coefficient was 0.763 ($p < 0.001$), but this correlation was largely due to the controls ($r = 0.933$, $p < 0.001$). The correlation in the nine Graves' patients between OKT8 and UCHT4 was poor ($r = 0.096$). Like OKT8, however UCHT4 was significantly reduced in the Graves' patients (18.4%) compared to controls (23.1%, $p < 0.05$).

ABSOLUTE LYMPHOCYTE NUMBERS Untreated Graves' disease was associated with a mild lymphocytosis - the absolute lymphocyte count in 13 cases was $2.33 \times 10^9/l$ compared to $1.80 \times 10^9/l$ in 22 controls ($p < 0.05$). In 13 patients in remission (five on carbimazole, eight post-treatment), the mean lymphocyte count was not elevated at $2.06 \times 10^9/l$.

FIGURE 7.3 RATIO OKT4/OKT8 POSITIVE CELLS IN TREATED AND UNTREATED

GRAVES' DISEASE

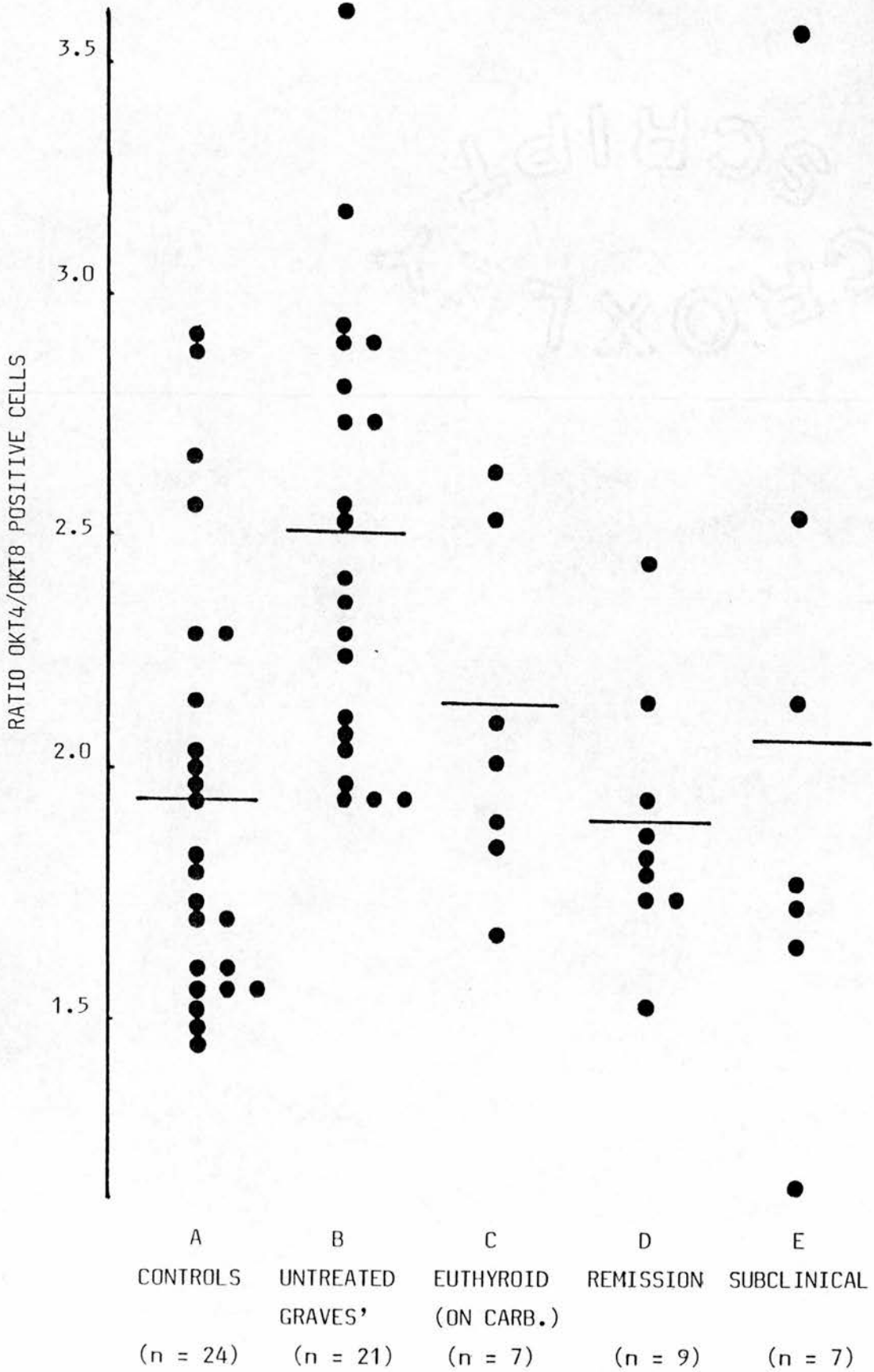
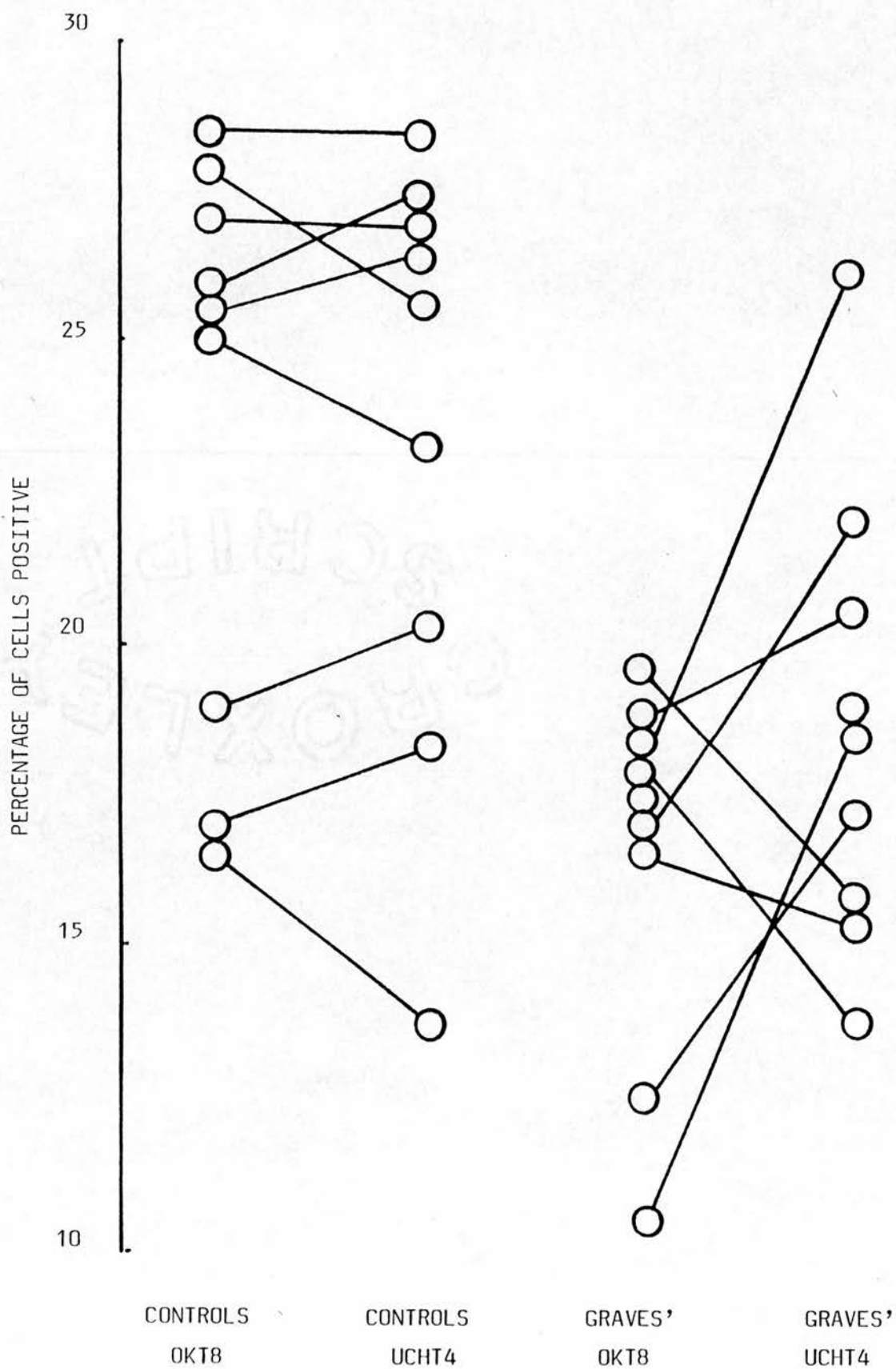


FIGURE 7.4 COMPARISON OF TWO MONOCLONAL ANTIBODIES FOR QUANTIFYING SUPPRESSOR CELLS



ABSOLUTE SUBSET NUMBERS See TABLE 7.1. The absolute count of OKT8 positive cells in 22 controls averaged $4.10 \times 10^8/1$ compared to $4.21 \times 10^8/1$ in 13 untreated Graves'. These were not significantly different although the percentage OKT8 positive cells in the patients was 18.6 compared to 22.5% for the controls ($p < 0.01$) and the OKT4/OKT8 ratios were 1.95 and 2.32 respectively ($p < 0.05$). There was a slight increase in the number of OKT4 positive cells in the untreated cases - $9.82 \times 10^8/1$ as against $7.62 \times 10^8/1$ for the 22 controls ($p = 0.05$).

The absolute OKT8 count for the euthyroid Graves' cases was $4.24 \times 10^8/1$ which is similar to the control value and the count of OKT4 positive cells was $8.89 \times 10^8/1$ which again is comparable to the control value.

CHANGES WITH AGE IN NORMAL FEMALE CONTROLS In the 20 female controls used in this study, there was a significant negative correlation between age and the percentage of OKT8 positive cells ($r = -0.641$, $p < 0.01$ - see FIGURE 7.5). There was a consequent positive correlation between age and OKT4/OKT8 ratio which is illustrated in FIGURE 7.6 ($r = 0.696$, $p < 0.001$). OKT4 did not change with age and there was no relationship between absolute subset numbers and age.

TABLE 7.1 ABSOLUTE LYMPHOCYTE SUBSET NUMBERS IN THYROTOXIC AND EUTHYROID GRAVES' PATIENTS AND IN CONTROLS

	OKT8 (%)	OKT4 (%)	RATIO 4/8	LYMPHOCYTE COUNT	ABSOLUTE OKT8	ABSOLUTE OKT4
CONTROLS (N = 22)	22.5	42.6	1.95	1.80 x 10 ⁹	4.10 x 10 ⁸	7.62 x 10 ⁸
EUTHYROID GRAVES' (N = 13)	20.6	42.0	2.06	2.06 x 10 ⁹	4.24 x 10 ⁸	8.89 x 10 ⁸
UNTREATED GRAVES' (N = 13)	18.6 **	42.0	2.32 *	2.33 x 10 ⁹ *	4.21 x 10 ⁸	9.82 x 10 ⁸ *

* = p < 0.05
** = p < 0.01

FIGURE 7.5 CORRELATION BETWEEN AGE AND PERCENTAGE OKT8 POSITIVE CELLS IN NORMAL WOMEN

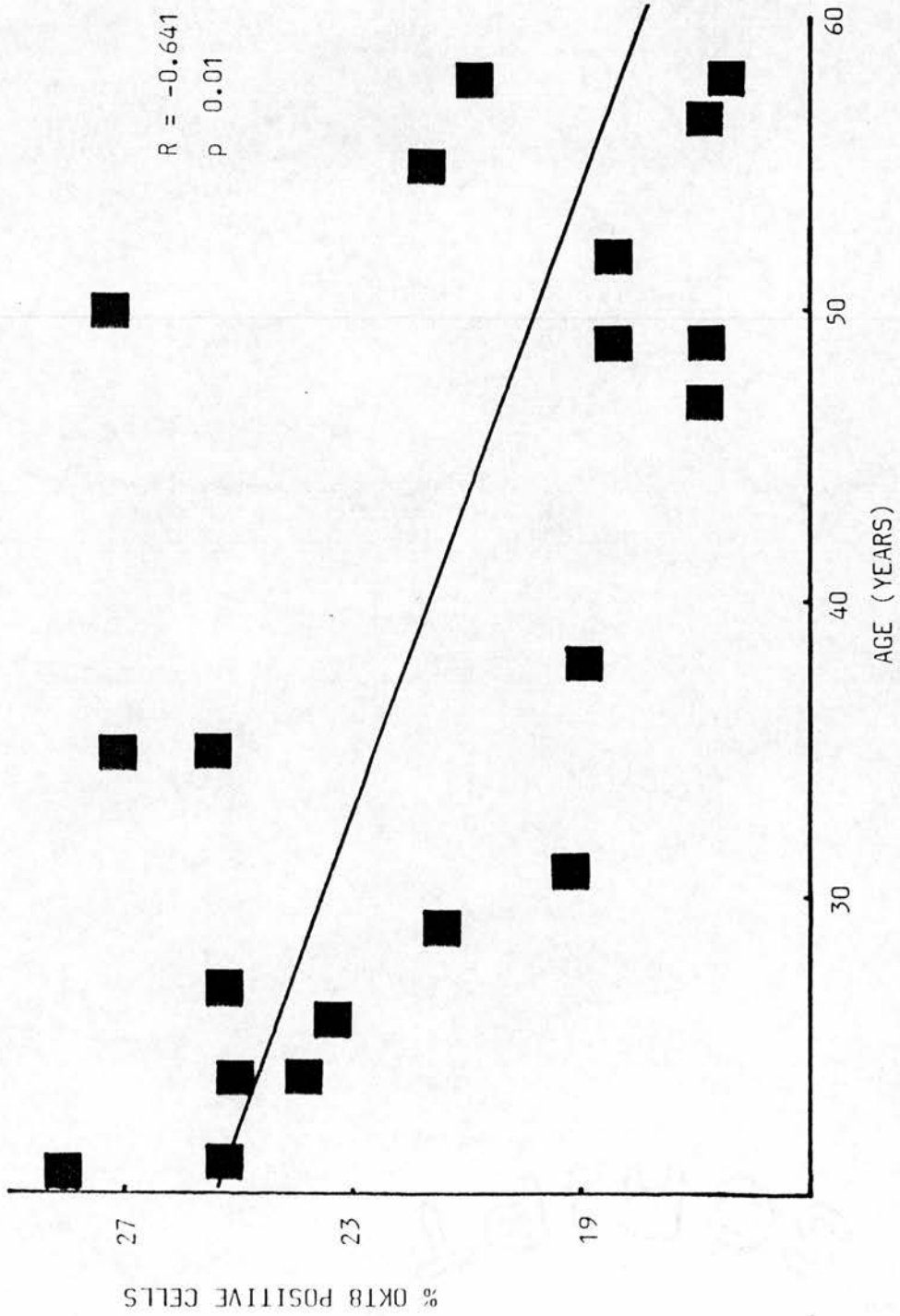
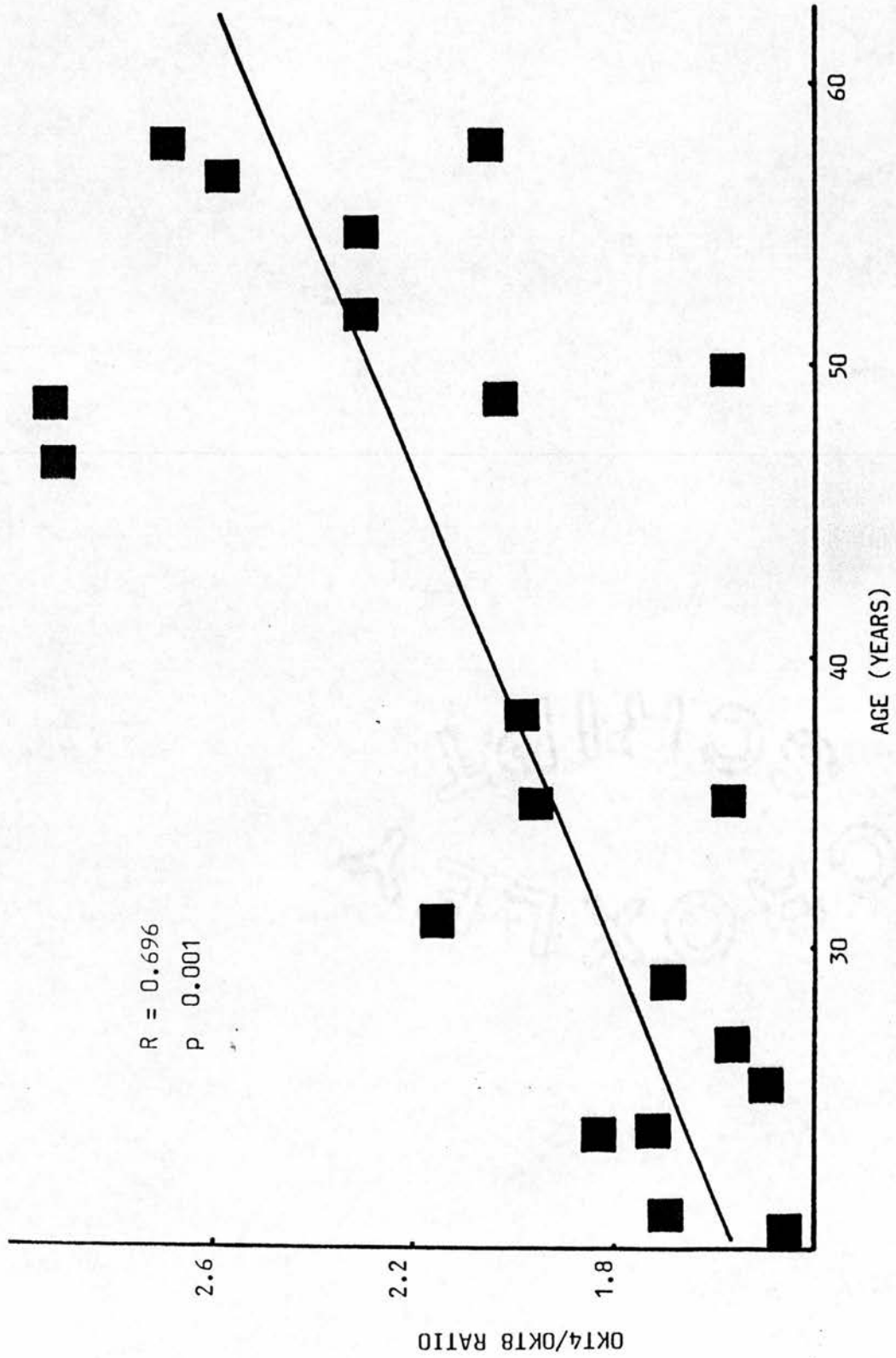


FIGURE 7.6 CORRELATION BETWEEN AGE AND OKT4/OKT8 RATIO IN NORMAL

WOMEN



DISCUSSION

Helper and suppressor lymphocyte subsets were quantified in Graves' disease using monoclonal antibodies. Untreated Graves' was associated with a mild lymphocytosis with a peripheral blood count of $2.33 \times 10^9/l$ in 13 patients compared to $1.8 \times 10^9/l$ in 22 controls. The proportion of total T lymphocytes (OKT3 positive cells) was, however, normal, in keeping with the findings of Mulaisho et al (1975), Hsu et al (1976), Calder et al (1976) and Lundell et al (1976). All of these authors employed rosetting techniques to enumerate T cells but normal T cells have also been found with monoclonal antibodies by Sridama et al (1982) and Ludgate et al (1984). By contrast, Wall et al (1983) found OKT3 positive cells to be reduced in active Graves'.

The percentage of OKT8 positive cells was reduced in the peripheral blood of 21 patients with untreated Graves' disease (17.2%) compared to 24 controls (22.4%) in agreement with the findings of Thielemans et al (1981), Sridama et al (1982) and Ludgate et al (1984). OKT8 percentage was normal in patients euthyroid on carbimazole and in patients who were in remission after therapy. Not all published studies with monoclonal antibodies have found suppressor cells to be reduced; both Wall et al (1983) and Iwatani et al (1983) found the proportion of these cells to be normal although the latter authors did report a qualitative change in the fluorescence of suppressor cells in Graves' patients.

Since other subsets of cells are undoubtedly raised in Graves' and other autoimmune diseases (see below and Chapter 8), the reduction in the percentage of OKT8 positive cells may result and it was therefore

of interest to measure the absolute count of OKT8 positive cells. In 22 controls the count of OKT8+ cells was $4.10 \times 10^8/l$ and this did not differ significantly from that of 13 untreated Graves' patients whose average count was $4.21 \times 10^8/l$. While the evidence for a functional suppressor cell deficit in autoimmune thyroid disease is good, both in animal and human studies (Rose et al 1981; Okita et al 1981b), this need not necessarily be reflected in cell numbers. Topliss et al (1983) have suggested that the suppressor defect in autoimmune endocrine disease is organ specific and that such a defect may therefore only affect a proportion of the total suppressor cells. It may also be that changes in the proportion of a cell subset in peripheral blood do not accurately reflect what is happening at the actual site of the autoimmune reaction. For example, in autoimmune thyroid disease, both Wall et al (1983) and Warford et al (1985) have found differences in proportion of lymphocyte subsets in the thyroid compared to the peripheral blood.

The proportion of OKT4 positive (helper/inducer) cells was normal in Graves' patients although there was a modest increase in the absolute number of OKT4+ cells ($9.82 \times 10^8/l$ in Graves' patients compared to $7.62 \times 10^8/l$ in controls). This increase may account for some of the reduction in the percentage of OKT8+ cells. It may be that the relative reduction in suppressor cells in relation to inducer cells is sufficient to allow the autoimmune reaction to proceed. OKT4% has generally been reported as normal in Graves' although Buschard et al (1983) did find OKT4% to be increased in newly diagnosed diabetes. Newer monoclonals may contribute to the study of helper cells. The antibody 5/9, for example, reacts with 15 to 20% of peripheral T cells and this subset contains the cells which promote pokeweed

mitogen-driven B cell differentiation and increase the activity on killer cells (Corte et al, 1982; Moretta et al, 1982). 5/9+ cells are known to be increased in patients with active Graves' disease (Canonica et al, 1983).

When the two monoclonal antibodies OKT8 and UCHL1 (both of which identify the suppressor/cytotoxic cell subset) were compared in nine Graves' patients and nine controls, the overall correlation in the percentage of positive cells was good ($r = 0.763$, $p < 0.001$). This was, however, almost entirely due to the correlation in controls ($r = 0.933$) while the two sets of values for the Graves' patients correlated poorly, although both antibodies confirmed a reduction in the percentage of suppressor cells. This phenomenon has not previously been reported and clearly requires further study. It does demonstrate that caution is necessary when comparing results of studies which use different monoclonal antibodies. Changes in the cell surface chemistry may occur during the development of an autoimmune disease and this could affect the reaction of cells with monoclonal antibodies. Pincus et al (1985) showed that some activated lymphocytes in patients with rheumatoid arthritis expressed both the OKT4 and OKT8 markers. Other methods of quantifying suppressor cells are laborious to apply and difficult to reproduce. There are, however, problems associated with the use of monoclonal antibodies and each antibody must be evaluated for use in individual disease states.

In 20 normal female controls, there was a negative correlation between age and OKT8% ($r = -0.641$, $p < 0.01$) with a corresponding positive correlation between age and OKT4/OKT8 ratio. Absolute subset

numbers showed no such correlation and the percentage reduction may reflect an increase in another cell subset. The onset of autoimmune disease is well known to be age-related (Burch and Rowell, 1963) and this might be a reflection of age-related changes in immunological functions which could be exaggerated in those individuals genetically predisposed to autoimmune disease.

