CIRCULATING IMMUNE COMPLEXES IN THYROID DISORDERS AND DIABETES MELLITUS

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DECLARATION

I hereby declare that the work for this thesis was carried out by me without assistance. This thesis has not been submitted for any other degree.

Sabah F. A.-W. Al-khateeb

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SUMMARY

Sera from patients with thyroid disorders or diabetes were tested for the presence of soluble immune complexes using a number of established or modified assay systems. In addition, a new and sensitive assay for the detection of complexes was developed.

The first assay system applied was based on the inhibition of the antibody-dependent cell-mediated cytotoxic (ADCC) activity of rat spleen cells by soluble complexes which compete with antibody-coated target cells for Fc receptors on the effector cells. The sensitivity of this assay was found to be approximately 25µg/ml of added aggregated IgG.

The C1q deviation test, an assay based on the deviation of binding of radiolabelled C1q to sensitized sheep erythrocytes by immune complexes, was found to be insufficiently sensitive or reproducible and accordingly rejected.

The Raji cell radioimmunoassay, which relies on the binding of complexes to complement receptors on Raji cells and the subsequent detection of radiolabelled anti-human IgG antibody bound to the cells, was found to have a sensitivity of 6µg/ml added aggregates. By referring to a standard curve of radiolabelled antibody uptake by cells previously incubated with varying amounts of aggregates, it was possible to quantitate the complexes detected in test sera. A modification of the assay using radiolabelled anti-human thyroglobulin antibody instead of radiolabelled anti-IgG antibody was used to characterize the complexes detected in the sera from patients with thyroid disorders.

A new sensitive and reproducible assay was developed during this study. This method, the rat spleen leucocyte (RSL) radioimmunoassay, is based on the binding of complexes to rat spleen leucocytes via Fc receptors and the subsequent detection of radiolabelled

anti-human IgG bound to the cells. The sensitivity of this assay was found to be 4µg/ml added aggregates. The complexes were quantitated by referring to a standard curve of radiolabelled antibody uptake by cells previously incubated with varying amounts of aggregated IgG. The results obtained using this assay were compared with those obtained using the Raji cell radioimmunoassay.

Using the ADCC inhibition assay, 17 out of 25 Hashimoto, 15 out of 29 hypothyroid, 8 out of 26 untreated thyrotoxic and 12 out of 27 treated thyrotoxic sera were found to be positive for the presence of immune complexes. The degree of inhibition could not be correlated with levels of serum IgG. In particular, the inhibitory activity of Hashimoto sera could not be attributed to the high levels of IgG found in these sera.

Using the Raji cell radioimmunoassay, 18 out of 23 Hashimoto, 10 out of 28 hypothyroid and 4 out of 20 untreated thyrotoxic sera were found to be positive for the presence of immune complexes. Thyroglobulin was demonstrated to be a component of the immune complexes detected in 8 out of 18 positive Hashimoto and 3 out of 10 positive hypothyroid sera. No correlation was found between the presence and titre of anti-thyroid antibodies and the presence of complexes by either the ADCC inhibition assay or the Raji cell radioimmunoassay.

The Raji cell radioimmunoassay was also used to detect and quantitate immune complexes in the sera of diabetics. 7 out of 13 newly diagnosed insulin-dependent diabetics were found to be positive. Islet cell antibodies were detected in 6 of the positive sera but in none of the negative sera. The titres for a wide range of viral antibodies were found to be similar in these 13 diabetic patients and in their age- and sex-matched controls. 17 out of 32 diabetics

treated with insulin and 5 out of 52 diabetics requiring oral hypoglycaemic agents or restrictions of diet had evidence of immune complexes in their serum. High titres of insulin antibodies in the sera of insulin-treated diabetics were found to correlate with the presence of immune complexes. In addition, there was a tendency for immune complexes to occur in the presence of moderate titres of insulin antibodies when the age at onset of insulin-dependent diabetes was less than 30 years. Out of 16 patients treated with insulin for 13 years or more, with late diabetic complications, 12 had immune complexes in their serum.

In both patients with thyroid disorders and those with diabetes, the results using the RSL radioimmunoassay were comparable to those obtained using the Raji cell radioimmunoassay. In general, the amount of complexes detected in a given serum was lower in the RSL than in the Raji cell radioimmunoassay. The RSL assay was also used to demonstrate that heat treatment of normal human serum induces the formation of aggregates.