Chapter Eight

**Activated T Lymphocytes,
Killer/Natural Killer Cells and
the Expression of HLA-DR
on Thyroid Cells**

SUMMARY

Subsets of peripheral blood mononuclear cells have been investigated using monoclonal antibodies in patients with either newly diagnosed or recurrent Graves' disease. T cells with early activation antigen were studied with the antibody 4F2 while those with late activation antigens were quantified using three separate monoclonal antibodies to class II histocompatibility antigens. Cells with killer/natural killer activity were measured as were helper and suppressor subsets of lymphocytes.

OKT8 positive cells were reduced in both new and recurrent Graves' with 16.2% and 15.9% respectively compared to 22.4% in controls (both $p < 0.05$). 4F2 positive cells were increased in new Graves' with 14 out of 28 patients having increased numbers of positive cells ($p < 0.01$) but they were normal in patients with recurrent Graves' disease. There was a negative correlation between TSH receptor antibodies and 4F2 positive cells in newly diagnosed Graves' disease ($r = -0.603$, $p < 0.05$).

Class II antigen positive T cells were increased in both new and recurrent Graves' - 12 out of 21 (58%) new and five out of eight (63%) recurrent cases having increased numbers of positive cells. Many patients showed increased levels of positive cells with one antibody but not with the others. Overall, increased numbers of activated T cells were seen in 15/21 (71%) new cases and in 6/8 (75%) patients with recurrent thyrotoxicosis.

Using the antibody H366, cells with natural killer activity were found to be increased in untreated Graves' (10.6% of peripheral blood mononuclear cells compared to 6.0% for controls) but normal levels were found in recurrent disease. Class II antigen expression on the surface of thyroid cells was confirmed in three Graves' thyroids.

In conclusion, changes in cell-mediated immunity are present in Graves' disease with reduced percentage of suppressor cells, increased activated T cells and increased cytotoxic cells. Class II antigen expression both on thyrocytes and on T lymphocytes may be involved in initiating and maintaining the autoimmune response. The negative correlation between 4F2 positive cells and TBII may account for some of the heterogeneity seen in the expression of immunological markers. Recurrent Graves' disease differs immunologically from new disease by being associated with normal K/NK cells and early activated T cells in the peripheral blood although changes in immunoregulatory T cell subsets and late activated T cells are comparable to those found in new disease.

INTRODUCTION

The use of new monoclonal antibodies has much to offer for the study of cell-mediated immunity in Graves' and other autoimmune diseases. A sequence of antigen expression on the surface of T lymphocytes as they become activated is now apparent, while novel antibodies readily allow us to study cells with effector functions such as killer (K) and natural killer (NK) cells. Monoclonal antibodies have also revealed the aberrant expression of class II histocompatibility antigens on the

surface of tissues which are the target for autoimmune attack. Studies of these phenomena will tell us much about the early events in the development of diseases such as Graves' and further reveal the multi-faceted nature of the autoimmune response.

EARLY ACTIVATION ANTIGENS ON T CELLS.

By using monoclonal antibodies to study the appearance of cell surface antigens relative to DNA synthesis when T cells are stimulated, Cotner et al (1982) have defined a sequence of activation antigens. Thus, early activation antigens include the transferrin receptor, Tac (T cell activation antigen), and that defined by the monoclonal antibody 4F2 while, most notable amongst the late activation antigens are the DR specificities of the major histocompatibility complex (class II antigens). 4F2 is a mouse monoclonal developed and characterised by Eisenbarth's group in Boston, USA (Eisenberth et al, 1980; Haynes et al, 1981). Although primarily a monocyte antigen, it is expressed on the surface of about 70% of activated T cells. It has been widely used to study these cells and is included in the panel of monoclonal antibodies used for the work presented in this chapter. It binds to a cell surface component of 120,000 Daltons, is not related to HLA antigens and does not block killer or natural killer activity. Increased circulating mononuclear cells bearing the 4F2 antigen have been reported in Crohn's disease (Fais et al, 1985) but Jackson et al (1982, 1984) did not find increased levels of such cells in either untreated Graves' disease or in patients with recently diagnosed type I diabetes. Raised levels of 4F2 positive cells were found in

Hashimoto's thyroiditis by Canonica et al (1982) and, more recently, Kennedy et al (1985) have reported increased numbers in thyrotoxic Graves' patients (see Note 1 at the end of introduction).

CLASS II ANTIGENS ON ACTIVATED LYMPHOCYTES

Class II antigens are normally detected on the surface of B lymphocytes, monocytes and other antigen presenting cells such as the Langerhans cells of the skin, endothelial cells and haematopoietic precursors. Resting T cells do however express a small quantity of class II antigen which can be detected using the highly sensitive technique of flow microfluorometry with a fluorescence-activated cell sorter (Mann & Sharrow, 1979). The amount of Ia antigen on the surface of T cells can be greatly increased by stimulation with antigen, mitogen or in a mixed lymphocyte reaction (Zimmerman et al, 1979; Yu et al, 1980; Charron et al, 1980). The latter authors further showed that the electrophoretic pattern of the class II antigen so produced was similar to that on the B cells of the same individual. That the antigen is actively synthesized by the T cells rather than being passively absorbed, was shown by Evans et al (1978) using ^{35}S -methionine added to lymphocyte cultures and was later confirmed by Pincus et al (1985) by RNA hybridisation studies with a cloned HLA-DR alpha chain gene probe. It is also known that physical changes in the cell membrane induced by cross linking cell surface antigens can unmask DR antigens without the need for de novo synthesis (Mittler et al, 1983).

In normal individuals, only a small proportion of the circulating T cells are usually found to express class II antigens (Fu et al, 1978). The number of these "activated cells" is greatly increased after infection, in autoimmune diseases (SLE and rheumatoid arthritis) and in response to immunization with tetanus toxoid or purified protein derivative of tubercle (Yu et al, 1980). In the peripheral blood of rheumatoid patients, for example, 16.7% of T cells were found to be class II antigen positive compared to 2.4% in controls (Pincus et al, 1985). Ia positive cells are also found in the synovial fluid and tissues of some patients (Burmester et al, 1981) and, although some were of the "blastoid" type described by Yu et al (1980), more were typical of the T lymphocytes seen in the peripheral blood. Increased Ia positive T cells have also been found in the circulation of patients with Sjogren's syndrome (Sanvezie et al, 1982) and in a young patient with a primary autoimmune disease similar to acute graft-versus-host disease (Reinherz et al, 1979).

Increased class II antigen positive T cells were found in the circulation of nine out of eleven patients with diabetes of recent onset by Jackson et al (1982). The same applied to all 33 of the thyrotoxic Graves' patients studied by these authors, while other thyrotoxic patients and patients euthyroid after antithyroid therapy for Graves' had normal levels of activated T cells (Jackson et al, 1984). These findings were similar to those of the previous study by Canonica et al (1983). In their study of a series of patients tested sequentially after starting drug treatment, Ludgate et al (1984) confirmed the elevated Ia cells at diagnosis and reported a progressive decrease during treatment except in those patients whose disease remained active. In a recent report by Kennedy et al (1985) where Ia

positive T cells were again increased, the importance of using more than one antibody was stressed since many patients had increased levels of cells staining with one antibody but not with another. There is some overlap between the results in this report and the work presented in this chapter and the panel of monoclonal antibodies used was the same for both (see Note 1 at the end of introduction). Some heterogeneity in the expression of antigens on lymphoblastoid cells bound by two of the monoclonal antibodies used had already been reported (Van Heyningen et al, 1982). The class II molecules on B cells and activated T cells have at least one epitope which is not expressed on haematopoietic precursors or monocytes (Torok-Storb et al, 1983) and differences in the binding of anti-DR antibodies by Ia positive cells are well recognised to occur (Lampson & Levey, 1980; Hurley et al, 1982) and it thus seems better, where possible, to employ more than one monoclonal in the search for activated T cells.

The expression of class II antigens on the surface of activated T lymphocytes is undoubtedly important in the interaction with other cell types including antigen presenting cells. It also gives rise to the phenomenon of restriction: for example, Rodey et al (1979) have shown that, when accessory cells and antigen are added to T cells in culture, a proliferative response if the former cell type shares an HLA region determinant with the T cells. Similarly, the antigen production of spleen cells in response to antigenic stimulation can be inhibited by adding an anti-Ia specific for the Ia type of the cells but not with an antibody against another Ia type (Hodes et al, 1980). Class II antigens are thus crucial to the cooperation which exists between antigen presenting cells, B cells and helper T cells, a relationship which must be central in the production of an autoimmune disease. Examining

the cell infiltrates in the synovial tissue of rheumatoid patients, Klareskog et al (1982) found that the bulk of the T cells were helper/inducer cells and were mostly found in close proximity to cells expressing HLA-DR. Pincus et al (1985) found that the activated T cells expressed the T6 (thymocyte-related) antigen suggesting that they may be young cells released from the thymus in response to an immune challenge. Most of the activated T cells in the circulation share surface markers with suppressor cells - thus they are enriched in T_g fractions and can be stained with the monoclonal antibody OKT8 (Speares et al, 1979; Yu et al, 1980; Burmester et al, 1981). Direct evidence of their suppressor activity comes from the study of Sauvezie and colleagues (1982) who, by depleting lymphocyte suspensions of Ia positive cells, showed enhancement of both the autologous and the allogeneic mixed lymphocyte reactions (MLR).

KILLER (K) AND NATURAL KILLER (NK) CELLS.

Not all effector cells in the immune system are either B cells or typical T cells. A subset of cells with spontaneous cytolytic activity are now recognised, the natural killer (NK) cells (for review of their properties, see Herberman & Ortaldo, 1981; and summary in TABLE 8.1). These cells circulate in normal individuals and are thought to be important in immune surveillance. Most NK cells have Fc receptors for IgG and are therefore capable of antibody-dependent cellular cytotoxicity (i.e. K cell activity). Morphologically, they are somewhat larger than T cells with a higher ratio of cytoplasm to nucleus, they are thus often identified as "large granular lymphocytes". Although they do not depend on the thymus for their

TABLE 8.1 PROPERTIES OF NATURAL KILLER CELLS

1. 12 to 15 micrometers in diameter with a high cytoplasm to nucleus ratio (large granular lymphocytes).
2. Have spontaneous cytolytic activity against tumour cell lines but are not phagocytic.
3. Proliferate in response to T cell mitogens and T Cell Growth Factor.
4. They are non-thymus dependent but share a number of T cell markers (50% have receptors for sheep red blood cells but NK cells do not bind OKT3).
5. Possess receptors for the Fc portion of IgG and may therefore function as killer cells.
6. Their action is not restricted by MHC products.
7. There is no evidence for immunological memory.
8. They produce interferon and possibly T Cell Growth Factor.
9. They are recognised by the monoclonal antibody H366 which is directed at a 96,000 molecular weight cell surface protein.

development, they do express a number of T cell markers. For example, receptors for sheep red blood cells are found on about half of them although they do not bind the monoclonal antibodies OKT3, OKT4 or OKT8. They are not phagocytic but share some surface markers with monocytes (e.g. OKM1). NK cells will proliferate in response to the T cell mitogens phytohaemagglutinin and concanavalin A and can also be stimulated by Interleukin 1 (T cell growth factor) and interferon. Recently, NK cells have been shown to display activation antigens similar to those on activated T cells (HLA-DR, 4F2, transferrin and interleukin 2 receptors) when they are stimulated (London et al, 1985). their activity is not restricted by HLA gene products and, as a result, they can respond to antigenic challenge much quicker than cytotoxic T cells (probably less than four hours in vivo). There is no evidence for immunological memory in this subset of cells.

As with subsets of T lymphocytes, a number of methods have been developed to study NK cells. By looking at the formation of low affinity sheep red blood cell rosettes, Pozzilli et al (1979) have found raised levels of NK cells in 13 out of 23 patients with type I diabetes and in five out of ten unaffected sibs. Assays which directly measure ADCC (usually by release of Cr⁵¹ from antibody coated target cells) are more satisfactory than this method although they are technically much more demanding. Using such a method, Calder et al (1976) demonstrated raised K cell levels in patients with Hashimoto's, primary hypothyroidism and Graves' disease. In Graves' patients, the K cell activity was most marked in those patients with small or absent goitres and in those with low titres of circulating thyroid antibodies. Feldman et al (1976) using the same method, reported reduced activity of K cells in systemic lupus and in two

patients with polyarteritis nodosa. The K cells in this study were inhibited reversibly by some factor in serum. In a similar fashion, Goto et al (1981) found reduced K cell activity in Sjogren's and hypothesised that anti-lymphocytic antibodies might have been responsible although this was not borne out in their experiments. The negative correlation between ADCC and circulating immune complexes was discussed in Chapter 5 (Calder et al, 1974) and this may provide an alternative explanation for the reduced NK activity reported in some autoimmune diseases. Monoclonal antibodies provide a simpler and more reproducible way of studying cell subsets although the presence of a surface marker does not always reflect functional activity of that subset of cells. Pozzilli and Andreani (1982) used the antibody 3A1 in a cytotoxic assay to show increased K/NK cells in Graves' patients. The problem with this study was that the K/NK cells were contained in the 3A1-negative cell population and could only therefore be deemed to be increased by inference. Two mouse monoclonals have recently been described which will bind to cells active in both K and NK cell assays. H25 (an IgG₁) binds to a 96,000 Dalton subunit of a cell surface polypeptide, while H366 (an IgG_{2b}) binds to the other, 53,000 Dalton subunit. Increased levels of K/NK cells was reported recently (Kennedy et al, 1985; see Note 1 at the end of introduction) using one of these monoclonals (H25) and the other antibody (H366) has been used for the work presented in this chapter.

ABERRANT HLA-DR ANTIGEN EXPRESSION ON TISSUES

On the basis of their studies with thyroid cells in culture, follicular suspensions and cryostat sections of thyroidectomy specimens, Bottazzo and his colleagues (1983) at the Middlesex Hospital in London have developed one of the most intriguing hypotheses to come out of immunological research in recent years. They suggest that the expression of DR antigens on the surface of diseased thyroid epithelium, coupled with increased antigen availability because of reversed epithelial polarity exposing microsomal and other antigens might be responsible for initiating autoimmune attack on the gland. DR expression on thyroid cells in culture can be induced by the lectins phytohaemagglutinin, concanavalin A and pokeweed mitogen as well as gamma interferon which is much more potent (Pujol-Borrell et al, 1983; Todd et al, 1984). When cryostat sections of normal thyroid are stained with appropriate monoclonal antibodies, no DR staining and only weak HLA-A, B and C staining are seen. By contrast, the Middlesex group (Hanafusa et al, 1983) found 16 out of 20 Graves' thyroids to have patchy HLA-DR staining at the vascular pole of the thyrocytes. The amount of HLA-A, B, and C staining was greatly increased and the capillary endothelium showed particularly strong HLA-DR staining. Thyrocyte staining could also be demonstrated in follicular suspensions and on cultured monolayers of cells from these thyroids. The thyroid from a patient with Hashimoto's was even more strongly positive than the Graves' thyroids and one out of five euthyroid multinodular goitres was also positive and an increase in capillary staining was also seen in the latter thyroids. By changing the concentration of foetal calf serum in the thyroid cell cultures, it is possible to produce a gradual movement of microvilli from the colloid edge to the vascular pole of

the thyrocyte (Hanafusa et al, 1984). The tendency for this to happen is much greater in Graves' glands and it has been suggested that this may increase the exposure of microsomal antigen which is normally concentrated at the colloid edge.

The expression of HLA-DR on the surface of thyrocytes in Graves' glands has been confirmed by Jansson et al (1984) who also found thyroids from patients with nodular goitres to be positive. In contrast to the studies of Bottazzo's group, in this study the DR expression was found in relation to infiltrating lymphocytes (particularly those of the helper/inducer phenotype) and the extent of DR staining correlated with the degree of lymphocytic infiltration implying a pathogenetic relationship between the two. HLA-DR staining has also been found on the surface of the beta cells in the pancreatic islets in a patient with diabetes mellitus (G.F. Bottazzo, personal communication) and is recognised to occur in acute graft-versus-host disease and in the colonic epithelium of patients with inflammatory bowel disease (Mason et al, 1981; Selby et al, 1983). While in normal skin the HLA-DR staining is confined to the antigen presenting dendritic cells, it has been detected on the keratinocytes of patients with dermatoses such as eczematous dermatitis and discoid lupus (Lampert, 1984). DR expression was usually seen in association with keratinocyte damage and lymphocytic infiltration. Eczematous skin also showed a marked increase in the number of DR-positive Langerhans cells. Weetman et al (1985) have recently published studies on factors affecting the DR expression on cultured thyrocytes: DR could be induced, even in non-diseased thyroids, by culture supernatant containing T cell-derived lymphokines and, more potently, by gamma interferon which could also induce DQ expression. Phytohaemagglutinin would

induce DR on thyrocytes only in the presence of T cells. Unlike the induction of DR on monocytes, DR expression on thyrocytes could not be inhibited by the addition of prostaglandin E₂ or dibutyryl cyclic AMP.

There are however several problems which have to be overcome before aberrant HLA-DR expression can be accepted as an initiating event in the pathogenesis of autoimmune diseases: 1) growth factor as well as B cell growth and differentiation factors. Weetman et al (1985) were unable to detect IL-1 production from DR positive thyrocytes in culture. Keratinocytes, on the other hand, are known to be capable of producing IL-1 (Oppenheim & Gery, 1982) and this may account for the association of lymphocytes with DR-expressing keratinocytes observed by Lampert in human dermatoses. 2) The DR expression may simply be secondary to cell damage. For example, perturbation of the cell membrane by cross linking cell surface antigens can unmask DR (Mittler et al, 1983). In the dermatoses, DR expression was only seen in damaged areas of skin, while, in Jansson's study of Graves' thyroids the lymphocytes associated with DR-expressing cells may have caused thyrocyte damage by producing autoantibodies or by cell-mediated cytotoxicity. 3) The aberrant DR expression is not confined to thyroids with established autoimmune diseases. Both Jansson and Bottazzo have found euthyroid nodular goitres which are positive. However, immunological abnormalities are relatively common in patients with multinodular goitre and some role for autoimmunity in the pathogenesis of this condition cannot be ruled out. 4) Bottazzo has argued that the change in antigen availability due to reversed epithelial polarity is important. He notes that in the only six completely DR negative thyroids in their study, there was no

circulating thyroid microsomal autoantibody. This antibody is not however the major pathogenetic antibody in Graves' disease and its correlation with DR expression may simply reflect cell damage by these cytotoxic antibodies. All Graves' patients probably have antibodies directed towards the TSH receptor (see Chapter 2) although these are not always picked up by the standard assays. Since the TSH receptor exists to interact with circulating TSH, it would seem highly likely that it is expressed mainly at the vascular pole of the thyroid follicle rather than at the colloid edge. A change in antigen availability therefore seems unlikely for this antigen although, no doubt DR expression may markedly alter its immunogenicity.

NOTE 1: The work on lymphocyte subsets presented in this chapter was performed as part of a cooperative project between the Endocrine/Immunology Unit, Royal Infirmary, Edinburgh and the Department on Endocrinology in the University of Rome, Italy. Data from the Italian patients is contained in the report by Kennedy et al (1985) while that from the Edinburgh patients is presented here. The combined results of the two parts of the study, including the relationship of activated lymphocytes with humoral immune markers has been presented both to the British Endocrine Societies (4th Joint Meeting, Oxford, March 1985; see Kadlubowski et al, 1985) and to the Italian Endocrine Society (Pisa, May-June, 1985; see Scardellato et al, 1985) and a full report of this work has now been submitted for publication.

PATIENTS AND METHODS

The study included 28 patients with newly diagnosed Graves' disease (six male and 22 female, mean age 40 years) and nine patients (three male and six female, mean age 42 years) with recurrent Graves' disease. None of the new patients had received any antithyroid therapy at the time blood was withdrawn for study while the recurrent cases had all been treated previously with antithyroid drugs although none had been treated with radioactive iodine or surgery. All recurrent patients had enjoyed a period of remission prior to their recurrence. The diagnosis of Graves' disease was made, as before, on the basis of raised T_3 and/or T_4 , flat TRH test, diffuse goitre and/or scintigram. The controls were 24 normal subjects of comparable age and sex distribution to the patient groups.

COLLECTION AND STORAGE OF SAMPLES

For TSH receptor antibody studies, fresh serum was obtained and stored at -20°C . prior to use. The assay for TBII was performed on neat serum as described in APPENDIX 1. For cell studies, 20 mls. of venous blood was collected in a sterile universal container with 20 units per ml. of preservative-free heparin and mononuclear cells were isolated by gradient centrifugation as described in APPENDIX 3. These cells were frozen at a concentration of $5 \times 10^6/\text{ml}$ and stored in liquid nitrogen until used. Control samples were stored under identical conditions. The cells were thawed in a 37°C . water bath, washed twice in medium and their viability checked. The concentration was adjusted to 6×10^6 cells per ml. in RPMI 1640 with 10% foetal calf serum.

ENUMERATION OF CELL SUBSETS

Suppressor/cytotoxic cells, helper/inducer cells and total T lymphocytes were enumerated respectively with the antibodies OKT8, OKT4 and OKT3 (Ortho Diagnostics). The other monoclonal antibodies were used as part of a cooperative study between the Endocrine Unit, Royal Infirmary, Edinburgh and the Department of Endocrinology, University of Rome and were donated for use by Dr. Umberto di Mario.

4F2 binds to a 120,000 molecular weight glycoprotein on the surface of monocytes and activated T lymphocytes (Eisenbarth et al, 1980). 4F2 antibody was a gift of Dr. G. S. Eisenbarth, Joslin Clinic, Boston, U.S.A. and he also donated the antibody L243 which is against a determinant of the DR locus and is thus a marker for late lymphocyte activation (Lampson & Levey, 1980). The antibodies DA6.164 and DA6.231 (Guy et al, 1982; van Heyningan et al, 1982) bind to different determinants of the non-polymorphic region of the beta chain of DR molecules. These antibodies were a gift of Dr. K. Guy of the M.R.C. Clinical and Population Cytogenetics Unit, Edinburgh. The antibody H366 defines a set of cells with killer and natural killer activity and was donated by Dr. P. C. L. Beverley, I.C.R.F. Human Tumour Immunology Group, University College, London.

For the assays, approximately 300,000 cells were incubated in polystyrene tubes with 50 microlitres of monoclonal antibody (suitably diluted as determined by previous titration) at 4°C. for 30 minutes. The cells were washed twice with medium and 50 microlitres of fluorescein conjugated goat anti-mouse IgG (Nordic) at a dilution of 1:10 was added. The cells were incubated once more for 30 minutes at 4°C. and then washed twice. For those incubations with the

antibodies OKT3, OKT4, OKT8, 4F2 and H366, the cells were mounted in 30% glycerol/PBS and duplicate 200 cell samples were counted. For the cells stained with anti-DR antibodies, double staining immunofluorescence was carried out (Di Mario & Guy, 1984); The cell pellet was resuspended in 50 microlitres of medium with 0.1 mg. of OKT3 and a previously determined amount of freeze-dried rabbit complement (Pel Freeze Biologicals). The cells were incubated at 37°C. for 30 minutes and five microlitres of 1mg/ml ethidium bromide was added for one minute prior to washing. The cells were mounted on microscope slides and 200 cell samples counted as before. Values above the 90th centile of the normal controls were considered to be high.

CLASS II ANTIGENS ON THYROID CELLS

Three Graves' thyroids were tested for class II antigen positivity using five micrometer cryostat sections. Monoclonal antibodies were applied at the appropriate dilutions for 30 minutes at 37°C.. After washing, goat anti-mouse IgG was applied for 30 minutes and the sections were washed then read by fluorescent microscopy.

RESULTS

HELPER AND SUPPRESSOR LYMPHOCYTES

The percentage of OKT4 positive cells in normal controls (n = 24), new Graves' (n = 28) and recurrent Graves' (n = 9) was 42.3%, 42.1% and 45.0% respectively (no significant difference). OKT8 positive cells in controls were 22.4% compared to 16.2% in new and 15.9% in

recurrent Graves' (both $p < 0.05$, see FIGURE 8.1). The corresponding OKT4/OKT8 ratios were 1.95 for controls, 2.79 for new Graves' and 3.05 for recurrent Graves'.

EARLY ACTIVATED T CELLS

The median percentage of 4F2 positive cells in controls ($n = 15$) was 8.3% compared to 11.1% in 28 newly diagnosed Graves' patients ($p < 0.01$, see FIGURE 8.2). 14 (50%) of the Graves' patients had elevated levels of 4F2 positive cells. By contrast, only two out of nine recurrent cases had increased 4F2 positive cells and the median value in this group was 6.4% (not significantly different from normal).

TSH receptor antibodies were measured in 14 of the new patients. Positive values were obtained in 13 (93%) and there was a negative correlation between the values in the TBII assay and the percentage of 4F2 positive cells ($r = -0.603$, $p < 0.05$ - see FIGURE 8.3).

LATE ACTIVATED T CELLS

The percentage of class II antigen positive T cells in normal controls, new and recurrent Graves' patients is shown in TABLE 8.2. In the normal controls, the 90th centile for the antibodies DA6.164, DA6.231 and L243 were 2.9, 3.1 and 3.1 respectively.

Increased class II positive T cells were seen in 12/21 (58%) new Graves' (see TABLE 8.3) and in five out of eight (63%) recurrent Graves'. Taking results for 4F2 and class II antigen positive T cells together, increased activated T lymphocytes were seen in 15 out of 21 (71%) new Graves' and six out of eight (75%) recurrent Graves'.

FIGURE 8.1 SUPPRESSOR/CYTOTOXIC (OKT8 POSITIVE) CELLS IN NEWLY DIAGNOSED AND RECURRENT GRAVES' DISEASE

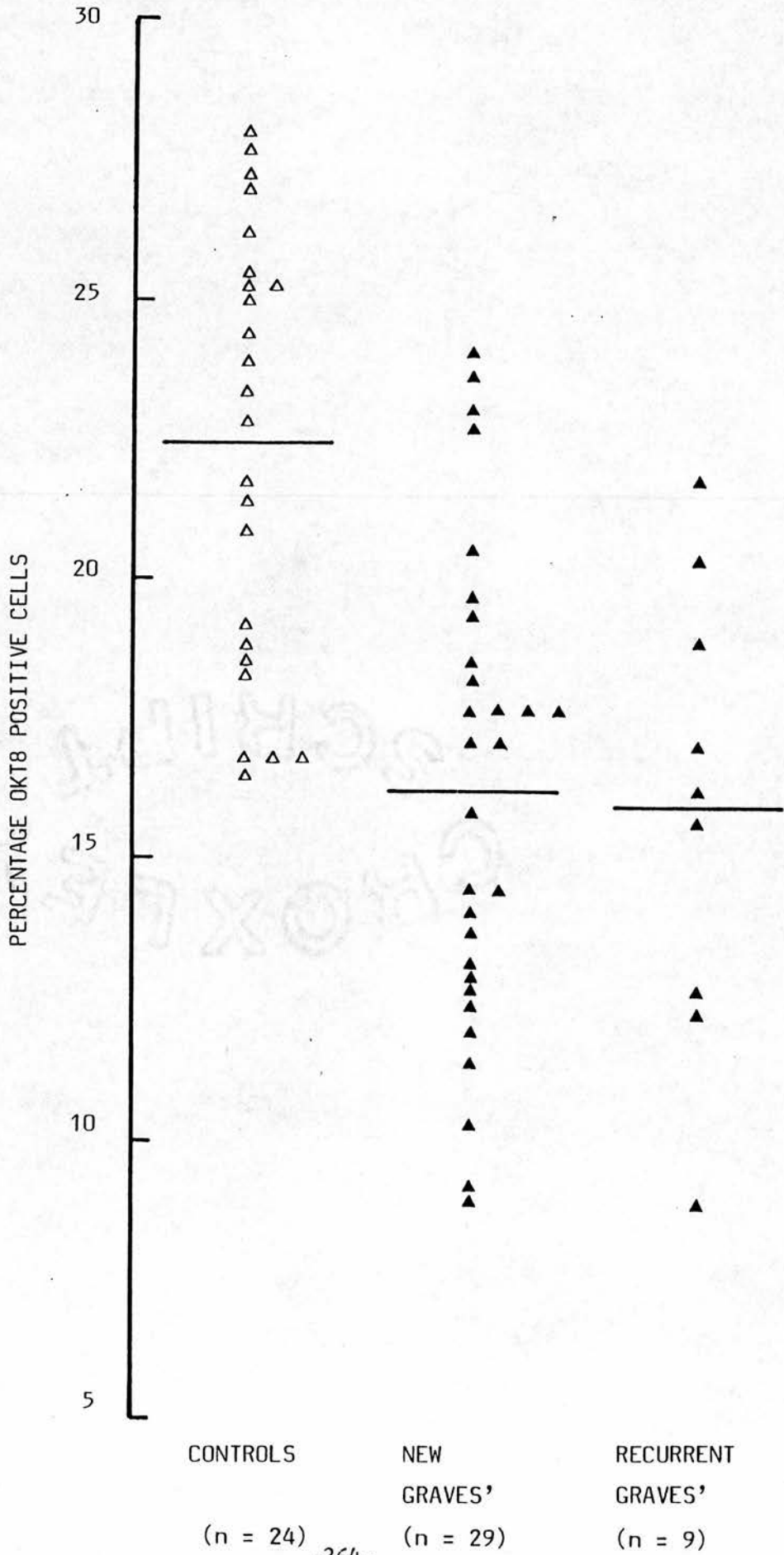


FIGURE 8.2 EARLY ACTIVATED T LYMPHOCYTES IN NEWLY DIAGNOSED AND RECURRENT GRAVES' DISEASE

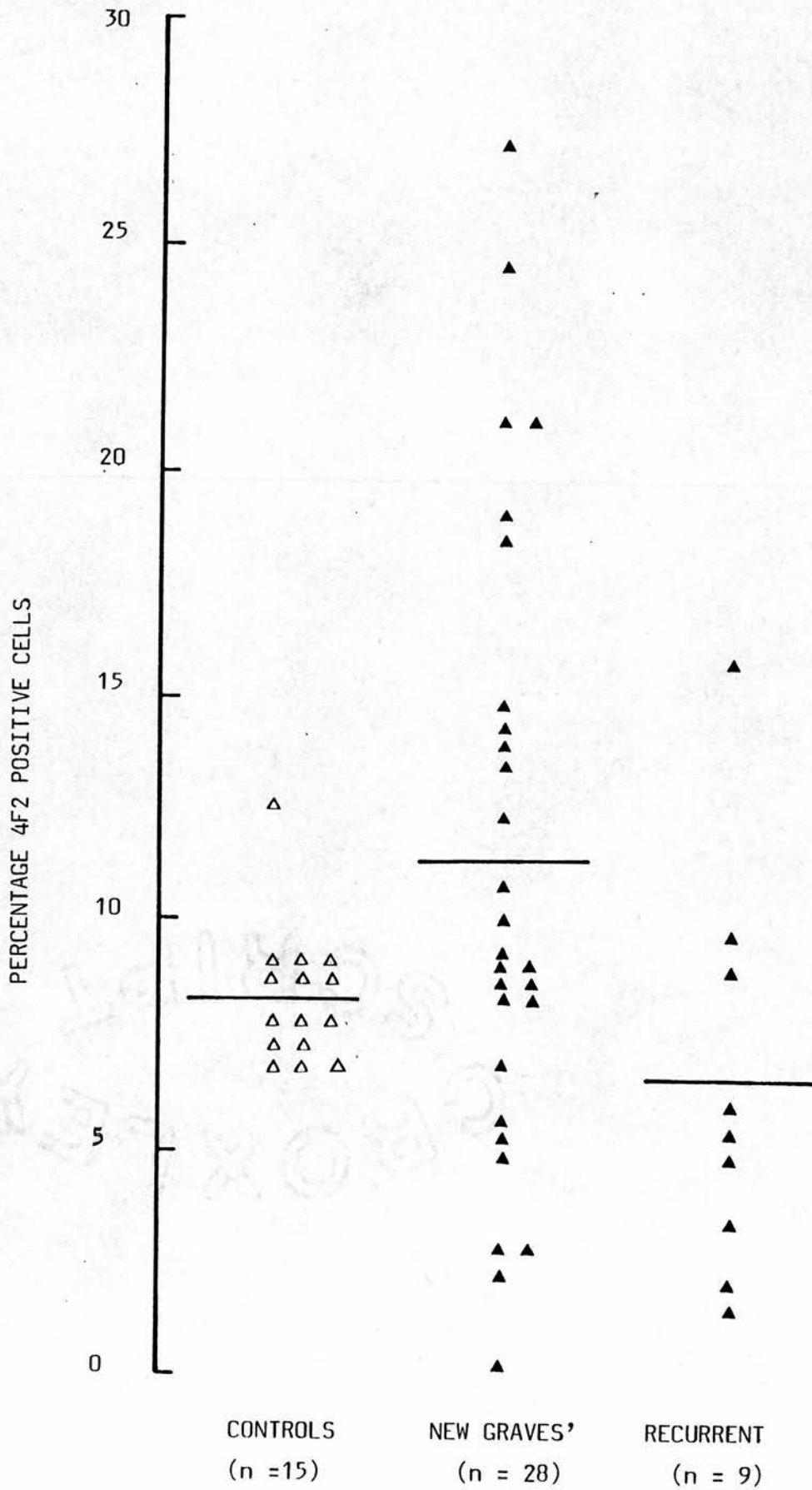


FIGURE 8.3 CORRELATION BETWEEN ⁴F2 POSITIVE CELLS AND TSH RECEPTOR ANTIBODIES IN NEWLY DIAGNOSED GRAVES' DISEASE

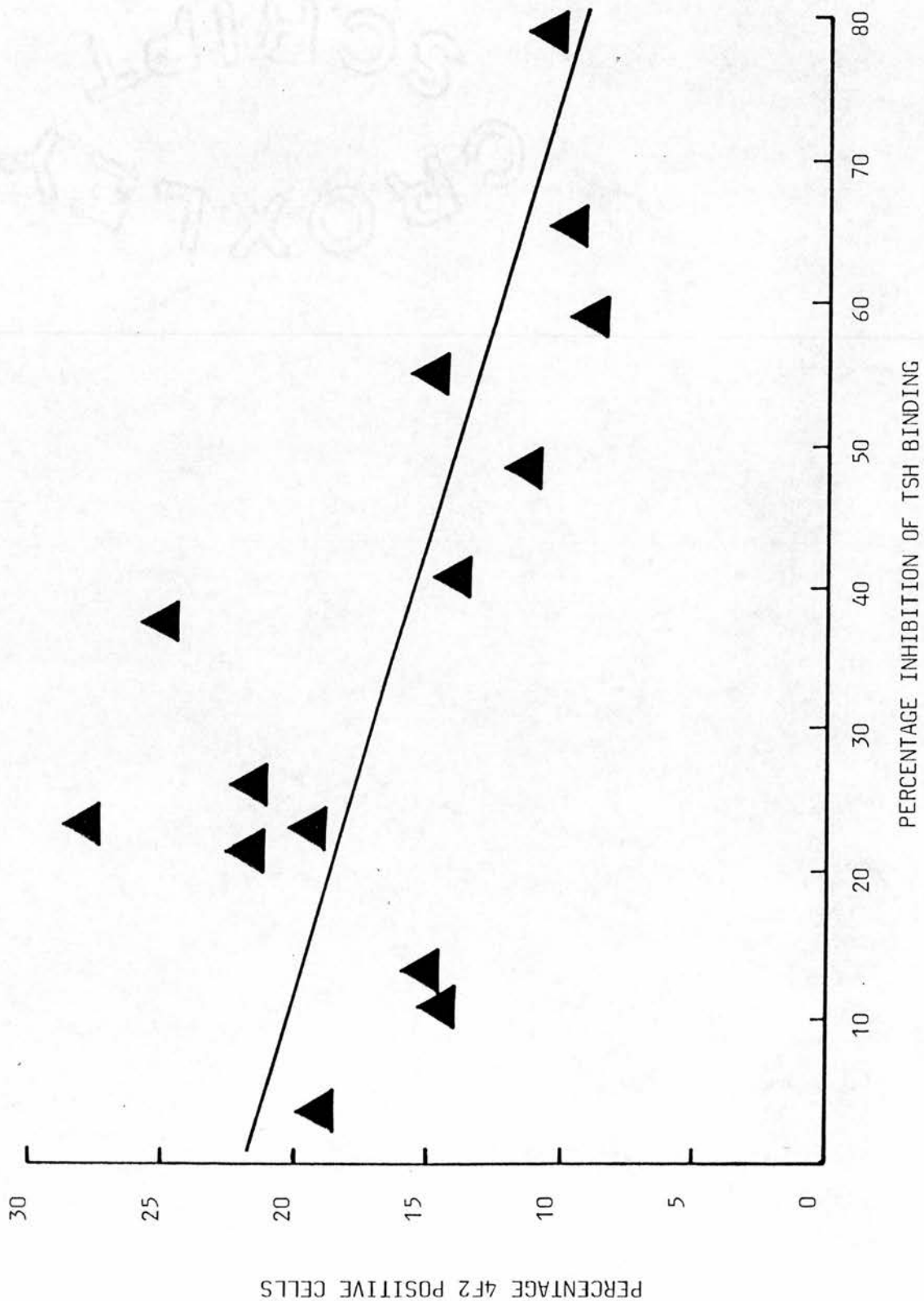


TABLE 8.2 CLASS II ANTIGEN POSITIVE T CELLS IN NORMAL CONTROLS AND IN PATIENTS WITH NEWLY DIAGNOSED OR RECURRENT GRAVES' DISEASE

	MONOCLONAL ANTIBODY		
	L 243	DA6.164	DA6.231
CONTROLS (No.)	1.6% (18)	0.5% (23)	1.1% (23)
NEW GRAVES' (No.)	5.1% (19)	1.8% (21)	2.7% (21)
RECURRENT (No.)	2.4% (8)	1.9% (8)	2.5% (8)

The figures given in the table refer to the percentage of peripheral blood mononuclear cells which are HLA-DR positive T lymphocytes. Absolute numbers of these cells were not calculated because of the small percentages involved.

TABLE 8.3 CLASS II ANTIGEN POSITIVE T CELLS IN INDIVIDUAL PATIENTS
WITH GRAVES' DISEASE

PATIENT	DA6.164	DA6.231	L.243	ABNORMAL?
1.	0	1.8	4.2	YES
2.	0	0	1.0	NO
3.	0	1.2	3.1	NO
4.	5.3	7.5	5.2	YES
5.	2.1	1.9	0.9	NO
6.	2.9	4.8	4.9	YES
7.	1.2	1.9	N.T.	NO
8.	0.8	0.8	8.7	YES
9.	0	0.9	5.7	YES
10.	0	0.9	0.9	NO
11.	6.6	11.9	3.6	YES
12.	1.9	1.8	2.7	NO
13.	2.6	0	N.T.	NO
14.	1.0	1.8	2.4	NO
15.	2.0	1.9	4.0	YES
16.	2.9	0	5.7	YES
17.	0	4.9	6.5	YES
18.	5.1	5.4	12.6	YES
19.	0	3.1	11.0	YES
20.	2.9	2.0	12.4	YES
21.	0.7	1.4	1.5	NO

90th centile values for normal controls:-

DA6.164	2.9%
DA6.231	3.1%
L 243	3.1%

N.T. = Patient's cells not tested against this antibody.

KILLER/NATURAL KILLER CELLS

Percentages of H366 positive cells with characteristic small lymphocyte appearance in controls and the two groups of Graves' patients are shown in FIGURE 8.4. The mean value for the 16 controls was 6.01% compared to 10.6% for 28 patients with untreated Graves' ($p < 0.05$). The value for recurrent Graves' was 6.24% which did not differ from normal controls.

CLASS II ANTIGENS ON THYROCYTES

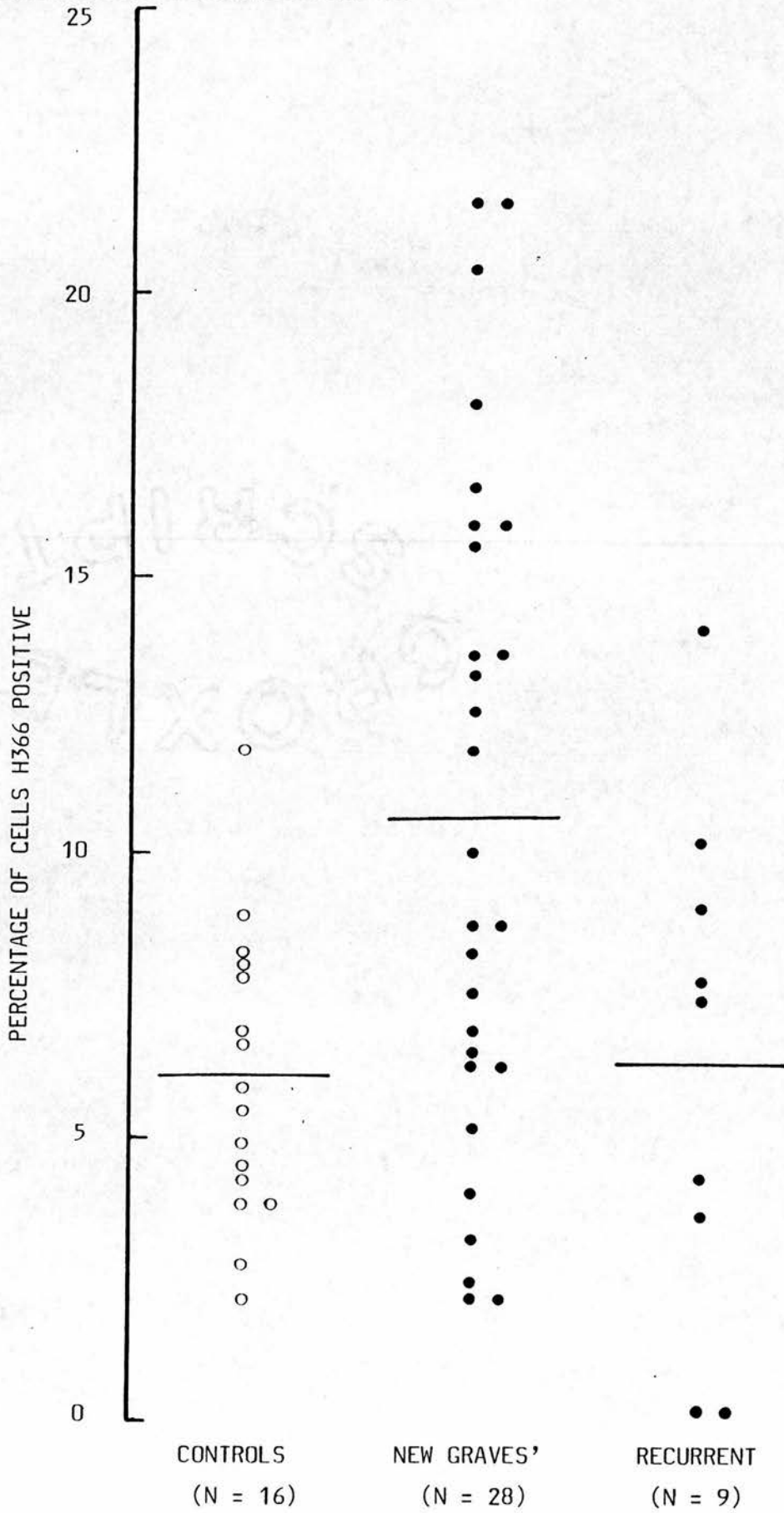
The monoclonal anti-DR antibodies were tested on cryostat sections of three Graves' thyroids. Similar results were obtained for all three glands, the fluorescence being seen as a thin rim round the vascular pole of the thyroid follicle (see below):-

DA6.164 NEGATIVE

DA6.231 NEGATIVE

L243 POSITIVE

FIGURE 8.4 PERCENTAGE OF KILLER/NATURAL KILLER CELLS IN NEWLY
 DIAGNOSED AND RECURRENT GRAVES' DISEASE



DISCUSSION

Activated T cells and cells with killer/natural killer activity were enumerated in the peripheral blood mononuclear cell preparations of patients with thyrotoxic Graves' disease. A distinction was made between patients at first presentation and those in whom the thyrotoxicosis was recurrent. A sequence of T cell activation antigens is recognised (Cotner et al, 1983). Early activation antigens include that recognised by the antibody 4F2 which binds to a 120,000 molecular weight glycoprotein on the surface of 70% of activated T cells (Haynes et al, 1981) while late activation antigens include class II histocompatibility determinants.

Jackson and colleagues (1982) found the proportion of 4F2 positive cells to be normal in the peripheral blood of Graves' patients and in newly diagnosed diabetics. Subsequently, however, increased 4F2 positive cells were found in Hashimoto's patients by Canonica et al (1982) and in Graves' patients by Kennedy et al (1985). In the study presented here, the mean percentage 4F2 positive cells in controls was 8.3% compared to 11.1% in newly diagnosed Graves' patients ($p < 0.01$). In patients with recurrent thyrotoxicosis, only 6.4% of cells were 4F2 positive. The early stages of activation of the cell-mediated immune system may have been superceded in these patients by memory phenomena or may have become confined to the thyroid gland. In newly diagnosed patients, there was a negative correlation between 4F2 positive cells and TBII. The possibility that TBII were masking the 4F2 antigen and thus preventing the antibody from binding was investigated (data not presented) by culturing normal lymphocytes with and without TSH receptor antibody

preparations and in the presence and absence of mitogens. TBII did not per se affect the expression of the 4F2 antigen and it is concluded that early activation of the cell mediated immune system and TSH receptor antibody formation may represent different stages in the pathogenesis of Graves' disease.

The percentage of T cells bearing class II antigens was raised in 12 out of 21 (58%) new Graves' patients and in five out of eight recurrent cases. The comparable incidence of increased cell numbers in new and recurrent disease is in contrast to the data for early activation antigen. Class II antigen positive T cells may be important in antigen presentation to other components of the immune system and the expression of these antigens could thus be regarded as an end point of cell activation. The work of Ludgate et al (1984) showing that activated T cells decline with antithyroid drug therapy would suggest that increases of these cells are related to recurrence of the disease rather than persistence of an underlying immunological abnormality. The work presented here shows the importance of using antibodies against different determinants of the DR molecules. Class II antigen expression at the vascular pole of the thyroid follicle has been confirmed although only one of the three antibodies tested gave consistently positive results.

Cells with K/NK activity as defined by the antibody H366 were elevated in new but not in recurrent Graves'. This again may be because the immune reaction is confined more to the thyroid in advanced disease. It is not clear why some patients have elevated cytotoxic cells in the circulation while others do not but one reason

may be the presence of circulating immune complexes which can decrease K cell numbers (as discussed in Chapter 5).

These studies with monoclonal antibodies provide further evidence for the role of the cellular immune system in the pathogenesis of autoimmune thyroid disease. The inverse relationships between K cells and immune complexes and between activated T cells and TSH receptor antibodies may underlie some of the heterogeneity which exists in the expression of immunological markers.

Chapter Nine

Autoantibodies in Feline Hyperthyroidism

SUMMARY

Thyroid autoantibodies have been demonstrated by indirect immunofluorescence in the sera of ten out of 29 (34.5%) cats with hyperthyroidism. Antinuclear factor, rare in normal cats, was found in a further four cats. 28 of the cats had a palpable goitre at first presentation - in 16 cases this was unilateral while in the others the goitre was bilateral. Lymphocytic infiltration was present in nine of the 27 (33%) thyroids examined histologically.