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1. INTRODUCTION

1.1. GENERAL: THE ROLE OF IMMUNE COMPLEXES IN DISEASE

Early in this century two different phenomena attracted the attention of the clinicians. The first was observed by Arthus (1903), who undertook an experiment with horse serum injected to animals. The first injection was found to effect the animals in such a manner that repeated injections were very dangerous. Von Pirquet (1911) observed that a single injection of horse serum which was used as a source of antitoxin in the treatment of diphtheria, tetanus, scarlet fever and epidemic meningitis, sometimes caused symptoms to which he gave the name of serum sickness. It was suggested that the symptoms were due to the formation of antibody to the injected material. An analogy between serum sickness and clinical events such as urticaria, hay fever, post scarlet fever nephritis, tuberculosis, syphilis, and actinomycosis was also suggested by Von Pirquet.

The observations of Longcope and Rackemann (1918), and those of Mackenzie and Leake (1921) on the morphological lesions which developed in humans receiving therapeutic serum lead them to suggest the involvement of antigen-antibody reactions within the circulation or on tissue cells. Rich and Gregory (1943), Hoppes and Wissler (1946), Rich (1947) and Ehrich and associates (1949), using animal models, demonstrated the effects of antigen-antibody reactions resembling those occurring in human diseases such as rheumatic fever, rheumatoid arthritis, glomerulonephritis, disseminated lupus erythematosus and polyarteritis nodosa.

Hawn and Janeway (1947) injected purified serum proteins into rabbits and demonstrated lesions similar to those induced using whole serum. They concluded that the lesions of serum sickness did indeed develop at the time of antigen-antibody interaction. Similar findings

were also reported by Germuth in 1953, who described an "immune phase" of antigen elimination in which all tissue lesions developed as a result of antigen-antibody combination.

The pathogenic mechanisms by which antigen-antibody reactions lead to tissue injury were not clearly defined. One concept was that the damage was caused by the reaction of antibody with the target tissue. This derived from the experiments of Lindemann (1900), Pearce (1903) and Smadel (1936) who used heterologous anti-kidney serum to produce experimental nephritis resembling glomerulonephritis in man. It is unlikely that this mechanism operates since although humoral antibody may induce damage to circulating cells or cells in monolayer (endothelium and vascular basement membrane), there is no evidence that solid tissue can be affected (Waksman, 1962). The alternative concept was that of Von Pirquet (1911), who ascribed the phenomenon of serum sickness to the formation of a toxic compound by antibody combined with the foreign serum antigen.

Germuth and McKinnon in 1957 demonstrated that soluble antigen-antibody complexes by themselves were pathogenic agents as features of anaphylactic shock could be elicited by injecting unsensitized guinea-pigs with these complexes. Tokuda and Weiser (1958) reported similar results using mice. Weigle and co-workers (1960) confirmed these results in guinea-pigs and rabbits. Similar findings were reported earlier by Dean and co-workers (1936), using intravenous injections of antigen immediately after an intravenous injection of antiserum.

Using isotopically labelled antigens, soluble antigen-antibody complexes could be demonstrated in the circulation during experimental serum sickness (Dixon, 1957). The development of the morphological manifestation of serum sickness could be shown to be accompanied by an almost simultaneous localization of antigen and in some cases antibody

in the site of the lesion, but there was no detectable localization or fixation of antigen in the tissue predisposed to the development of lesion prior to the formation of antibody (Dixon et al., 1958).

Many workers have used a variety of techniques in order to establish the pathogenicity of complexes. The early work of Kulka (1942) showed that antigen-antibody mixtures induce smooth muscle contraction and that this effect depends on a quantitative relationship between antigen and antibody. Similar results were demonstrated by Trapani and co-workers (1958), who found that only complexes in slight to moderate antigen excess were active in inducing smooth muscle contraction in vitro.

The induction of increased cutaneous vascular permeability by complexes was used by Ishizaka and associates (Ishizaka and Campbell, 1958, 1959; Ishizaka et al., 1959a, b) to study the molecular characteristics of the pathogenic complexes. These authors were able to conclude that the activity of a complex depends upon its composition with respect to antigen/antibody ratio and upon the properties of the antibody and not the antigen. Human, rabbit and guinea-pig antibodies but not bovine, chicken or horse could form active complexes. The toxicity of complexes was due to a molecular change brought about in either the antigen or antibody molecule as a result of combination. The ability to fix complement depends on the properties of the antibodies e.g. complexes of either horse or chicken antibodies do not fix complement while rabbit antibodies have this ability. In addition they found that human and rabbit IgG as well as inactive antigen-antibody complexes inhibit the cutaneous permeability usually induced by an active complex.