Five of the sera gave a particularly strong reaction on immunofluorescence for thyroid antibodies. Four of these cases had bilateral goitres and lymphocytic infiltrates were found in four of the five thyroids ($p < 0.05$). 21 of the cats were followed up for a mean period of 10.3 months after operation, during which time three cats developed recurrent hyperthyroidism: Two of them had bilateral goitres with lymphocytic infiltration at first presentation and the serum was strongly positive in both for thyroid autoantibodies. The third recurrence was in a cat with unilateral goitre and lymphocytic infiltration and the serum in this case was positive for antinuclear factor, the recurrence involving the same lobe which had been previously operated on.

Some cases of feline hyperthyroidism may be immunologically mediated and the condition is thus a potential model for some aspects of autoimmune thyrotoxicosis in Man.

INTRODUCTION

The study of animal models of thyroiditis has greatly contributed to our knowledge not only of this condition but that of autoimmune disease in general. However, no satisfactory model exists either for Graves' disease or for toxic nodular goitre. Attempts to induce the former condition in animals have been unsuccessful while the pathogenesis of the latter condition is poorly understood. Spontaneous hyperthyroidism is uncommon in the animal kingdom and, when it does occur, it is usually due to malignant neoplasms of the thyroid. The one exception is the domestic cat in which hyperthyroidism has been found recently to be a relatively common occurrence. Although the lesion is more akin histologically to toxic nodular goitre, a role for autoimmunity in the pathogenesis of the condition has not yet been ruled out.

Feline hyperthyroidism was first described in 1979 by Cotter and by Peterson et al. The common occurrence of benign thyroid lesions in the cat was previously well recognised (Lucke, 1964) and, in reviewing 52 cases of enlarged thyroid, Leav et al (1976) found 22 to have typical benign adenomas while a further 17 had multinodular adenomatous goitres. Over 90% of palpable neoplasms in the cat thyroid are benign in contrast to, for example, the canine thyroid where 90% of clinically apparent tumours are carcinomas and 25% or less produce hyperthyroidism (Kirk, 1982). Hyperthyroidism is equally common in male and female cats and shows no particular breed predisposition. The clinical presentation is entirely comparable to the human condition with weight loss, hyperactivity, increased temperature and heart rate, cardiac failure, anorexia, vomiting, diarrhoea with bulky

stool, thirst polyuria, hyperaemia of the skin and mucous membranes and muscle weakness. The feline thyroid consists of two separate lobes which are not usually joined by an isthmus (Lucke, 1964). Goitre in the cat may thus be unilateral or bilateral.

Radioisotope scanning using 99m technetium as pertechnetate is a useful adjunct to diagnosis and to assess the extent of thyroid involvement prior to surgery (Hoenig et al, 1982). With the use of scintiscans, many cases which were thought to be unilateral initially turn out to have bilateral involvement: In Peterson's review (1984) of 135 cases which had been scanned, 38 (27%) had unilateral involvement while the remaining 97 (73%) were bilateral. The treatment of choice for hyperthyroidism in the cat is, of course, surgery with complete removal of the affected lobe or lobes. After operation, the thyroid hormone levels return to normal within 24 hours (Peterson et al, 1980). Propylthiouracil has also been shown to be an effective treatment (Peterson, 1981) but, apart from obvious difficulties of administration, side effects are quite common and, in particular a syndrome with autoimmune haemolytic anaemia and thrombocytopenia has been described in nine out of 105 cats treated (Peterson et al, 1984).

Animal Models of Hypothyroidism

It seems appropriate here to discuss the contribution that the study of animal models of thyroiditis has made to our understanding of the pathogenesis of autoimmune thyroid disease in Man. Of particular interest are the studies on murine thyroiditis and the disease which

occurs in Obese Strain chickens and these are both discussed in detail below. Thyroiditis in the dog is also of interest because it occurs naturally in a domestic animal. These three models are not however the only ones: Thyroglobulin administration to guinea pigs in appropriate dosage schedules may induce thyroiditis and a similar disease can be induced in the Rhesus monkey injected with crude thyroid extract (Kite et al, 1966). Rats may also be induced to develop thyroiditis by immunization with thyroglobulin although certain strains such as the BUF strain will develop the disease spontaneously. The genetic influence has been studied in the rat to some extent and the antibody response to thyroglobulin is not thought to be linked to the major histocompatibility complex (Lillehoj et al, 1981). An influence of genes on the X chromosome has been postulated to account for the enhanced response to thyroglobulin in female animals. The T cell proliferative response correlates better with the severity of the lesions than does antibody level suggesting that there may be separate genetic influences on the humoral and cell mediated immune abnormalities (Lillehoj & Rose, 1975). Microsomal antigen as well as thyroglobulin may induce thyroiditis in the rabbit and, indeed this was the first animal model of thyroiditis to be described (Witebsky & Rose, 1956; Mangkornkanok et al, 1972).

Susceptibility to murine thyroiditis is known to be related to the major histocompatibility complex of the mouse and, in particular, to the H-2K locus (Vladutiu & Rose, 1971) although an influence of the H-2D end of the gene complex is also recognised and may be related to the control of suppressor cell function (Kong et al, 1979). Although B lymphocytes are clearly required for autoantibody production and thence the induction of thyroiditis - indeed the

thyroid lesions may be induced by the passive transfer of immune serum (Tomazic & Rose, 1975) - the susceptibility to thyroiditis following immunisation with thyroglobulin is transferred with T lymphocytes rather than B lymphocytes (Vladutiu & Rose, 1975). Evidence for the role of immunoregulatory T cell subsets in the induction of thyroiditis also comes from other animal models (for review of T cell regulation in murine and Obese Strain chicken thyroiditis, see Rose et al, 1981). For example, the thymectomised, irradiated rat has been used as a model (Penhale et al, 1975). Thyroiditis is much less common in rats subjected only to sublethal total body irradiation but not thymectomised (Penhale et al, 1973) and the autoallergic response may be suppressed by reconstituting the animals with normal lymphoid cells (Penhale et al, 1976). In the Obese Strain chicken, thymectomy also greatly enhances the development of thyroiditis (Wick et al, 1970; Welch et al, 1973). Inhibition of suppressor cells with cyclophosphamide has been shown to convert low responder mice to high responders (Vladutiu, 1982) while activation of suppressors by appropriate immunization schedules may induce tolerance to thyroglobulin (Okayasu et al, 1980; Kong et al, 1982).

Although the production of autoantibodies and the formation of complement fixing immune complexes are early and necessary events in the evolution of thyroiditis (Clagget et al, 1974), they are not the only mechanisms involved: Poor responder strains may produce very high levels of autoantibody with little evidence of tissue damage and no reason to suspect deficiency of a complement fixing subclass of immunoglobulin (de Carvalho & Roitt, 1982). Good responder strains have T cells reactive to thyroglobulin (Esquivel et al, 1978) and

stimulation with polyadenylic:polyuridylic acid shows them to have helper function which may be important in the development of thyroiditis. In experiments with thyroid cell monolayers, depletion of T cells from the lymphocyte suspensions reduces the degree of sensitisation to the thyrocytes (Charriere, 1982) whereas there is enhanced reactivity with a T cell enriched fraction and, indeed, a pure T lymphoblast suspension can be immunologically sensitised to the thyrocytes (Charriere & Michel-Bechet, 1982). More recently, a direct cytotoxic effect of T lymphocytes has been demonstrated and is specific for the inducing antigen (thyroglobulin) and is also H-2 restricted (Creemers et al, 1983).

Obese Strain (OS) chickens develop severe thyroiditis, circulating anti-thyroglobulin antibodies and hypothyroidism within a few weeks of hatching. Three factors are thought to be important in the development of the lesion in these animals (Wick et al, 1982): Firstly, genetic susceptibility related to the major histocompatibility complex, although this is less well characterised than for the mouse. Secondly, abnormal thymic maturation with a relative deficiency of suppressor cells and thirdly, intrinsic abnormalities of the thyroid gland. The genetic influence is related to the b locus of the MHC (Bacon et al, 1974) although this is not the only genetic factor involved (Bacon et al, 1981). Further recent evidence for the role of non-MHC genes has come from the work of Boyd et al (1983) who have compared two inbred strains of chicken which are identical as far as the MHC is concerned but one was selected for only on the basis that it developed hypothyroidism while the other strain was specifically bred because it had high levels of circulating anti-thyroglobulin and this strain also had high levels

of thyroid-specific cytotoxic T cells and antibody dependent cellular cytotoxicity. The role of thymic maturation of T cells in the pathogenesis of the disease is apparent from the enhancing effect of thymectomy in the development of thyroiditis (Wick et al, 1970; Welch et al, 1973). However, in this model too, T cells are clearly necessary for the development of the thyroid lesion since combination of thymectomy with treatment with anti-T cell serum (which completely depletes the animal of T cells) prevents the thyroiditis evolving (de Carvalho et al, 1981). Furthermore, the disease may be produced in T cell depleted normal animals by reconstitution with T cells from histocompatible OS chickens (Livezey et al, 1981). The conclusion is that a relative defect in suppressor cells in these animals allows helper and cytotoxic T lymphocytes to initiate the autoimmune response.

The disease is, however, multifactorial and abnormalities are not confined to the immune system. Incomplete suppression of ^{131}I uptake by administered thyroxine has been shown (Sundick et al, 1979) as has increased thyroid uptake of ^{131}I when glands are transplanted to the chorioallantoic membranes of normal chickens (Sundick & Wick, 1976). Susceptible chickens may thus go through an initial stage of hyperthyroidism before thyroiditis is initiated and this is supported by the observation that thyroglobulin levels are high in the early weeks of life prior to the onset of thyroiditis (Sanker et al, 1983). The release of antigens from an overactive gland may stimulate an immune reaction in genetically susceptible animals and, in keeping with this would be the observed temporary suppression of anti-thyroglobulin antibody synthesis when thyroxine is administered in doses sufficient to suppress endogenous thyroid activity (Sanker

et al, 1983). The possibility that this effect may not be due to reduction in antigen release but to a direct effect on the immune system must be borne in mind. Long and Shewell (1955) showed that T₄ administration enhanced the immune response to injected diphtheria toxin and, more recently, work on experimentally hyperthyroid animals has suggested that T₄ may specifically inhibit suppressor cell function while retaining normal B cell function (Wall et al, 1979). The presence of a generalised autoimmune disturbance in OS chickens has been confirmed by the demonstration that some animals have antibodies not only to thyroid components but also to the proventricular glands of the stomach (analogous to parietal cells) and occasionally to pancreas and adrenal glands (Khoury et al, 1982).

Naturally occurring canine thyroiditis is well described (Mueller et al, 1983). It has an equal sex incidence and may affect practically any breed of dog although some breeds are particularly predisposed. Two types of pathology are recognised to lead to thyroid failure in the dog (Gosselin et al, 1981): Firstly, idiopathic follicular atrophy, where the functioning thyroid tissue is replaced by fatty fibrous tissue. It appears to be a degenerative condition with no immunological component. Secondly, lymphocytic thyroiditis which is an autoimmune condition similar to human Hashimoto's thyroiditis. Here, infiltration of the gland by lymphocytes, plasma cells and macrophages precedes fibrosis and is associated with circulating antithyroid antibodies. The condition was first described in 1968 by Beierwates and Nishiyama in a colony of purebred beagles where thyroiditis occurred in over 10% of animals. Only dogs with the most severe thyroid lesions showed evidence of low PBI on a low iodine diet, low ¹³¹I uptake and lack of response of uptake to injected TSH.

Antibodies against thyroid microsomes, thyroglobulin and a non-thyroglobulin colloid antigen have been demonstrated in these dogs (Micejewski et al 1971). Systemic lupus erythematosus has also been well characterised in the dog and produces a spectrum of clinical abnormalities similar to the human condition. It is associated with circulating antinuclear antibodies and there is often a family history of other autoimmune disease including lymphocytic thyroiditis (Grinden & Johnstone, 1983). The overlap between autoimmune thyroid disease in Man and non-organ specific autoimmune disease is also documented (Hijmans et al, 1961).

ANIMAL MODELS FOR GRAVES' DISEASE

There is at present no animal model for human Graves' disease. One experimental approach which has been tried involves the use of transplanted tissue. Gittes (1966) used transplanted isologous thyroid tissue to study thyroiditis in inbred strains of guinea pig and found that the lesion produced in transplanted tissue was identical to that in the in situ thyroid when the animals were immunized with thyroid antigen. The use of athymic (nude) mice has been suggested by Smeds et al (1981) as a suitable recipient for transplanted Graves' tissue but, although transplanted thyroid tissue survives in this animal, it does not continue to hyperfunction (Leclare et al, 1984). Part of the problem is undoubtedly the lack of T cells in these animals, particularly helper T cells which are necessary for the immune reaction and thus autoantibody production to

continue. In support of this, Wick et al (1978) found that thyroiditis did not develop in homozygous nude mice when injected with thyroid antigen but did develop in Nu/+ heterozygotes.

The work presented in this chapter is concerned with the search for autoantibodies to the thyroid gland in a series of 29 hyperthyroid cats which were investigated, treated and followed up in the Department of Veterinary Medicine of the University of Edinburgh. It was hypothesised that immunological abnormalities might be found especially in cats with bilateral goitre and particularly if this was associated with lymphocytic infiltration of the thyroid.

MATERIALS AND METHODS

The study comprised 29 consecutive cases of feline hyperthyroidism presenting either directly to the University of Edinburgh's Department of Veterinary Medicine or to veterinary surgeons in private practice who agreed to take part in the study. The diagnosis was made on the basis of history and clinical findings and confirmed by the presence of elevated total thyroid hormone levels. The clinical assessment of the patients, their treatment, follow up and thyroid hormone measurements were all carried out by Mr. Keith Thoday of the above department.

Thyroxine (T₄) and triiodothyronine (T₃) were measured by double antibody radioimmunoassays optimised and validated for use with cat serum as described by Thoday et al (1984). Normal ranges were

established by the nonparametric method of percentile estimates, using measurements from 318 normal cats for T_4 and 299 normal cats for T_3 . For T_4 this was 8.5 to 46.2 nmol/l (Mean \pm SD = 26.1 \pm 10.1) and for T_3 0.13 to 1.26 nmol/l (Mean \pm SD = 0.69 \pm 0.29).

Two cats died soon after diagnosis was made. Euthanasia was requested by the owners in two further cases. One cat was treated medically with propylthiouracil and one cat was lost to follow up. The remaining 23 cases were treated surgically by unilateral or bilateral thyroidectomy after one week's treatment with potassium iodide (ten milligrams, three times daily) and propranolol (2.5 milligrams, three times daily). Because radionuclide imaging facilities were not available, the decision whether to perform unilateral or bilateral thyroidectomy was a clinical one based on the distribution of goitre and the appearance of apparently uninvolved lobes at operation. Affected lobes were removed, taking care to preserve at least one parathyroid gland and excised tissue was preserved in 10% formal saline for histological examination. Tissue for immunological studies was snap frozen in liquid nitrogen and subsequently stored at -40. Where possible, animals were followed up at regular intervals after the operation.

AUTOANTIBODY MEASUREMENTS

Thyroid microsomal and antinuclear antibodies were measured by indirect immunofluorescence on normal cat thyroid. The tissue was removed from a normal cat after euthanasia using intravenous injection of 20% pentobarbitone sodium, and frozen in liquid

nitrogen. For the assay, sera were diluted 1:4 with phosphate buffered saline pH 7.2 (PBS) and applied to five micrometer sections of thyroid for 30 minutes at room temperature. These sections were then washed in PBS for 30 minutes with two changes of buffer. Goat anti-cat IgG conjugated with fluorescein isothiocyanate (Miles-Yeda) was then applied at a dilution of 1:8 for 30 minutes after which slides were washed for two hours with several changes of buffer.

The sections were coded and read under ultraviolet light. The intensity of fluorescence was scored as follows:

- 0 = No fluorescence.
- + = Borderline fluorescence (not counted as positive).
- +
- ++ = Strong positive.

Sera from fifteen healthy cats acted as controls and autoantibodies were not detected in any of their sera. TSH receptor antibodies were sought using the radioreceptor assay with neat serum as described in Southgate et al (1984). The non-organ specific nature of the antinuclear factor detected in some cases was confirmed by repeating the experiment using rat kidney as a substrate.

STATISTICS

Continuous variables were analysed using the Student's t test (T_3 and age). The Wilcoxon Rank Sum test was used for T_4 since two of the very high values were not precisely quantified, and for TSH receptor antibodies. All other data was analysed using the Fisher Exact test.

RESULTS

There were 28 domestic short haired and one domestic long haired cats in the study. Thirteen (45%) were male (one intact and 12 castrated) and fifteen were female (three intact and 13 ovariohysterectomised). The mean age was 12.8 years with a range of six to seventeen years. The symptoms and signs at presentation were as follows: Weight loss (92%), polyphagia (81%), polydipsia and polyuria (78%), hyperactivity (58%), diarrhoea (57%), polypnoea 39%), tachycardia (39%), skin changes (mats, scaling and hyperaemia - 38%), increased faecal bulk (35%), vomiting (24%), elevated temperature (18%), reduced activity (17%), cardiac murmur (14%), dyspnoea (8%) and anorexia (8%). Twenty eight cats had a palpable goitre and 16 of these were unilateral while the remaining 12 were bilateral.

Thyroid antibodies were detected in ten cases - five of which were strongly positive. Antinuclear factor was found in a further four cats. Clinical and laboratory data from the fifteen antibody negative cats is shown in TABLE 9.1 and that for the fourteen antibody positive cats in TABLE 9.2. In all cases, the total T_4 was elevated,

TABLE 9.1 CLINICAL AND LABORATORY DATA ON ANTIBODY NEGATIVE CATS

NO.	SEX	AGE (years)	T ₃ (nmol/l)	T ₄ (nmol/l)	GOITRE	LYMPHO. INFILT.	FOLLOW- UP
1	M (c)	12	5.57	297.6	UNILAT	NONE	30 mths.
2	M (c)	12	1.27	71.6	BILAT	NONE	died
3	F (o)	15	2.40	150.0	UNILAT	NONE	11 mths.
4	F (o)	-	3.60	145.0	UNILAT	NONE	9 mths.
5	F (o)	11	4.00	241.0	UNILAT	NONE	10 mths.
6	F (o)	15	2.50	152.0	UNILAT	PRESENT	4 mths.
7	M (c)	10	1.73	160.0	UNILAT	PRESENT	died
8	M (c)	-	4.00	440.0	BILAT	PRESENT	euth
9	F (o)	10	6.20	168.0	BILAT	-	euth
10	F (o)	17	5.80	299.0	UNILAT	NONE	8 mths.
11	F	9	1.65	107.8	BILAT	NONE	12 mths.
12	M	13	6.70	338.0	NONE	-	-
13	F	16	1.70	92.0	UNILAT	NONE	3 mths.
14	F	15	-	136.0	BILAT	NONE	3 mths.
15	F (o)	15	9.00	654.0	BILAT	NONE	died

M = Intact male

F = Intact female

M (c) = Castrated male

F (o) = Ovarohysterectomised female

died = Death in immediate pre- or post-operative period

euth = Euthanased

TABLE 9.2 CLINICAL AND LABORATORY DATA ON ANTIBODY POSITIVE CATS

No.	SEX	AGE (years)	T ₃ (nmol/l)	T ₄ (nmol/l)	GOITRE	LYMPHO. INFILT.	FOLLOW- UP
A: THYROID ANTIBODY +							
16	M (c)	13	1.91	146.3	UNILAT	NONE	9 mths.
17	M (c)	13	4.70	250.0	UNILAT	NONE	3 mths.
18	M (c)	11	2.10	86.0	UNILAT	NONE	9 mths.
19	M (c)	15	3.80	109.0	BILAT	NONE	died
20	F (o)	14	4.20	205.0	BILAT	NONE	3 mths.
B: THYROID ANTIBODY ++							
21	F (o)	12	7.41	> 200	BILAT	PRESENT	24 mths.*
22	M (c)	15	1.40	86.0	BILAT	NONE	5 mths.
23	M (c)	16	1.06	65.1	UNILAT	PRESENT	9 mths.
24	F (o)	14	2.40	159.0	BILAT	PRESENT	19 mths.
25	F (o)	9	10.30	388.0	BILAT	PRESENT	7 mths.*
C: ANTINUCLEAR FACTOR ++							
26	F (o)	6	6.57	> 200	UNILAT	NONE	24 mths.
27	F (o)	7	2.00	121.0	UNILAT	PRESENT	-
28	M	17	2.20	206.0	UNILAT	PRESENT	10 mths.*
29	M (c)	11	2.20	70.0	UNILAT	NONE	3 mths.

* = Recurrent hyperthyroidism

while the T_3 was elevated in all but one cat. The T_4 was not precisely quantified in two cats with very high levels and was reported at >200 nmol/l. In the remainder, the T_4 ranged from 65.1 to 654.0 nmol/l (mean 197.9) and the T_3 ranged from 1.06 to 10.3 nmol/l (mean 3.87).

THYROID ANTIBODIES

Cat thyroid incubated with normal serum and serum positive for thyroid antibodies is shown in FIGURES 9.1 and 9.2 respectively. The ten thyroid antibody positive cats consisted of six castrated males and four ovariectomised females. The mean age was 13.3 years at diagnosis compared to 12.5 years for the other seventeen cats. The mean T_3 and T_4 in the thyroid antibody positive cats was 3.93 nmol/l and 166 nmol/l respectively compared to 3.84 nmol/l and 213 nmol/l in the thyroid antibody negative cats. None of these differences was statistically significant. Thyroid tissue from thyrotoxic cats could also be used to detect thyroid antibodies although the background staining was much higher making it harder to distinguish negative from weakly positive reactions.

Of the five cats scored ++ for thyroid antibodies, four had bilateral goitres compared to only eight of the other 24 cats and lymphocytic infiltrates were found in four of the five thyroids. Lymphocytes were seen in only five out of the remaining 22 thyroids examined histologically ($p < 0.05$) and two of these cats were positive for antinuclear factor.

FIGURE 9.1 CAT THYROID INCUBATED WITH NORMAL CAT SERUM

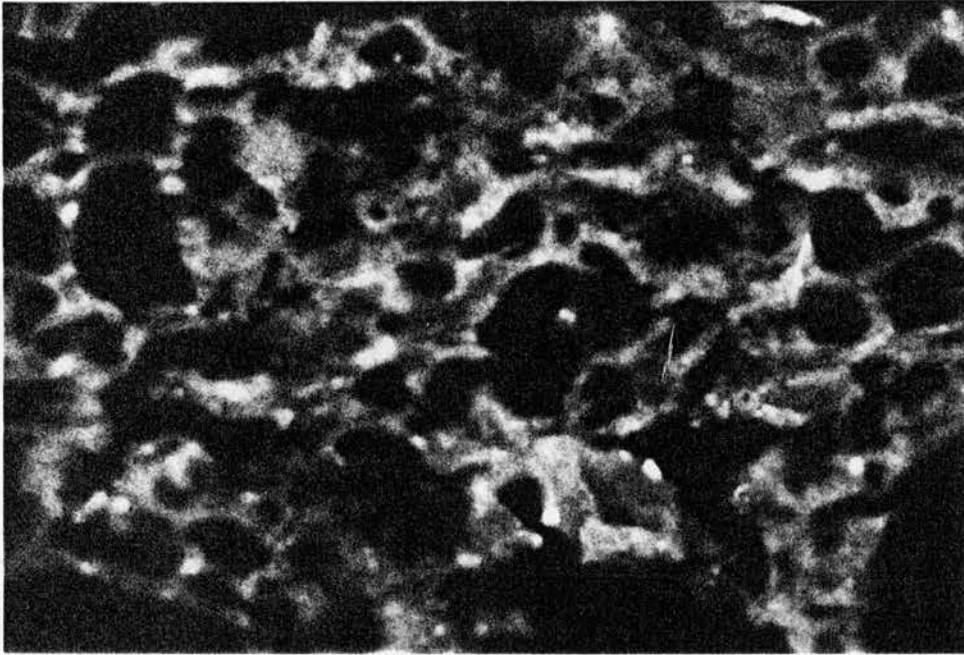
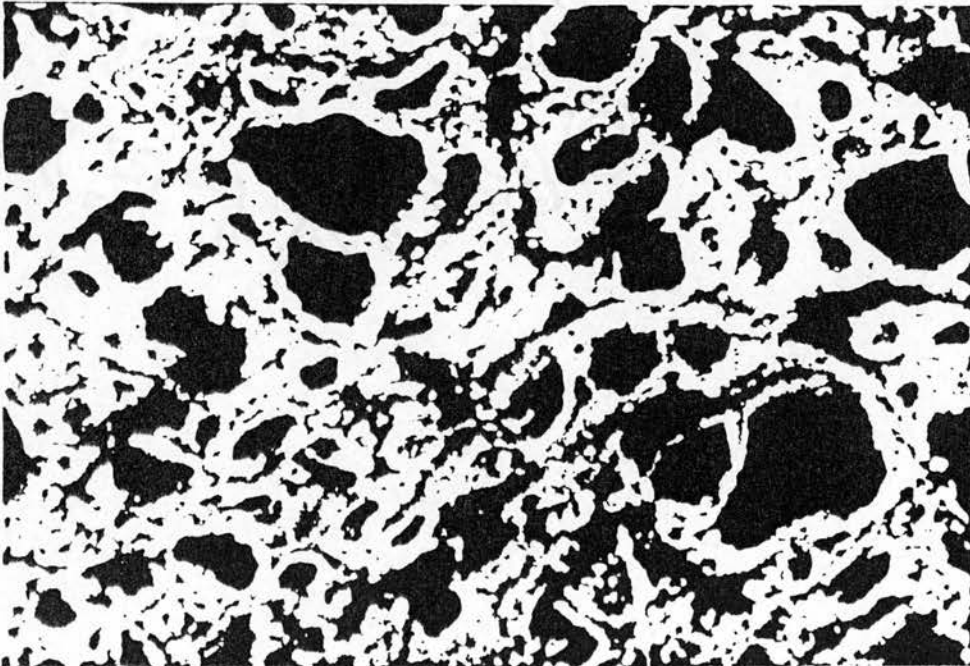


FIGURE 9.2 REACTION OBTAINED WITH THYROID ANTIBODY POSITIVE SERUM



TSH receptor antibodies were not detected in any of the twelve sera tested in the radioreceptor assay nor in any of the sera from twelve control cats.

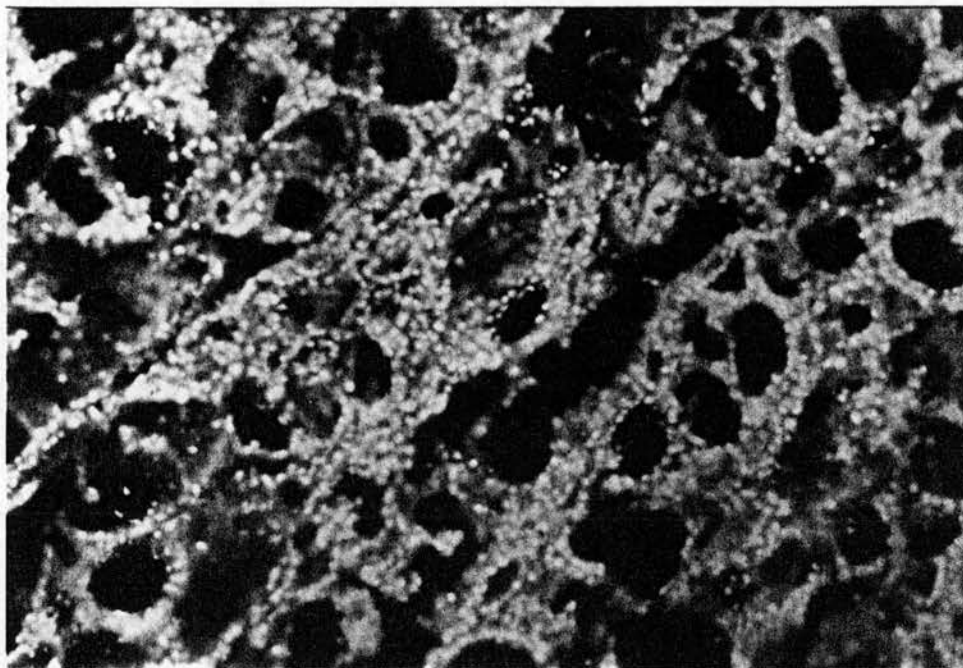
ANTINUCLEAR FACTOR

Four cats were positive for antinuclear factor, an example of which is shown in FIGURE 9.3. All of these cats had unilateral goitres and lymphocytic infiltrates were found in two of the thyroids. The two youngest cats in this series, aged six and seven and a half years, are in this group. The mean T_3 and T_4 in these four cats did not differ from that in the antinuclear factor negative cats. The non-organ specific nature of the antinuclear factor was demonstrated by using rat kidney as a tissue substrate.

FOLLOW UP

Twenty one cats were followed up for a mean period of 10.3 months. During this time, three cats developed recurrent hyperthyroidism which was confirmed biochemically in all cases. Two of these had bilateral goitre at initial presentation and the thyroid contained lymphocytic infiltrates. The serum of both cats was strongly positive for thyroid microsomal antibodies. The third recurrence was in a cat with unilateral goitre, with lymphocytic infiltration in the gland and the serum was positive for antinuclear factor. The recurrence involved the lobe which had already been operated on. Further resolution of thyrotoxicosis followed surgery in all cases.

FIGURE 9.3 REACTION OBTAINED WITH ANF POSITIVE SERUM



Figures 9.1, 9.2 and 9.3 are reproductions of photomicrographs obtained from the experiments described in the text. The final magnification was x400.

DISCUSSION

The 29 cats described in this study have clinical features comparable to other series reported in the literature. Using normal cat thyroid as a tissue substrate, autoantibodies have been detected in 14 (48%) of these cats. Ten showed thyroid antibodies with a pattern of staining similar to that of the microsomal antibody seen in human thyroid diseases and four had antinuclear antibodies. The non organ specific nature of the latter was demonstrated by incubating the positive sera with rat kidney.

The five sera with strong reactions for microsomal antibody included four cats with bilateral goitre and four of the five thyroidectomy specimens contained lymphocytic infiltrates which were found in only five of the other 22 thyroids examined (and two of these cats were positive for antinuclear factor). The correlation between lymphocytic infiltration of the gland and circulating antibodies to the thyroid is well recognised in human disease (Goudie et al, 1959; Schade et al, 1960) and the presence of both correlates with the development of post-operative hypothyroidism which presumably, therefore has an autoimmune basis (Whitesell & Black, 1949; Greene, 1950; Young et al, 1975). Despite adequate surgical therapy with removal of affected thyroid lobes, thyrotoxicosis recurred in three cases during a follow up period ranging from three to thirty months (mean 10.3 months). Two of the cases which recurred had bilateral goitres with lymphocytic infiltrates at the initial presentation and their sera were both strongly positive for thyroid microsomal antibodies. Autoantibodies may thus have prognostic significance as they do in human thyroid disease. The third cat with recurrence had a unilateral goitre but

there was lymphocytic infiltration and the recurrence involved the side which had been previously operated on (remnants of the thyroid gland may have been left with the parathyroid glands). The serum in this case was strongly positive for antinuclear factor.

Antinuclear factor is rare in normal cats but may occur in systemic lupus, feline leukaemia virus infections and liver disease. Two of the antinuclear factor positive cats in this series (aged six and seven and a half years) are amongst the youngest cases of feline hyperthyroidism reported. If thyroid autoantibody production is secondary to thyroid damage rather than part of a primary immunological disturbance, the formation of non-organ specific antibodies would perhaps not be surprising although ANF is also found in human autoimmune thyroid diseases.

Histologically, the changes in the thyroids of hyperthyroid cats are usually described as adenomas or adenomatous hyperplasia and the condition has been proposed as a potential model for the toxic nodular goitre of Man. Autoantibodies to the thyroid are found in a high proportion of cases of nodular goitre in Man, and although they may simply reflect thyroid damage or overactivity, a variety of other immunological disturbances has been described in the condition (see Chapter 1). An autoimmune aetiology is not necessarily excluded by the finding of a nodular goitre and one case of particular interest was reported by Benezu et al (1977) where radioactive iodine treatment of an adenoma was succeeded by thyrotoxicosis associated with a diffuse goitre. Tissue damage by the therapy may have stimulated autoantibody production including TSH receptor antibodies.

The equal sex incidence in feline hyperthyroidism, unlike the female predominance in Graves' disease, again does not preclude an autoimmune pathogenesis. Neither systemic lupus or primary hypothyroidism in the dog have a particular sex predisposition (Muller et al, 1983; Scott, 1981) while diabetes in the dog has only a slight female preponderance (Feldman, 1983). The fact that feline hyperthyroidism is a disease of older cats makes it more like toxic nodular goitre with respect to age but autoimmune diseases in general tend to become more common with advancing years. The absence of TSH receptor antibodies is not surprising since the assay used in this study has been developed for use with human serum and even so is not positive in all human patients with Graves'.

The observation that many cases of feline hyperthyroidism have autoantibodies may indicate the presence of an underlying immunological disturbance. Pathologically the condition is not similar to Graves' but the role of the immune system in its pathogenesis certainly merits further study.

Chapter Ten

DISCUSSION

A: IMMUNOLOGICAL CHANGES IN AUTOIMMUNE THYROTOXICOSIS

The work presented in this dissertation has confirmed the variety of immunological mechanisms which may operate in the pathogenesis of Graves' disease and these are summarised in FIGURE 10.1. Both humoral and cellular mechanisms are involved and interaction between the various components of the immune system may alter the presentation of the disease.

I) HUMORAL IMMUNE MECHANISMS

Hypergammaglobulinaemia has been demonstrated in the autoimmune diseases including Graves' disease (Briones-Urbina et al, 1982) and has been taken as evidence of altered humoral immunity. Indeed, changes in B lymphocyte function have been documented in both systemic lupus erythematosus and in rheumatoid arthritis (Budman et al, 1977; Kallenberg et al, 1983; Slaughter et al, 1978). However, when complement and immunoglobulin G, A and M levels were investigated by radial immunodiffusion in patients with Graves' or nodular goitres (data not presented), no difference was found from normal controls. Similarly B lymphocyte numbers are generally normal, and the major evidence of disturbed humoral immunity is the presence of circulating autoantibodies. The pathognomonic change in Graves' is the presence of antibodies to the TSH receptor. When different assays for these antibodies are combined, they can be detected in most patients with active Graves' disease (Marcocci et al, 1983).

FIGURE 10.1 IMMUNOLOGICAL MECHANISMS INVOLVED IN THE PATHOGENESIS OF GRAVES' DISEASE

Immunological Mechanisms in Graves' Disease

Genetic

DR3 Associated

Immunoregulatory

- Relative Deficiency of Suppressors
- Increased Nos. Helper/Inducer Cells
- Activated T Lymphocytes

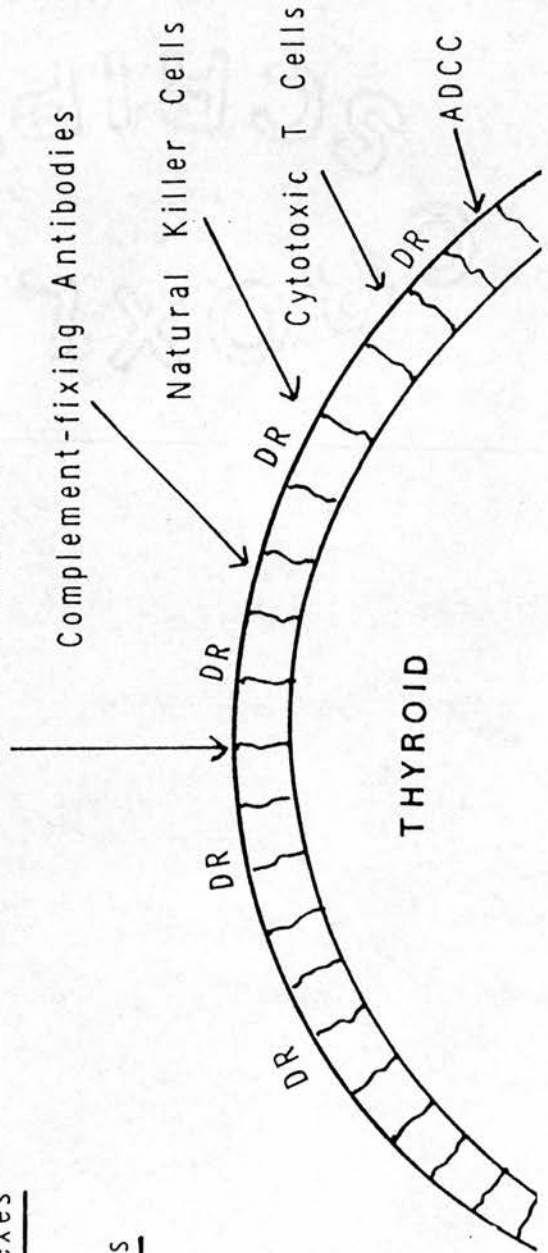
Immune Complexes

Anti-TSH Receptor Antibodies

Effector T Cells

Autoantibodies

DR Expression



Functional assays for TSH receptor antibodies are too demanding for routine clinical laboratories and the method which measures TSH binding inhibitory immunoglobulins (TBII) has found wide usage. TBII were found in 84 out of 138 (61%) patients when immunoglobulin concentrates were used for the assay and in 121 out of 150 (81%) patients when neat serum was used, in keeping with other large series (Schleusener et al, 1978; Teng et al, 1980; Bliddal et al, 1982; Biro, 1980). The greater sensitivity of the assay using neat serum was reported by Southgate and colleagues (1984). TBII were not found in other goitrous thyroid diseases except for a low incidence in multinodular goitre - in all, one out of 57 patients with euthyroid, and five out of 60 patients with toxic nodular goitre were mildly positive. It is not clear whether these are true Graves' patients with nodular goitres or whether there is, in fact, autoimmune thyroid stimulation in some cases of multinodular goitre as suggested by Brown et al (1978).

That the level of TBII does not correlate with thyroid hormone or radioactive iodine uptake values shows that other factors both in the thyroid and in the immune system (such as coincident thyroid damage from thyroiditis) affect the response of the gland to stimulatory immunoglobulins. In fact, not all antibodies detected by the TBII assay are stimulatory: For example, non-stimulatory antibodies develop after ^{131}I therapy. Indeed, TBII correlate poorly with the level of thyroid stimulators measured in vivo by the LATS assay (Clague et al 1976; Endo et al, 1978) and in vitro by cyclic AMP accumulation (Kuzuya et al, 1979; Sugeno et al, 1979; Macchia et al, 1981; Shishiba et al, 1982; Hardisty et al, 1983). TBII assay is not, therefore, a useful means of gauging the degree of thyroid

stimulation but is useful in confirming the diagnosis of Graves' although multiple determinations may be necessary (Hensen et al, 1984) and immune complexes may interfere with the expression of TBII.

Thyroid microsomal antibodies and anti-thyroglobulin antibodies are found in a proportion of Graves' patients (38% and 17% respectively in the series of 150 patients reported here) and, while immune reactions to these antigens may be important in initiating the disease and determining its prognosis, they are not specific markers for any aspect of Graves' disease. Their presence does serve to illustrate the polyclonal nature of the autoimmune response. That over 90% of patients with primary hypothyroidism had these antibodies testifies to their role in mediating thyroid destruction. A positive correlation ($p < 0.001$) was found between TBII level and microsomal antibody titre in untreated Graves'. This may either be an indicator of the degree of the immune response or because of a cross reaction between the two assays.

Immune complexes (ICs) were detected with the C1q solid phase assay in patients with autoimmune thyroid disease and Addison's disease and their relationship to circulating pathogenetic antibodies was explored. In untreated Graves' disease, ICs were found in 12 out of 55 (22%) and this low incidence is in keeping with the findings of Brohee et al (1979) and Endo et al (1983). A similar incidence was found when ICs were measured with the conglutinin binding assay (data not presented). There was a negative correlation between TBII and ICs - one out of twelve IC positive patients was also positive for TBII compared to 28 out of 42 (67%) IC negative patients ($p < 0.001$). Van der Heide et al (1980) reported a similar phenomenon with the C1q

fluid phase assay. ICs did not correlate with other autoantibodies. A negative correlation between ICs and microsomal antibodies was seen in euthyroid patients with Hashimoto's thyroiditis. In idiopathic Addison's disease, ICs were found in 41 out of 65 (63%) patients at diagnosis and a similar incidence was seen with the conglutinin assay (data not shown), the correlation between the two assays being good. There was no difference in ICs between adrenal antibody positive and negative cases. However, in the 31 antibody negative cases studied, only eight out of 18 (44%) without non-adrenal antibodies were positive compared to 11 out of 13 (85%, $p < 0.05$) patients with other antibodies or autoimmune diseases. It seems, therefore, that in Graves' disease, euthyroid Hashimoto's and Addison's with multiple autoimmune diseases that ICs may prevent circulating antibodies from being detected.

The situation is different in patients with primary atrophic hypothyroidism and in those hypothyroid with Hashimoto's thyroiditis where the level of ICs showed a positive correlation with microsomal antibody titre. Five out of 21 hypothyroid patients with Hashimoto's were positive for anti-thyroglobulin antibodies and all had ICs while, in euthyroid patients with Hashimoto's, only one of the five anti-thyroglobulin antibody positive patients also had circulating ICs. The mean C1q binding in the two groups of anti-thyroglobulin antibody positive patients was 7.7% and 5.4% respectively ($p < 0.05$). This is comparable to the relationship between anti-microsomal antibodies and ICs in Hashimoto's thyroiditis. In Addison's patients tested on follow up, ICs did not decline in parallel with adrenal antibodies with time from diagnosis but persistence of autoantibodies correlated with the presence of non-adrenal autoantibodies or

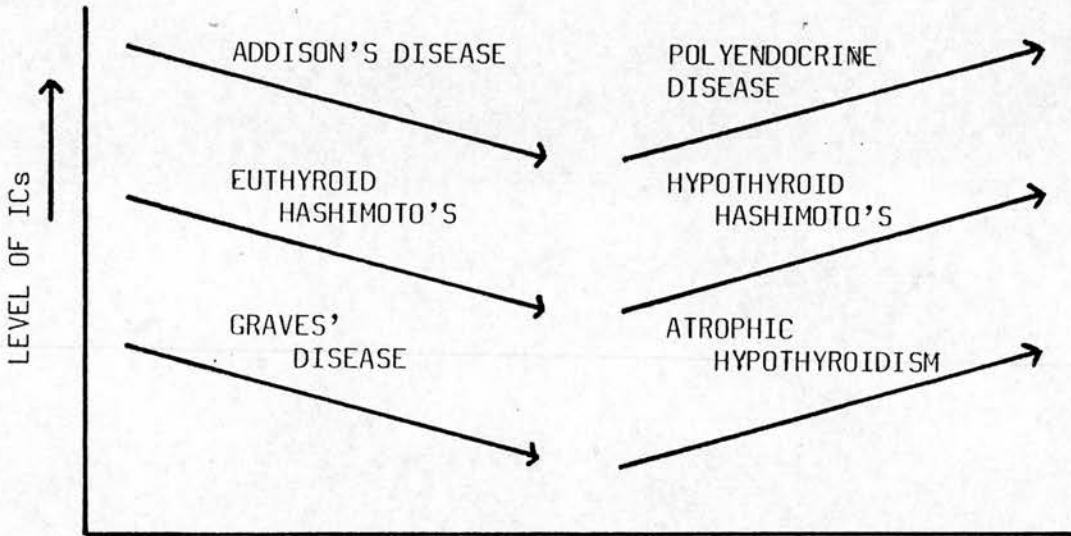
autoimmune diseases. The correlation between pathogenetic antibodies and ICs in autoimmune endocrine diseases is illustrated in FIGURE 10.2.

In the early stages of the diseases, a relative excess of antigen may lead to the formation of complexes with antigen excess which the C1q solid phase assay is particularly useful for detecting. The relative lack of free antibody in this situation may prevent its detection by insensitive methods such as immunofluorescence. Thus, in Graves' disease and the early phase of Hashimoto's or polyendocrine disease associated with Addison's, a negative correlation exists between the antibody marker and ICs detected by the C1q solid phase assay. With prolonged autoimmune attack on the thyroid and other endocrine glands, a predominance of antibodies may exist, both in the circulation and in ICs. A positive correlation was found between ICs and antibodies in these circumstances. A study of circulating immune complexes using different methods to take account of the relative antigen and antibody composition and to relate this to the natural history of the diseases would be of relevance. ICs are not therefore, simple antigen-antibody combinations but are dynamic structures, the changing nature of which may underlie the presentation of an autoimmune disease and its relationship with other diseases.

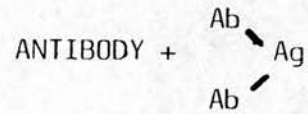
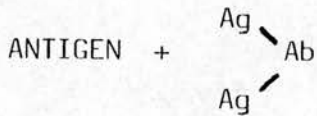
II) CELLULAR IMMUNE MECHANISMS

The major evidence for disordered immunoregulation has come from studies on the helper (T_h) and suppressor (T_s) subsets of T lymphocytes. There is good evidence from animal studies and from

FIGURE 10.2 THE RELATIONSHIP BETWEEN IMMUNE COMPLEXES AND PATHOGENETIC AUTOANTIBODIES IN ENDOCRINE DISEASES



CORRELATION WITH AUTOANTIBODIES



CHANGE IN COMPOSITION OF IMMUNE COMPLEXES

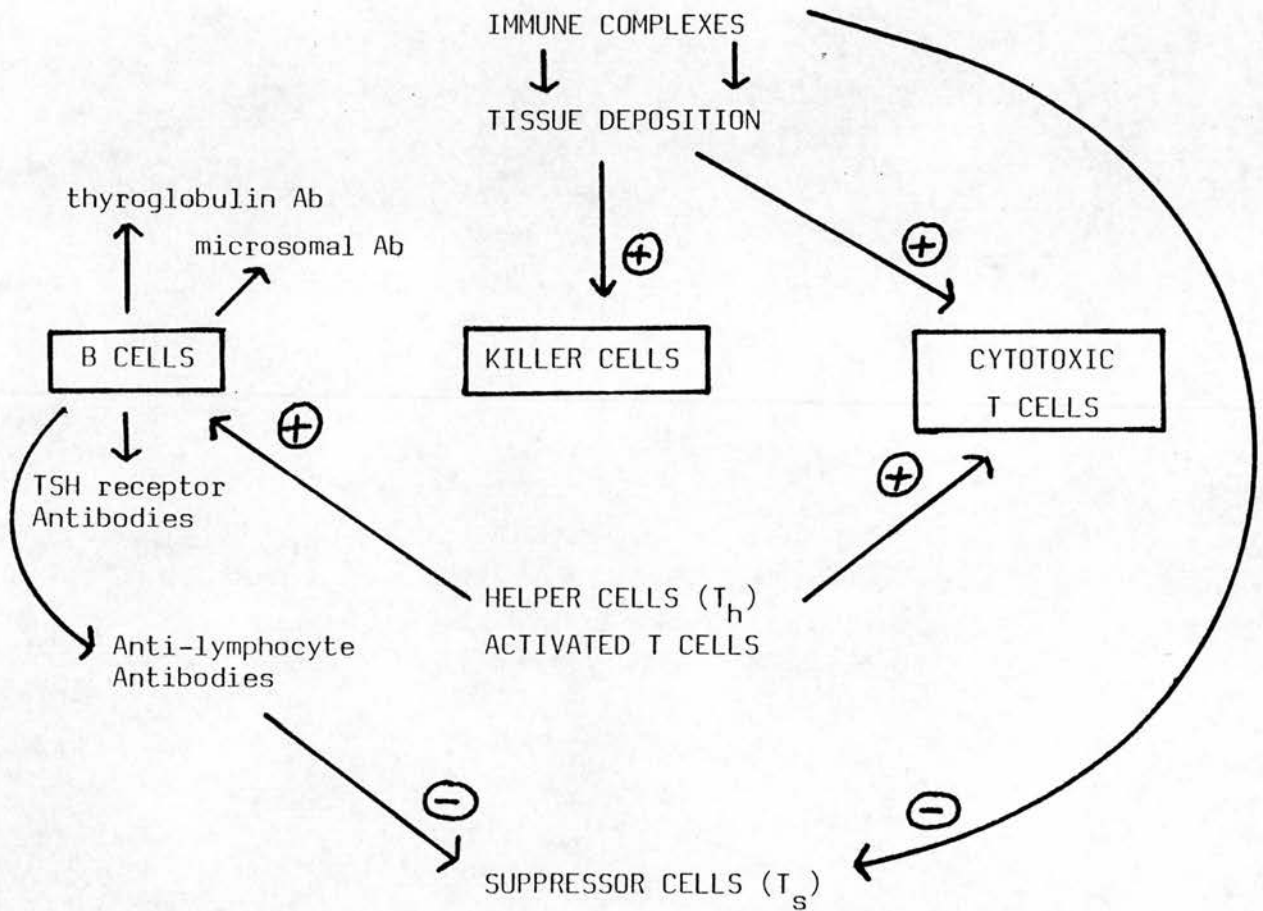
limited work on human patients that the function of T_S is defective in autoimmune thyroid disease (Rose et al, 1981; Balazs et al, 1984). In recent years, it has become possible to quantify these cells by using monoclonal antibodies. However, there are two notes of caution in interpreting these studies. Firstly, the reduction in T_S is in the proportion of cells (Thielemans et al, 1981; Sridama et al, 1982; Ludgate et al, 1984) rather than in their absolute numbers. This may be due to changes in the numbers of other subsets of peripheral blood mononuclear cells. For example, a mild increase in OKT4 (helper/inducer) cells was found ($9.28 \times 10^8/l$ in Graves' patients compared to $7.62 \times 10^8/l$ in controls, $p = 0.05$). It may be that the relative deficiency of T_S is sufficient to allow effector mechanisms to become activated, or the defect may be qualitative rather than quantitative (Iwatani et al, 1983). Alternatively, the defect may be specific for a particular antigen or organ and might have little effect on the overall cell numbers in this subset. The second caution relates to comparing work with different monoclonal antibodies. The antibodies OKT8 and UCHT4 gave a good correlation in cell numbers measured in normal individuals, but the correlation was poor in Graves' patients although both antibodies showed reduced proportions of T_S .

Other potentially important immunoregulatory mechanisms include the network of anti-idiotypic antibodies and the regulation of lymphocyte function by antibodies against these cells. The capacity of the former to regulate the expression and function of other components of the immune system, both humoral and cellular, is only just becoming appreciated. Anti-lymphocytic antibodies are recognised to occur in systemic lupus erythematosus (Morimoto et al, 1980), myasthenia

gravis (Mishak & Dau, 1981), juvenile rheumatoid arthritis (Morimoto et al, 1981) and in Graves' disease (Pacini et al, 1983). They have mainly been found to be directed at the suppressor/cytotoxic subset. It is not clear whether this phenomenon is part of the normal immunoregulatory mechanism or whether it represents part of a polyclonal response in individuals susceptible to autoimmune disease. Immune complexes may also have an immunoregulatory role. They may bind via Fc receptors to T_S , K cells and activated T lymphocytes. Complexes with an antibody excess can arm K cells thus initiating antibody dependent cellular cytotoxicity (ADCC), while other complexes might be responsible for the reduced numbers of K cells detected in some cases of systemic lupus (Schneider et al, 1975), diabetes mellitus (Pozzilli et al, 1981) and Graves' disease (Endo et al, 1983). ICs may reduce T_S although such a correlation was not seen here using the C1q solid phase assay (data not presented) - possibly because complexes with a relative antibody excess would be much more likely to bind to cells expressing Fc receptors. ICs may also have a part to play in the initiation of the disease since their deposition is known to precede cellular infiltration (Kofler et al, 1983). The potential interactions between the humoral and cellular immune systems are summarised in FIGURE 10.3.

Evidence for increased activation of the cellular immune system comes from the presence of raised numbers of self-reactive, antigen-specific cells found in SLE and thyroid disease (Bankhurst & Williams, 1973; Bankhurst et al, 1975). Using monoclonal antibodies against early activation (4F2) and late activation (class II) antigens, increased numbers of activated lymphocytes have been demonstrated in over 70% of new Graves' patients. All patients studied had either increased activated T lymphocytes or TSH receptor

FIGURE 10.3 INTERACTIONS BETWEEN HUMORAL AND CELLULAR IMMUNITY IN THE PATHOGENESIS OF AUTOIMMUNE ENDOCRINE DISEASE



antibodies (TBII). There was a negative correlation between TBII and 4F2 positive cells which was not due to TBII masking the expression of the 4F2 antigen. It was concluded that the two abnormalities might represent different stages in the immunopathogenesis of Graves' disease. Enhancement of cellular effector mechanisms was shown using an antibody directed at a determinant on the surface of K/NK cells. There was no relationship between the numbers of these cells and circulating autoantibodies or immune complexes.

Both humoral and cellular immune abnormalities exist in Graves' disease. Their interactions may promote or limit the autoimmune reaction. The system is subject to a dynamic regulatory process and not all abnormalities need be present simultaneously.

B: THE NATURAL HISTORY OF GRAVES' DISEASE

Immunological changes in the circulation are useful in diagnosis and in monitoring the progress of an autoimmune disease but they do not necessarily tell us much about the lesion which initiates the disease. In type I diabetes, a pathological heterogeneity exists with some cases being induced by viral infection and others being purely of autoimmune origin. The relative contribution of the immune system in the pathogenesis of the condition may be genetically determined (Cahill & McDevitt, 1981). The evidence for virus infection as a triggering factor is good, both from pathological studies of individual patients who have died soon after diagnosis was made and from epidemiological studies. There is only very limited epidemiological evidence to suggest a viral trigger for Graves'

disease (Joassoo et al, 1975) although, by causing local increases in the concentration of interferon, viral infection may contribute to the increased expression of class II antigens on the surface of thyrocytes seen in the disease. Class II antigen expression on thyrocytes was confirmed here by immunofluorescent studies on three Graves' thyroids. Other variable features in the pathogenesis include iodine status and the relative contributions of autoimmune thyroid stimulation and destruction.

Both thyroid autoantibodies and thyrotoxicosis may develop after iodine refeeding in an iodine deficiency region (Boukis et al, 1983) although it is not at all clear whether the pathological entity which arises is true Graves' disease. Indeed, the recent study by Phillips et al (1985) in the United Kingdom showed an inverse correlation between TSH receptor antibody positive thyrotoxicosis and previous endemic iodine deficiency. The balance between thyroid stimulation and destruction may be crucial in determining the presentation of the disease. The response of the gland to thyroid stimulators may be limited in patients with significant thyroiditis, while it is possible that hyperstimulation with Graves' immunoglobulins may increase microsomal antibody expression and thus ultimately increase the tendency for thyroiditis to develop - it is known, for example, that TSH stimulation increases the expression of microsomal antigen on the surface of rat FRTL5 thyroid cells (Chiovato et al, 1985). The expression of histocompatibility antigens on the thyrocyte in response to thyroid stimulators requires full study and may be an important determinant of the subsequent immunological reaction.

Graves' is a disease of relapses and remissions with approximately 10% of patients developing hypothyroidism spontaneously in the absence of destructive anti-thyroid therapy (Doniach, 1980). In an attempt to obtain some information on the natural history of the immune changes in Graves' patients with subclinical disease have been studied as have those in remission and those with recurrent Graves' thyrotoxicosis. The information obtained from the different patient groups is summarised in TABLE 10.1. In subclinical disease (flat TRH test, high normal levels of thyroid hormones and mild symptoms) - a group of patients difficult to define clinically or to study epidemiologically - TBII were not found in any of the twelve patients studied, confirming that this is a highly specific marker for the onset of true Graves' although a low level of thyroid stimulation must be present within the thyroid gland. Other immunological abnormalities were present with microsomal antibodies in four cases and immune complexes in four cases. Both exophthalmic patients and three of the four with microsomal antibody were ICs positive. This relationship between ICs and the presence of other autoantibodies and autoimmune diseases may explain the reduced expression of autoantibodies in IC positive patients as was found in newly diagnosed Addison's disease. T_h and T_s were only studied in a few cases (data not presented) and were normal. Other lymphocyte subsets were not studied. It is not known whether there is a large pool of patients with subclinical disease with only a small number, under the appropriate genetic or environmental influences, going on to develop the full blown syndrome. One group which is of interest is the first degree relatives of patients with thyroid disease. These individuals have an increased incidence of thyroid autoantibodies (Mather et al, 1980) and biochemical thyroid abnormalities (Tamai et

TABLE 10.1 IMMUNOLOGICAL ABNORMALITIES AT DIFFERENT STAGES IN THE
NATURAL HISTORY OF GRAVES' DISEASE

	SUBCLIN.	ACTIVE	REMISSION	RECURRENT
MICROSOMAL ANTIBODY	++	++	+	+++
TSH RECEPTOR Ab.	NEGATIVE	++	NEGATIVE	+
IMMUNE COMPLEXES	+	+	+	?
SUPPRESSOR CELLS	NORMAL	REDUCED	NORMAL	REDUCED
EARLY ACTIVATED T CELLS	?	RAISED	NORMAL	NORMAL
LATE ACTIVATED T CELLS	?	RAISED	NORMAL	RAISED
K/NK CELLS	?	RAISED	NORMAL	NORMAL
HLA MARKER	? B8	DR3 (65%)	-	DR3 (77%)

al, 1979). One study has found an increased incidence of HLA-B8 (and from this we may infer DR-3: Mather et al, 1980) in contrast to the work by Chopra et al (1979) where a normal HLA distribution was found.

TBII were not generally found in patients who were in remission after a course of drug therapy although persistent microsomal antibodies and immune complexes are frequent (data not presented). Lymphocyte subset abnormalities are also not present apart from a slight, but non significant, reduction in the proportion of T_S .

The immunological abnormalities in patients suffering a relapse of thyrotoxicosis were impressively different to those of new Graves' patients. TBII were found in 64% of 14 recurrent cases compared to 82% of age and sex matched new cases, the median TBII in the two groups being 16 and 28 respectively ($p < 0.02$). Strongly positive titres of microsomal antibody were found in 35% of new and 85% of recurrent cases ($p < 0.01$). The percentage reduction in OKT8 positive cells was comparable in the two groups but 4F2 (early activated) T cells were not increased in recurrent disease. There was, however, the expected increase in T cells bearing class II antigens. K cells were elevated in new but not in recurrent disease. The incidence of HLA-DR3 was only slightly higher (77%) in recurrent disease than in new cases (65%) confirming recent observations that the marker is of limited use in predicting recurrence (Schernthaner et al, 1980; Dahlberg et al, 1981; McKenna et al, 1982; Young et al, 1985). Thus, while an underlying immunoregulatory defect is still present in recurrent disease, the lower levels of circulating TBII and cytotoxic cells may indicate that the immunological reaction is becoming

confined to the thyroid. The higher levels of microsomal antibody may represent the greater tendency to develop thyroiditis and hypothyroidism in long standing disease.

Class II antigen expression, both on the thyrocyte and on activated T lymphocytes, may be important in the pathogenesis of Graves' disease. The immunological markers associated with the disease are largely a feature of the active thyrotoxic phase of its natural history. Recurrent disease shows marked immunological differences to newly diagnosed disease and these patients should be considered separately in studies on the immunopathogenesis of Graves' disease.

C: AGE-RELATED CHANGES IN IMMUNOLOGICAL PARAMETERS

The prevalence of many autoantibodies and thus of autoimmune diseases increases with age (Doniach & Bottazzo, 1983). Graves' is one exception where the disease most commonly affects young to middle aged women. The incidence of TBII in Graves' patients fell off with age when the assay was performed using IgG concentrates ($p < 0.001$). The negative correlation between ICs and TBII has already been alluded to. The mean age in IC positive Graves' patients was 55.5 years compared to 42.8 for the IC negative patients ($p < 0.01$). The increasing incidence of ICs with age in the normal population is well recognised (Di Mario et al, 1981) and could account for the decline in TBII positivity with age in Graves' patients. Age related changes in suppressor cell function are documented in animals (De Kruyff et al, 1980). While no correlation between age and OKT8+ cells was seen in Graves' patients, there was a negative correlation between age and

OKT8⁺ cells in normal female subjects ($r = 0.64$, $p < 0.001$). It has been suggested that the decrease in suppressor cells in autoimmune disease may relate to the presence of the DR-3 allele (Lawley et al, 1981) and one may therefore expect that DR-3 positive patients would develop Graves' at a younger age than those without this allele. The mean age of onset in seven DR-3 homozygotes was 35.2 years compared to 37.6 in 46 DR-3 heterozygotes and 40.3 years in 29 DR-3 negative patients - a trend in the expected direction although it did not reach statistical significance.

There are changes in immune parameters with age (TSH receptor antibodies, immune complex formation and the proportion of suppressor cells) which may affect the onset of Graves' disease. The age of onset is not clearly related to HLA status.

D: HETEROGENEITY WITHIN GRAVES' DISEASE

It has been suggested in two previous published studies (Schleusener et al, 1983; Stenszky et al, 1983) that different forms of Graves' disease may exist. The former study confirmed the expected high incidence of HLA-DR3 in patients with either ophthalmopathy or TSH receptor antibodies but also identified another group of patients with goitrous thyrotoxicosis who had neither eye disease or receptor antibodies and had a high incidence of HLA-DR5. Stenszky's study used a complex mathematical technique to identify three possible subgroups. The first of these had low indices of autoimmunity, a tendency to remit with medical therapy and a low incidence of HLA-B8 (9%). The second group had high antibodies and marked lymphocytic

infiltration of the gland but again a relatively low incidence of HLA-B8 (21%). They proposed that this group had a combination of Hashimoto's thyroiditis and Graves' disease. The third group had high titres of antibodies, a high incidence of ophthalmopathy and relapse after drug treatment, a strong family history and a high incidence of HLA-B8 (87%).

In this dissertation, three putative subgroups have been studied. Their clinical and immunological data is shown in TABLE 10.2. It was hypothesised that patients with a low four hour uptake of radioactive iodine might have limited thyrotoxicosis because of the presence of thyroiditis and may therefore be similar to Stenszky's second group. Patients with no goitre have recieved some attention in recent literature and they were also considered as a possible immunologically distinct subgroup.

It is well known that Graves' and Hashimoto's may coexist in the same gland (Eason, 1928) and it has been suggested that patients with this combination have a strong personal or family history of other autoimmune disease (Fatourechí et al, 1971). Patients studied here with a low ^{131}I uptake and goitre had less severe thyrotoxicosis than the majority of patients with classic Graves' disease and they also had a later age of onset. These patients showed a decreased incidence of TBII but had a slight increase in the level of microsomal antibody and a marked increase in the incidence of non-thyroidal autoantibodies (particularly parietal cell antibodies). The prevalence of immune complexes was also slightly increased. Because of the potential overlap with Hashimoto's thyroiditis and the possible risk of pernicious aneimia in this group of patients, an

TABLE 10.2 CLINICAL AND IMMUNOLOGICAL DATA FROM THREE GROUPS OF

PATIENTS WITH GRAVES' DISEASE

	A	B	C
	GOITRE +	GOITRE +	NO GOITRE
	UPTAKE > 40%	UPTAKE < 40%	
	(N = 89)	(N = 21)	(N = 29)
AGE (years)	41.0	51.6	52.6
T ₃ (nmol/l)	6.6	4.2	5.4
¹³¹ I UPTAKE	62%	32%	46%
TBII (No. positive)	72%	43%	31%
TBII (median)	24.8	12.0	8.4
MICROSOMAL > 20 ²	34%	48%	28%
THYROGLOB. > 320	10%	14%	3%
PARIETAL CELL Ab.	10%	43%	7%
NON-THYROID Abs.	16%	57%	10%
IMMUNE COMPLEXES	12%	43%	36%
HLA-DR2	48%	25%	33%
HLA-DR3	62%	75%	57%
HLA-DR5	17%	17%	29%
HLA-DR3/3+4	21%	33%	19%
HYPOTHYROIDISM IN			
RESPONDERS TO ¹³¹ I	46%	88%	60%
(No.)	(28)	(8)	(15)

association with HLA-DR5 was proposed but was not, in fact, confirmed. The prognosis of this group following ^{131}I therapy differed from other Graves' patients with a high incidence of hypothyroidism presumably reflecting either pre-existing thyroid damage or a genetic predisposition to form destructive antibodies after treatment.

Thyrotoxicosis without goitre is a common and important clinical entity which has been neglected in immunological studies. Hegedus et al (1983b) confirmed normal thyroid volume by ultrasound in 23% of 96 consecutive thyrotoxic patients, and a similar incidence was reported in a large retrospective study by Greenwood et al (1985). The latter study showed that this group had an incidence of thyroid microsomal antibodies and ophthalmopathy comparable to that of classic Graves' disease. Once again the patients in this group who were described in Chapter 4 were older and had less severe thyrotoxicosis than Graves' patients with goitre and high ^{131}I uptake. The incidence of TBII in this group suggests that some, at least, of these patients are suffering from true Graves' disease. Growth promoting antibodies can now be readily measured (Drexhage et al, 1980; Valente et al, 1983) but their relationship to TSH binding inhibitory antibodies remains to be clarified. It may well be that, like other autoantibodies, their expression is age-related.

Agoitrous Graves' disease may thus be a form of autoimmune thyrotoxicosis where the immunological stimulus to thyroid growth is lacking. Studer et al (1978) have, however, shown that thyroid autonomy can be confined to individual follicles or groups of follicles in the early stages. Nodules may not, therefore be

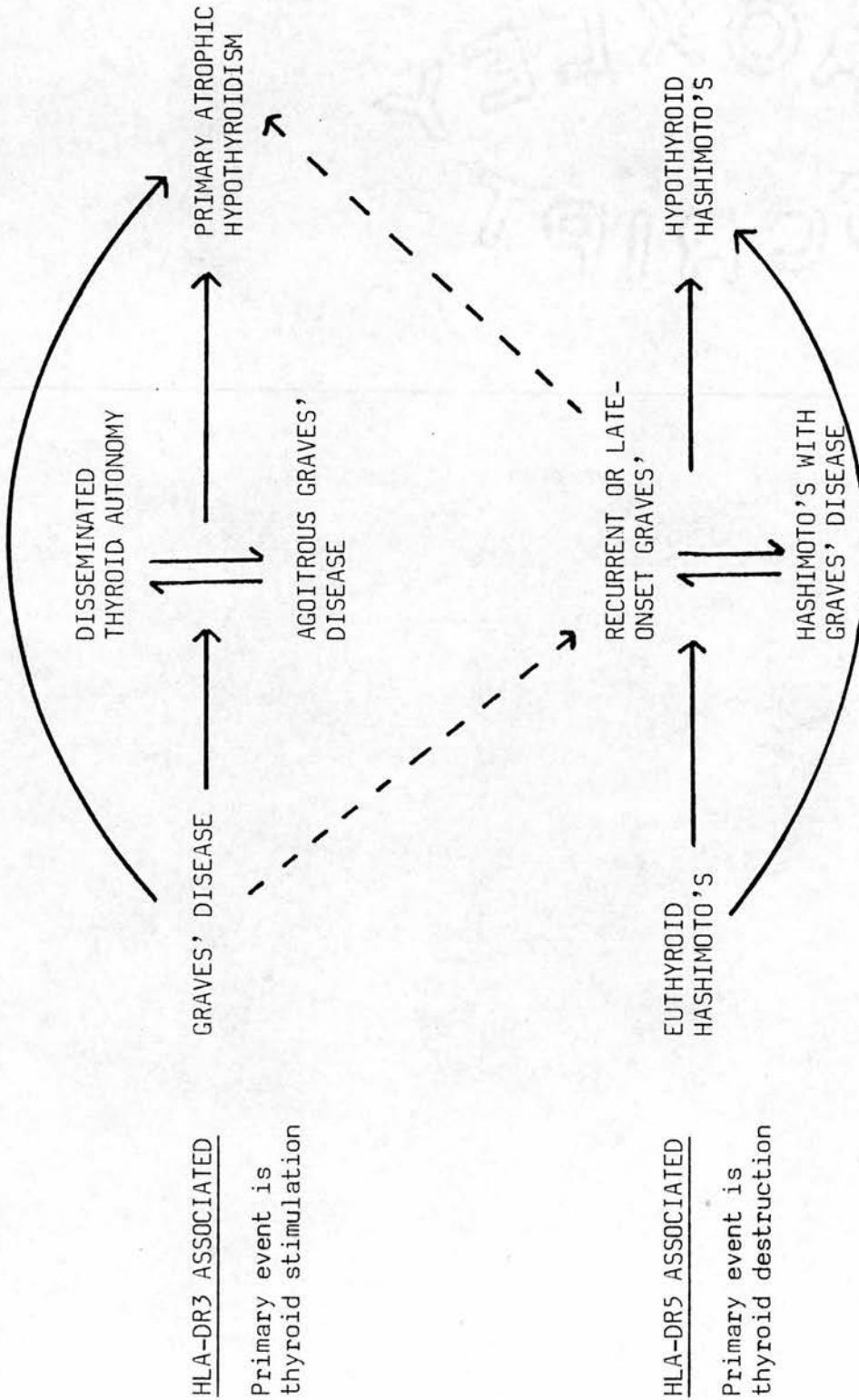
identified either clinically or scintigraphically and an autonomous thyroid condition could be mistaken for Graves' disease. Hyperfunction of parts of the gland may increase antigen expression or release and could account for the appreciable incidence of thyroid abnormalities including microsomal antibodies in such patients (Mota et al, 1980; Kuy et al, 1981). TSH receptor antibodies may be part of a polyclonal autoimmune response in some patients although they are not usually detected in the serum of patients with nodular goitres using the TBII assay (Bolk et al, 1972; Mukhtar et al, 1975; Strakosh et al, 1978). Changes in iodine status may initiate antibody formation in patients with thyroid autonomy (Boukis et al, 1983).

It has been proposed, on the basis of HLA-DR typing studies reported in Chapter 6, that thyroid stimulation by TSH receptor antibodies and thyroid destruction by cytotoxic antibodies might be under separate genetic control. Thyroid stimulation may relate to the DR-2 locus. The median TBII in DR-2 positive patients studied was 25.2 compared to 17.5 in other patients ($p < 0.05$). In DR-2 homozygotes, the median TBII was 36.2 compared to 19.9 in DR-2 heterozygotes ($p < 0.01$). Several DR-2 positive patients were encountered with unusual resistance to therapy and TBII took significantly longer in DR-2 positive patients to decline to normal values on initiating antithyroid drug therapy. Thyroid autoaggression, on the other hand, may be more related to the HLA-DR3 locus. The persistence of TBII after ^{131}I therapy was commoner in DR-3 positive patients. Patients who were DR-3 homozygotes or who possessed the DR-3+4 phenotype

tended to have high titres of thyroid microsomal antibodies. The possession of DR-3+4 is associated with a high risk of type I diabetes (Thomsen et al, 1979).

An interaction between Graves' and related thyroid disorders is proposed in FIGURE 10.4. The overlap between the various disease states was suggested in a pathological study by Levitt (1950) on a large number of thyroidectomy specimens and aspects of this interaction have been confirmed by many subsequent clinical and immunological investigations. It is hypothesised that the diseases can broadly be divided into two groups. The first includes classic Graves' disease with goitre, high levels of TSH receptor antibodies and an early age of onset. This group has a high incidence of HLA-DR3 and includes patients with primary atrophic hypothyroidism and agoitrous Graves' disease. Patients with disseminated thyroid autonomy overlap clinically and some immune changes may be present but the condition probably does not have an autoimmune aetiology. The second group of conditions shows an association with HLA-DR5 although this is probably not as strong a marker as DR-3 is for the first group. Recurrent Graves' disease is presented on the basis of data which has already been discussed and the observation by Schleusener et al (1983) that some cases of Graves' disease may be associated with this allele. Genetic factors may determine whether the primary event in autoimmune thyroid disease is thyroid stimulation or destruction while environmental factors such as viral infection or changes in iodine intake may relate to the timing of onset. Age related changes in immune function could then dictate the nature and severity of the presentation.

FIGURE 10.4 RELATIONSHIP BETWEEN THE VARIOUS THYROID AUTOIMMUNE DISEASES RELATED TO GRAVES'



The primary event in the DR-3 associated group may be thyroid stimulation by TSH receptor antibodies. It is well known that hypothyroidism develops spontaneously in Graves' disease (Doniach, 1981) although the natural history is frequently changed by destructive antithyroid therapy. In the obese strain (OS) chicken, a phase of subclinical thyrotoxicosis may well precede the development of thyroiditis (see Chapter 9). The expression of thyroid microsomal antigen in rat FRTL5 cells is known to be TSH dependant (Chiovato et al, 1985) and it will be of interest to see whether the antigen expression is increased during the course of autoimmune thyroid stimulation. The primary event in the second, DR-5 associated, group is the development of cytotoxic antibodies. Thyroid stimulation may develop as part of the polyclonal immune response to thyroid damage. The existence of cases of thyrotoxicosis succeeding hypothyroidism was discussed in Chapter 4. The relationship between immune complexes and pathogenetic antibodies has already been discussed. In the early phases of autoimmune diseases, ICs may decrease the expression of antibodies and, because of the relative antigen excess in complexes, they are unable to arm K cells and their interaction with other Fc bearing cell subsets may be similarly limited. Furthermore, the clearance of immune complexes may be more effective in young patients thus limiting the extent of tissue damage. As the immune response develops with advancing years, complexes of antibody excess may form and be associated with progressive tissue damage.

Heterogeneity may exist in Graves' disease. It may partly depend upon age-related changes in the immune system and thyroid function. The genetic basis is not clear although separate genetic control of thyroid stimulation and destruction may exist. The recognised thyroid

autoimmune disease states are part of a complex network of interacting pathologies and the presentation of individual cases may vary with time and relate to environmental trigger factors.

E: IMPLICATIONS FOR DIAGNOSIS

Whatever other immunological abnormalities play a part in the pathogenesis of Graves' disease, the final common pathway is thyroid stimulation by immunoglobulin molecules binding to the TSH receptor. However, using standard assay methods, these antibodies are not always detected in the circulation. The assay in widest use is the radioreceptor assay. Even with the modified assay proposed by Southgate et al (1984), only 121 out of 150 unselected Graves' patients were positive. Although impractical in routine clinical laboratories, the use of multiple methods to detect TSH receptor antibodies greatly increases the yield of positive results (Biro, 1982; Marcocci et al, 1983; Hardisty et al, 1983). The development of reproducible methods for directly measuring thyroid stimulation in vitro has made an impact already on clinical studies of the disease. The measurement of iodide uptake by rat FRTL5 cells, for example, is a convenient assay which has found wide application in the past two or three years.

The yield of positive tests in assays for TSH receptor antibodies may also be decreased by including subgroups of patients which have a low incidence of antibodies. The possibility that the immune reaction becomes confined to the thyroid gland in cases of Graves' disease of long standing has already been postulated to account for the lower

levels of TBII found in the circulation of such patients. In the series of patients presented in Chapter 4, only 43% of patients with low uptake of ^{131}I and goitre were TBII positive and only 31% of patients with no goitre had antibodies compared to 72% of patients with classic Graves' disease with high ^{131}I uptake and goitre. In scientific studies on the immunopathogenesis of Graves' disease, the diagnostic criteria used must be clearly stated and abnormalities found must be related only to this defined population of patients.

HLA-DR3 was found in 53 out of 82 patients with thyrotoxic Graves' disease and this is in keeping with other published studies (Bech et al, 1977; Farid et al, 1979; Allanic et al, 1980; Dahlberg et al, 1981; McKenna et al, 1982). It is not a marker for any particular aspect of the disease and its role in predicting the response to antithyroid drug therapy has now been disputed. Graves' patients are commonly found who are negative for both TBII and DR-3, and, in view of the demanding nature of the latter test, it cannot be considered to have a useful part to play in the routine diagnosis and management of autoimmune thyroid disorders. It remains to be seen whether more newly characterised HLA phenotypes will prove to be better markers - for example, DC-3 has recently been shown to be present in a high proportion of patients with coeliac disease.

The importance of T cell abnormalities in the pathogenesis of autoimmune thyroid disease has been amply documented from animal studies. For example, Vladutiu and Rose (1975) have shown that susceptibility to murine thyroiditis following thyroglobulin immunization is transferred with T rather than B lymphocytes. The changes in T lymphocyte subsets discussed in this dissertation are

not specific for Graves' disease and are not, therefore, helpful in diagnosis. The inverse correlation found between 4F2 positive cells and TBII demonstrates that studying patients in different stages of the disease may reveal different immunological abnormalities.

The measurement of TSH receptor antibodies is the only true immunological test for Graves' disease. Assays of functional activity of these antibodies are now becoming widely available and the use of multiple tests improves diagnostic yield.

F: IMPLICATIONS FOR THERAPY

The possibility that antithyroid drugs exert an immunosuppressive action which is specific to the thyroid was discussed in Chapter 3. The presence, or otherwise, of this action has important implications for the way drug treatment is monitored. By studying 24 patients after carbimazole therapy was started, it was clear that different time courses existed for the reduction in thyroid hormones and for the decline in thyroid antibodies including TBII. The latter took much longer to become normal than the former. One conclusion is that the primary action of these drugs is antithyroid and the secondary reduction in autoantibody synthesis is brought about by reduced autoantigen presentation or expression when the thyrocyte is no longer hyperstimulated.

HLA-DR3 is not particularly helpful in predicting the response to drug therapy (Schernthaner et al, 1980; Dahlberg et al, 1981; McKenna et al, 1982; Young et al, 1985). Studies reported in Chapter 6

suggested that DR typing may help to identify those cases resistant to therapy. DR-2 positive patients took longer for their TBII to normalize with carbimazole when compared to other patients, an observation which needs to be tested in a larger series of patients.

The relationship of immunological changes to the outcome of radioactive iodine therapy was extensively studied. 18 patients tested sequentially after treatment showed a rise in the median TBII from 35 at diagnosis to 55 at four months after ^{131}I . The levels of antibody steadily declined thereafter to reach normal levels by two to three years. Persistent TBII beyond this correlated strongly ($p < 0.01$) with the presence of exophthalmos. Patients with a genetic predisposition to multiple autoimmune diseases may have a greater capacity to form autoantibodies after an immune challenge such as that provided by ^{131}I . Early changes in TBII following treatment related to the ultimate outcome. Nine patients becoming hypothyroid showed an increase in TBII from 26.7 (median) at diagnosis to 33.1 at two to four months ($p < 0.05$). In eight euthyroid patients, TBII were not only lower at diagnosis but showed a tendency to decline following treatment.

Of 52 patients followed up after ^{131}I therapy, six required further treatment 19 were euthyroid at six months and 27 were hypothyroid. There was a trend towards increasing TBII with increasing response to ^{131}I with median values for TBII in the three groups of 6.5, 10 and 18 respectively ($p < 0.01$). The cytotoxic or TSH receptor blocking properties of antibodies might be of importance. The sequestration of

such antibodies in immune complexes could account for the observation that seven out of 17 IC negative cases became hypothyroid while none of seven IC positive cases did so.

Feline hyperthyroidism is a recently recognised condition which may provide a model for some aspects of thyroid disease in Man. Autoantibodies to the thyroid (microsomal and antinuclear factor) were found in 14 out of 29 cases. The five cases with high levels of thyroid microsomal antibody included particularly cats with bilateral goitres and lymphocytic infiltration of the gland. These cases in particular may have an underlying immune disturbance. On follow up, three cats developed recurrent thyrotoxicosis and all three had circulating autoantibodies and lymphocytic infiltration of the gland. Immunological changes in feline hyperthyroidism may thus influence the presentation of the disease and its prognosis as they do in human thyroid disease.

The time courses for thyroid hormone and TSH receptor antibody reduction during the early months of carbimazole treatment of Graves' thyrotoxicosis do not support the hypothesis that the drug has a direct immunosuppressive action. TSH receptor antibodies may help the prediction of outcome of radioactive iodine therapy. Feline thyrotoxicosis is associated with a high incidence of anti-thyroid antibodies and these may be of prognostic importance in the condition.

G: PERSPECTIVES FOR FUTURE RESEARCH IN GRAVES' DISEASE

The defects in humoral immunity, cell-mediated immunity and immunoregulation which occur in Graves' and related diseases are far from completely understood. The antibody activities which stimulate the thyroid are being characterised by in vitro assays using cultured thyroid cells and it is likely that differences in the actions of different populations of antibodies will account for the varying presentations of the disease. The genetic basis for the production of these antibodies will become clearer and this along with the recognition of further HLA specificities may increase the value of tissue typing. The cell mediated immune system will be studied further with monoclonal antibodies and the relationship of cell surface markers to function of the cells in vivo will undoubtedly become clearer.

Monoclonal antibodies have not, thus far, elucidated the role of suppressor cells in the initiation of autoimmune diseases and a return to assays of functional activity is required. Other developments in our understanding of immunoregulation may come from the study of anti-idiotypic antibodies. Their role in animal models for myasthenia gravis and insulin resistant diabetes mellitus has been examined (Wasserman et al, 1982; Shechter et al, 1982) and such antibodies may occur in a high proportion of patients with myasthenia gravis (Dwyer et al, 1983). Naturally occurring antibodies binding TSH have been recognised (Kajita et al, 1983; Akamizu et al, 1984; Copping et al, 1985) and may be of relevance to anti-TSH receptor autoimmunity.

While changes in circulating immune parameters are of clinical importance, the thyroid gland is ideal for the study of a target organ in autoimmune disease. The discovery of changes in the expression of histocompatibility antigens on the thyrocyte and the characterisation of factors controlling this phenomenon is one of the most fundamental areas of research at present (Hanafusa et al, 1983; Pujol-Borrell et al, 1983; Weetman et al, 1985). No doubt further work will reveal much about the interaction of changes in target cell antigen expression with other components of the immune system.

Clinical and epidemiological studies will help us to define the condition, its variants and its overlap with other autoimmune diseases. The contribution from research on other receptor antibody diseases will increase and, new diseases of this group may be recognised. For example, a role for receptor antibodies in some cases of SLE has recently been proposed (Bennett et al, 1986) and receptor antibodies have been suggested to account for familial cases of Cushing's syndrome (van Berkhout et al, 1986).

It is now thirty years since Adams and Purves described the activity of the Long-Acting Thyroid Stimulator. Nobody could have predicted the advances which have taken place in our understanding of Graves' disease during this time. It will be fascinating to see what develops in the next thirty years of research.

SCRIPT

APPENDICES

CROXLEY

SCRIPT

APPENDIX 1: THE MEASUREMENT OF TSH RECEPTOR ANTIBODIES USING THE TSH BINDING INHIBITORY ASSAY

The assay was performed using either neat serum or immunoglobulin concentrates prepared from serum by precipitation with polyethylene glycol. All haemolysed or grossly lipaemic samples were excluded. Serum was separated and stored at -20°C prior to use. Reagents (including the TSH receptor preparation) were obtained in kit form from R.S.R. Ltd., Cardiff.

A) ASSAY BASED ON IMMUNOGLOBULIN CONCENTRATES

This assay was performed broadly as described in Shewring and Rees Smith (1982): immunoglobulins were precipitated from serum using a solution of 15% polyethylene glycol (PEG) in water. The precipitate was harvested by centrifugation at 2000g for 45 minutes after which 0.5 mls. of pre-warmed assay buffer (Tris/sodium chloride containing 1mg/ml bovine serum albumin) was added. The pellet was resuspended using a vortex mixer and allowed to stand for 15 minutes at room temperature. Undissolved material was removed by centrifugation at 2000g for 10 minutes.

Duplicate 100 microlitre aliquots of IgG preparation were added to 100 microlitres of a solution containing Lubrol-solubilized TSH receptors (porcine) and allowed to stand for 15 minutes at room temperature. 100 microlitres of a solution containing ^{125}I -labelled TSH was added and the resulting solution was incubated at 37°C for one hour, following which 0.7 mls of ice cold assay buffer and 1 ml of ice cold 30% PEG in 1M NaCl were added. After mixing, the tubes were centrifuged for one hour at 2000g, 4°C . The supernatant was

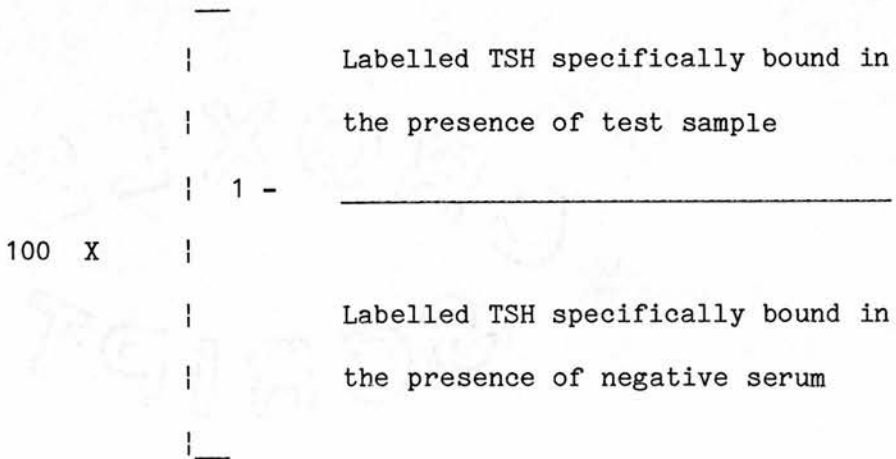
discarded and the pellet counted. Each assay included positive and negative control sera.

B) ASSAY BASED ON NEAT SERUM

This uses the same method and reagents as above and its use was described by Southgate et al (1984): duplicate 50 microlitre aliquots of serum were added to 50 microlitres of receptors and incubated as before prior to the addition of labelled TSH. After a further hour at 37°C, 800 microlitres of assay buffer and 1 ml of 30% PEG in 1M NaCl (to give a final PEG concentration of 15%) were added. The precipitate was harvested and counted as before.

EXPRESSION OF RESULTS

Non-specific binding was taken as the amount of labelled TSH precipitated with 1% Lubrol in the presence of negative serum. results were expressed as a binding inhibitory index calculated as follows:-



For the assay using immunoglobulin concentrates, the normal range was -10 to +10 (see Chapter 2, FIGURE 2.1 and Shewring & Rees Smith, 1982).

The normal range of the assay using neat serum was obtained from 98 normal controls in whom endocrine and autoimmune disease had been excluded (see FIGURE A1.1). The mean binding inhibitory index in these controls was -1.23 with a standard deviation of 4.7. The mean plus two standard deviations was 8.2 but nine was taken as the upper limit of normal since none of the controls had a value for TBII higher than this.

TSH receptor antibodies (TBII) were measured in neat serum from 150 patients with untreated thyrotoxic Graves' disease. The results are shown in FIGURE A1.2. Positive values were found in 121 (81%) patients. By contrast, there were very few positives in other goitrous thyroid diseases (see FIGURE A1.3). None of 16 patients with simple goitre or 25 patients with hypothyroidism secondary to Hashimoto's thyroiditis was positive. One out of 47 patients with euthyroid multinodular goitre was positive as were three out of 40 patients with toxic nodular goitres.

FIGURE A1.1 NORMAL CONTROLS FOR THE TBII ASSAY

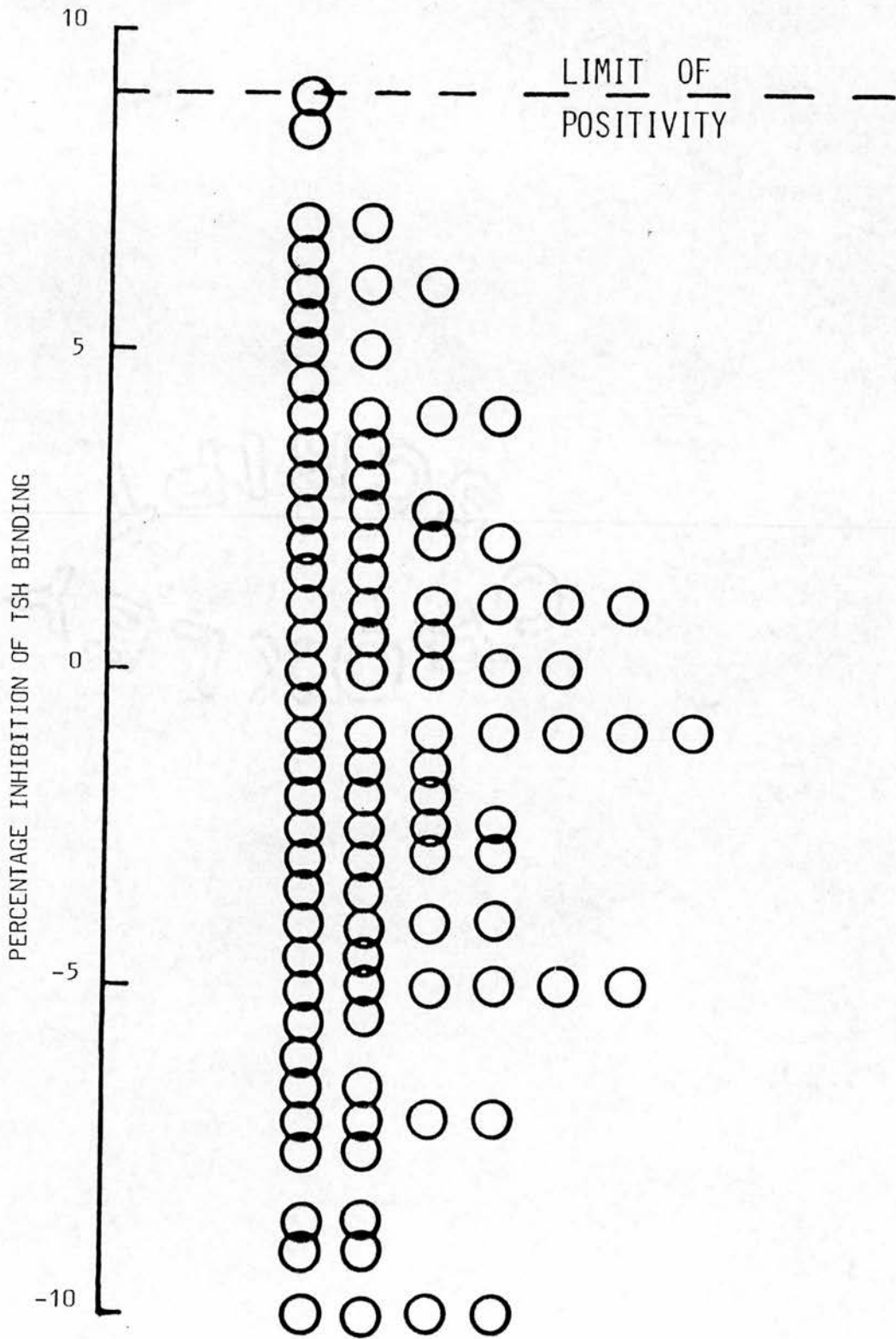


FIGURE A1.2 TSH BINDING INHIBITORY IMMUNOGLOBULINS IN PATIENTS WITH NEWLY DIAGNOSED GRAVES' DISEASE

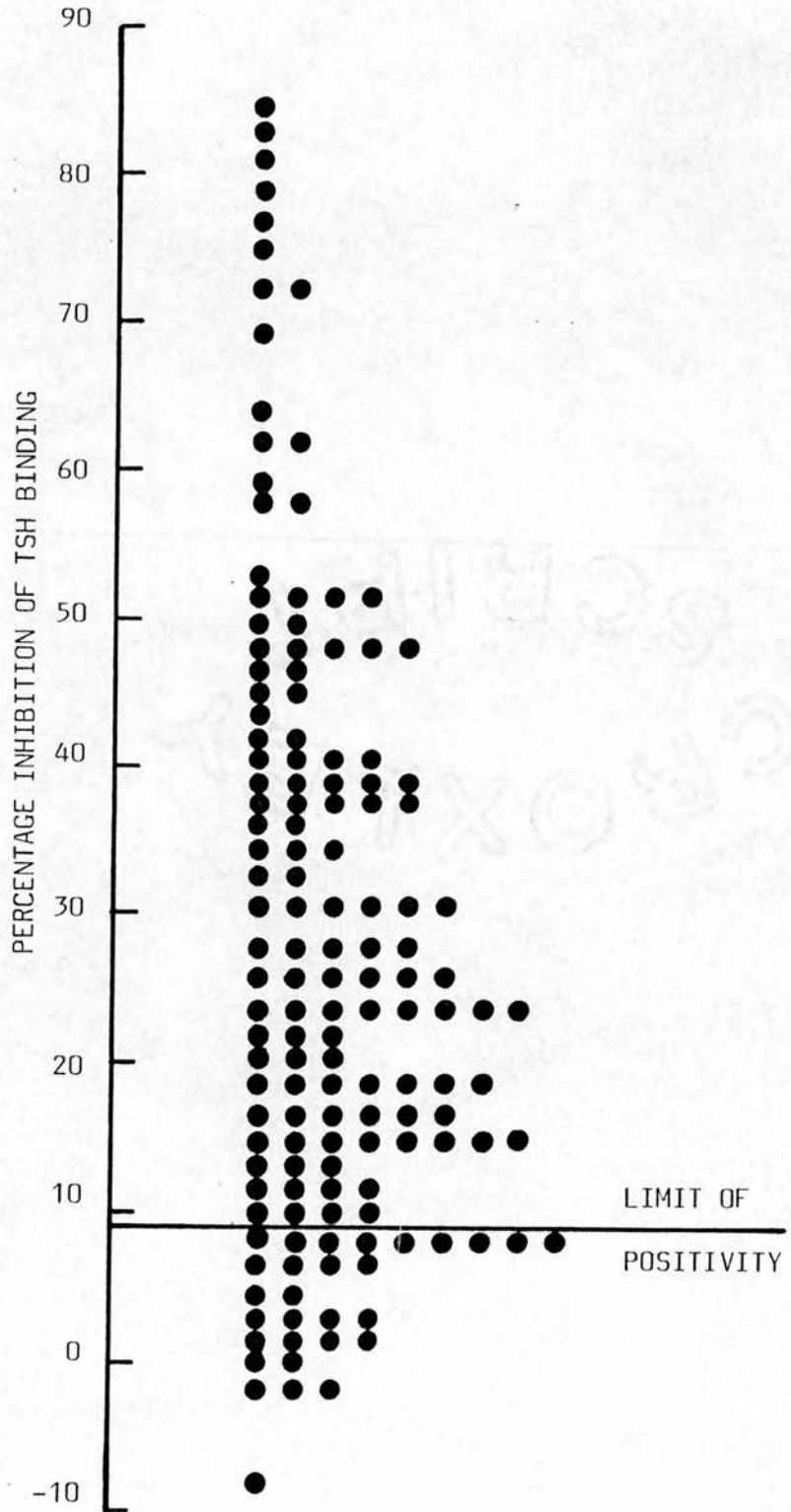
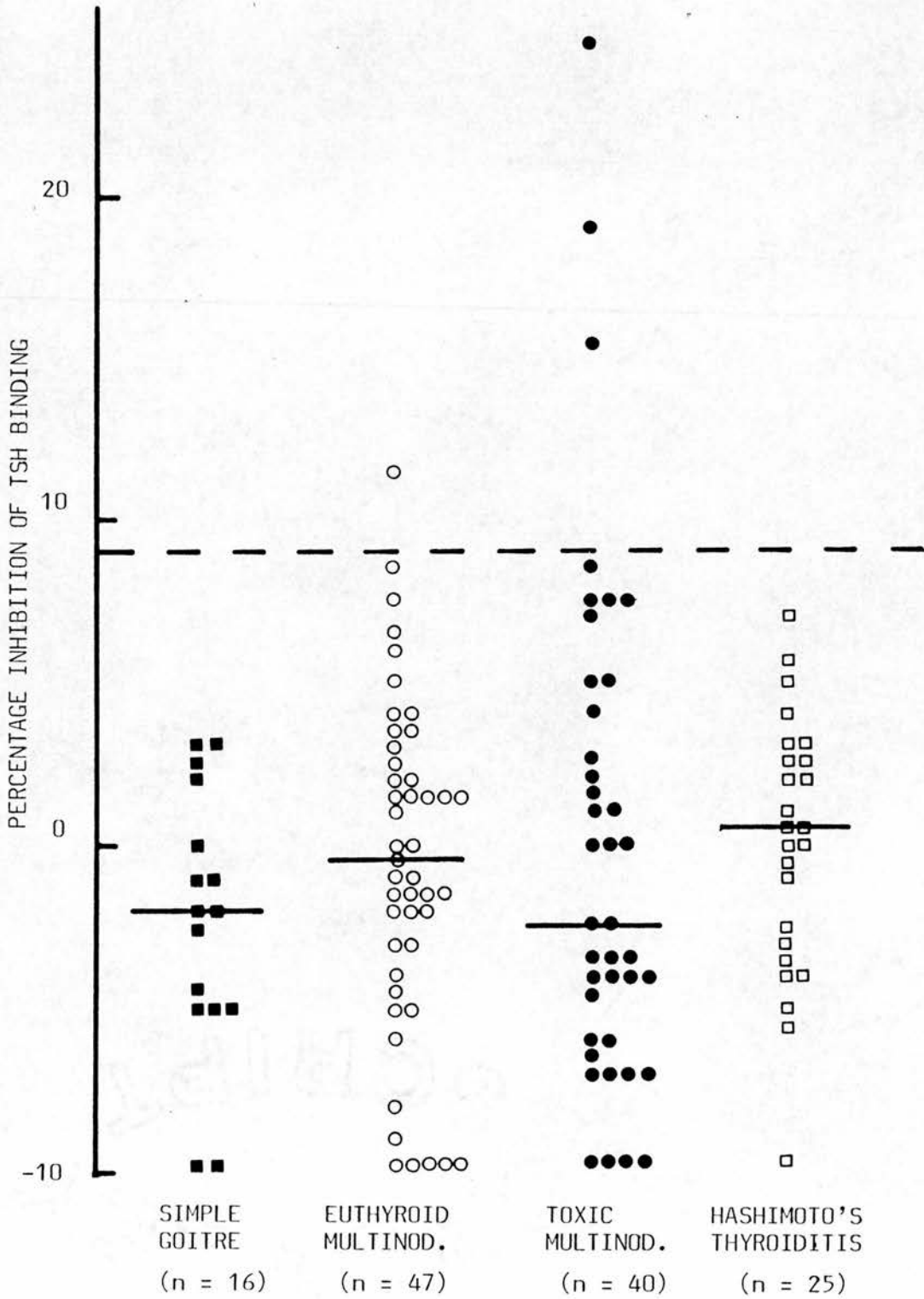


FIGURE A1.3 TSH BINDING INHIBITORY IMMUNOGLOBULINS IN GOITROUS
THYROID DISEASES OTHER THAN GRAVES'



APPENDIX 2: THE C1q SOLID PHASE ASSAY FOR IMMUNE COMPLEXES

A: THE PURIFICATION OF HUMAN C1q

The first component of complement consists of three subunits, C1q, C1r and C1s which only function in the presence of Ca^{++} . C1q is a heat-labile, 11S protein which will precipitate antigen-antibody complexes. It can be obtained in a highly purified form with a 40 to 60% yield by repeated precipitation in the presence of chelating agents to remove C1r and C1s. The method set out has been developed in the Department of Endocrinology/Immunology, Royal Infirmary, Edinburgh from that of Yonemasu & Stroud (1971).

Reagents

The C1q was prepared from 150 millilitres of fresh serum from a normal blood donor, stored at -40°C pending the procedure outlined below. The following solutions were required:-

BUFFER 1:	0.026M EGTA*	2.2 litres, pH 7.5
BUFFER 2:	0.75M sodium chloride	
	0.02M sodium acetate.3H ₂ O	
	0.02M acetic acid	
	0.01M EDTA*	500 mls., pH5
BUFFER 3:	0.06M EDTA	4 litres, pH5

BUFFER 4: 0.005M KH_2PO_4
 0.005M Na_2HPO_4
 0.75M sodium chloride
 0.1M EDTA 500 mls., pH 7.5

BUFFER 5: 0.035M EDTA 4 litres, pH 7.5

BUFFER 6: 0.75M sodium chloride
 0.02M sodium acetate.3H₂O
 0.02M acetic acid
 0.01M EDTA 500 mls., pH 7.5

* ABBREVIATIONS:-

EDTA = Trisodium ethylenediamine tetra-acetate (Sigma).

EGTA = Ethyleneglycol bis-(aminoethyl)-tetra-acetic acid (Sigma).

First Precipitation

- 1: Fresh serum was dialyzed against 1 litre of buffer for four hours at 4°C. The buffer was changed and dialysis proceeded for a further eleven hours.
- 2: Spin 10,000g for 15 minutes.
- 3: Wash precipitate with 50 mls. of Buffer 1.
- 4: Spin 10,000g for 15 minutes.
- 5: Dissolve in 3 mls. of Buffer 2.
- 6: Spin 5,000g for 5 minutes to remove undissolved material.

Second Precipitation

- 7: Dialyze solution against 4 litres of Buffer 3 for four hours at 4°C.

- 8: Spin at 10,000g for 15 minutes.
- 9: Wash precipitate with 50 mls. of Buffer 3.
- 10: Spin at 10,000g for 15 minutes.
- 11: Dissolve in 32 mls. of Buffer 4, stirring overnight if necessary.
- 12: Spin at 5,000g for five minutes.

Third Precipitation

- 13: Dialyze the above solution against 4 litres of Buffer 5 for five hours at 4°C.
- 14: Spin 10,000g for 15 minutes.
- 15: Wash precipitate with 50 mls. of Buffer 5.
- 16: Spin 10,000g for 15 minutes.
- 17: Dissolve with 16 mls. of Buffer 6 and store at -40°C. in aliquots.

The presence and the approximate concentration relative to reference solutions of C1q could be checked at each stage by the agglutination of IgG-coated latex beads (Hoechst, after Elwald & Schubart, 1966).

THE C1q SOLID PHASE ASSAY

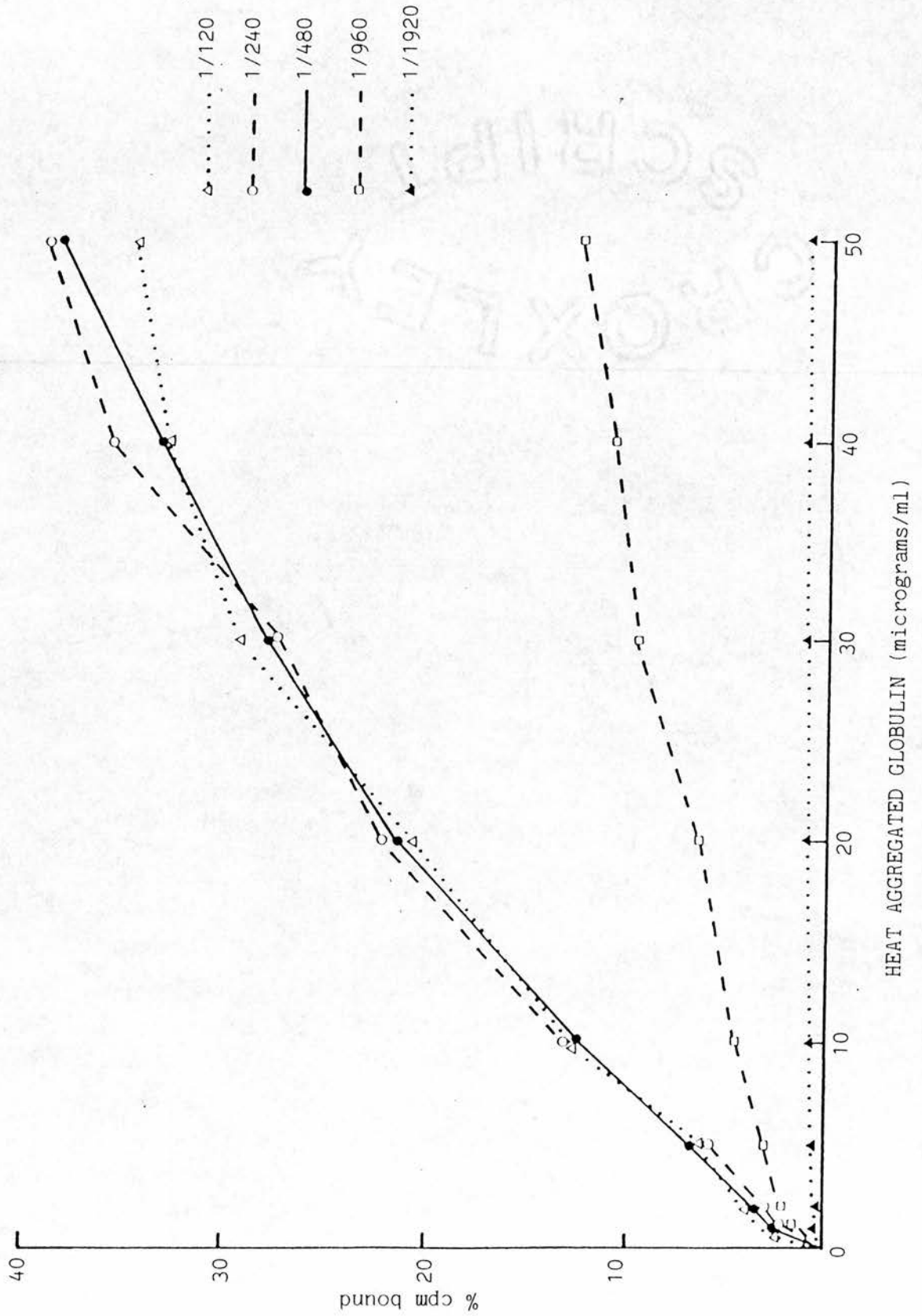
The assay was performed according to a modified method of Hay, Nineham and Roitt (1976). C1q coated polystyrene tubes (Luckhams, LP3) were prepared by adding 0.5 mls. of a solution of C1q purified as above and diluted appropriately (see below). These tubes were left at 4°C. for 24 to 78 hours. The incubation time and the C1q concentration were standard for each set of experiments. Immediately

prior to the assay, the tubes were washed three times with 0.5% Tween 20 in phosphate-buffered saline, pH 7.2 (PBS/Tween) and once with 0.1% gelatin in PBS.

In order to determine the working dilution of C1q, a series of standard curves was constructed with heat aggregated human globulin (Sigma, Cohn fraction II) using C1q at various dilutions as shown in FIGURE A2.1. For each curve, heat aggregated globulin (HAG) was added to a final concentration of 1, 2, 5, 10, 20, 30, 40 and 50 microlitres per ml. The assay was performed as described below. The total volume of solution added to each tube was 0.5 ml. - 500 mcl. of PBS for the reagent blank, 475 mcl. of PBS and 25 mcl. of EDTA-treated normal serum for the control and, for the standard curve, 375 mcl. of PBS, 100 mcl. of HAG in PBS at various dilutions and 25 mcl. of treated normal serum. For the experiment shown in FIGURE A2.1, a dilution of 1:480 was taken as the working concentration. The curve obtained was similar to that of more concentrated solutions and it was approximately linear in the range 3 to 20 mcl./ml. of HAG in which most immune complex positive sera fall. This process was repeated for each new batch of C1q.

All sera, including the normal serum used as a control were pretreated by incubating 25 mcl. of serum with 50 mcl. of EDTA (0.2M, pH 7.5) for 30 minutes at 37°C. These were then stored at 4°C until the start of the assay. 25 mcl. of the solution thus prepared was added to a C1q coated tube. All incubations were performed in duplicate. Each experiment included a reagent blank and control as above as well as a standard curve with heat-aggregated globulin. The tubes, each containing a total volume of 0.5 ml. were incubated at

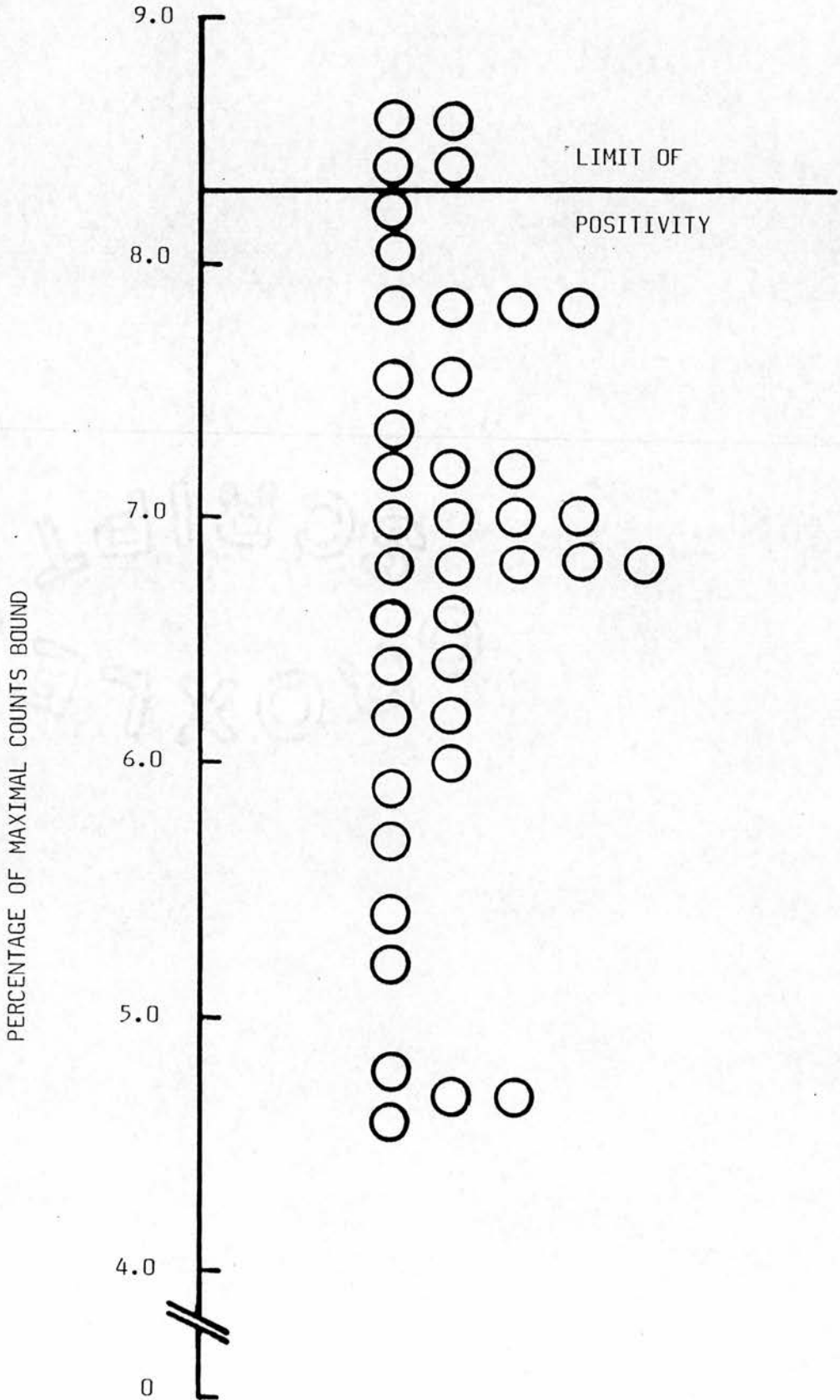
FIGURE A2.1 BINDING CURVES FOR HEAT-AGGREGATED GLOBULIN AT
DIFFERENT CONCENTRATIONS OF C1q



37°C for one hour and then at 40°C for 30 minutes. Each tube was then washed three times with PBS/Tween. Immune complexes bound to the surface of the tube were detected using staphylococcal protein A (Sigma) labelled with ^{125}I using the chloramine T method of McConahey & Dixon (1966).

The results were expressed as a percentage of the maximum counts bound on the standard curve and the normal range was calculated for each batch of C1q by testing the sera from 40 to 50 normal volunteers. An example of the results thus obtained is shown in FIGURE A2.2. The 90th centile was taken as the limit of positivity since the scatter of results in a normal population conforms to a skewed normal distribution (Di Mario et al, 1981). This limit of positivity was checked for each experiment by including at least ten sera from the normal donors. Also included in each assay were five positive controls, usually from patients with connective tissue diseases and ten sera which had already been tested in previous assays. The only test sera which had been stored long-term were those from Addison's patients and the limit of positivity was checked in these experiments by including 50 controls which had been stored with the test sera under identical conditions.

FIGURE A2.2 NORMAL CONTROLS FOR THE C1Q-SOLID PHASE ASSAY



APPENDIX 3: METHOD FOR HLA-DR TYPING

A: PREPARATION OF LYMPHOCYTES

Venous blood (20 mls.) was collected in a sterile plastic universal container with 20 units per ml. of preservative-free heparin. Separation of peripheral blood mononuclear cells was achieved using a modified method of Boyum (1966) within two hours. Blood was diluted with an equal volume of RPMI 1640 medium (Flow Laboratories) supplemented with 20 units per millilitre of preservative-free heparin and 20% foetal calf serum. This was divided into three aliquots of approximately 14 mls., each of which was underlaid with Ficoll-Hypaque, S.G. 1.077 (Lymphocyte Separation Medium, Flow Laboratories) and centrifuged at 400g for 30 minutes at 20°C. Cells were harvested by centrifugation, washed twice at 100g to remove contaminating platelets and resuspended in 2 mls. RPMI/FCS.

B: PREPARATION OF DESIALATED SHEEP RED BLOOD CELLS

Sheep's blood in Alsever's solution (Gibco) was diluted 1:2 with 2.5 mM MOPS buffer/150 mM sodium chloride (MOPS/Saline, pH 7.3) and white blood cells were removed by centrifugation over Ficoll-Hypaque. The red cell pellet was washed twice in MOPS/Saline and resuspended to a final concentration of 6%. To each 5 mls. of this suspension, was added 150 microlitres of a 1 unit per ml. solution of neuraminidase (Sigma, type V). After incubation at 30°C for 30 minutes, the cells were harvested, washed twice with MOPS/Saline and resuspended to a concentration of 2.5% in 10mM EDTA/100mM sodium chloride/2.5mM MOPS, pH 7.3 containing 0.1% gelatin. Sheep red blood cells prepared in this way were stored for up to one week prior to use at 4°C.

C: SEPARATION OF B LYMPHOCYTES.

One millilitre of a 5% solution of papain (Sigma, type II) was added to the 2 mls. of lymphocyte suspension prepared as described above and incubated at 37°C. for three minutes. Cells were harvested and washed twice in MOPS/saline/20% FCS and then resuspended to a density of 3×10^6 /ml. This suspension was mixed with an equal volume of desialated sheep red blood cells containing 0.001 ml. per ml. of 5% polybrene (Sigma) and agitated gently on a roller mixer for ten minutes before being left overnight at 4°C. to rosette. The cells were harvested the next morning by gentle centrifugation (approximately 500 rpm on a MSE bench centrifuge). Rossetted and unrosetted cells were separated on Ficoll-Hypaque, the B cell rich fraction being collected from the interface. The B cells were washed twice with MOPS/saline and the concentration adjusted to two million cells per ml.

D: PREPARATION OF PLATES FOR TISSUE TYPING

The antisera used for tissue typing defined the specificities HLA-DR 1, 2, 3, 4, 5, and 7. They included commercially available sera (Hoechst) and sera donated to the laboratory (Blood Transfusion Centre Sheffield; Department of Clinical Immunology, Royal Infirmary, Glasgow and Blood Transfusion Centre, Newcastle). 60 well Terasaki plates were filled with 10 mls. Liquid paraffin and into each well was injected one microlitre of antiserum. In all, four antisera were available to define the DR-4 locus, five for DR-3, two for each of DR-4 and DR-5, four for DR-7 in addition to one for DR-1, one cross reacting with DR-1 and DR-4, two cross reacting with DR-4 and DR-7 and one with DR-5 and DR-8. Each plate also included negative control serum, AB serum as a complement control and anti-lymphocyte serum as

a positive control. All antisera were preabsorbed with platelets to remove A, B, and C specificities. Prepared plates were stored at -40°C.

E: THE LYMPHOCYTOTOXICITY TEST FOR DR-TYPING

The microcytotoxicity assay was performed according to the method of Bodmer et al (9176). One microlitre of B cell suspension was added to each well in the Terasaki plate and left at 20°C. for one hour. Five microlitres of a complement solution specifically marketed for DR-typing (Pel-Freez Biologicals) was added and incubated for a further two hours. Three microlitres of 5% eosin was added, followed three minutes later by five microlitre of a 30 - 40% solution of formaldehyde, pH 7.0. The plates were allowed to settle and scored blindly as below. Each plate was reread after a few days and any discrepant test was repeated.

SCORING OF MICROCYTOTOXICITY TEST

PERCENTAGE DEAD CELLS

UP to 30%	NEGATIVE
31 to 50%	WEAK POSITIVE
51 to 75%	POSITIVE
ABOVE 75%	STRONG POSITIVE

Patients and normal volunteers previously typed in the laboratory and in the regional tissue typing laboratory functioned as controls and such subjects were typed repeatedly to ensure quality control. Four patients tested could not be assigned a DR type from the typing as

described above. Time and resources did not permit a large control population to be typed although, ideally controls should be typed from the same geographical location and with the same antisera. Data for the normal UK population was taken from that issued to transplant service users and was derived from over 2000 normal controls. The HLA-DR antigen frequencies in the normal UK population are shown in TABLE A3.1.

TABLE A3.1 HLA-DR ANTIGEN FREQUENCIES IN THE NORMAL U.K. POPULATION.

<u>HLA ANTIGEN</u>	<u>FREQUENCY IN POPULATION</u>
DR1	16.56
DR2	30.38
DR3	27.14
DR4	36.94
DR5	13.82
DRw6	22.20
DR7	25.72

Data from a report "HLA-A, B and DR antigens in the British Isles" issued to U.K. Transplant Service users, November 1985 by P.T. Klouda, J. Molnar, A. Wasik and J.G. Bodmer.

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