Ishizaka and Ishizaka (1959) demonstrated that heat-aggregated human gammaglobulin induced increased vascular permeability and that

the aggregates were comparable to antigen-antibody complexes on a weight basis with respect to both skin reactivity and complement fixation. Christian (1960b) found marked polymorphonuclear and mononuclear infiltrations in the skin of guinea-pig, rat and man injected with aggregated human gammaglobulin. In addition, the intravenous injection of such aggregates, which were anticomplementry in vitro, rendered the sera of recipient animals deficient in haemolytically active complement for several hours.

Dixon (1962) suggested the possibility that aggregation of gammaglobulin can occur in vivo as well as in vitro and could have the same biological activity. If this can occur, gammaglobulin complex-induced diseases may not necessarily have an immunological basis.

Other workers have investigated other aspects of the biological activity of soluble immune complexes. Arocha and McIlreath (1959) reported the release of histamine from isolated guinea-pig lung or rat small intestine when these tissues were perfused with soluble antigen-antibody complexes. Mota in 1961 using a similar assay system could not detect any damage to mast cells, and suggested that soluble complexes do not act by preferentially releasing histamine from the mast cells. Fibrinolysin was demonstrated to form in serum from its inactive precursor by addition of complexes (Unger and Mist, 1949). Lepow and co-workers (1956) showed that antigen-antibody aggregates could convert the C1 component of complement present in fresh serum to an active esterase. Antigen-antibody interaction in fresh serum was associated with loss of haemolytic potency of the serum and the appearance of anaphylotoxin as judged by its effect on capillary permeability and smooth muscle contraction (Osler et al., 1959).

Many workers have studied the effects of complexes on a variety of cell types. Using fluorescence and electron microscopic studies,

Feldman in 1958 showed that antigen-antibody complexes initiated injury to the endothelial cells of rabbit kidney glomeruli in serum sickness. Miescher and Straessle (1956), and Straessle and Miescher (1956), demonstrated that soluble antigen-antibody complexes agglutinate leucocytes and that this effect depends upon the nature of the antigen and antibody and upon the ratio of the two. Boyden (1962) demonstrated that antigen-antibody mixtures were chemotactic to rabbit polymorphonuclear leucocytes in the presence of fresh but not heated serum. The biological source of the test material was important in the initiation of chemotaxis in that plant or bacterial protein were much more effective than serum albumin of human or bovine origin. Immune complexes have also been shown to induce the release of lysosomal material from neutrophils (Hawkins and Peeters, 1971; Henson, 1971; Henson et al., 1972).

It has been demonstrated that immune complexes induce platelet aggregation (Siqueira and Nelson, 1961; Mueller-Eckhardt and Luscher, 1968) and the release of amines from platelets in the presence of plasma (Gocke, 1965; Henson and Cochrane, 1969). Henson and Spiegelberg (1973) demonstrated that human platelets bear receptors for the Fc portion of immunoglobulin and that the platelets clump and release nucleotides and vasoactive amines in response to immune complexes or aggregated immunoglobulin.

Immune complexes have been found to enhance lymphocyte activation as determined by morphological changes and the stimulation of DNA synthesis (Bloch-Shtacher et al., 1968; Moller, 1969). Moller and Lundgren (1968) reported that lymphocytes stimulated by antigen-antibody mixtures of various types acquired cytotoxic activity against fibroblasts of autochthonous and allogeneic origin.

Pathological manifestations induced by complexes could result from one or more of their demonstrated biological activities. The simplest complex-induced lesion is the Arthus reaction, a localized acute necrotizing vasculitis (Arthus, 1903; Arthus and Breton, 1903). Opie (1924) demonstrated that this phenomenon occurs when an animal immunized against a protein is reinjected with the same antigen and occurs when antigen and antibody meet in the tissue. A similar reaction was shown to occur at the site of injection when an injection of antigen was followed by injection of serum from an immunized animal.

Cannon and Marshall (1941) found a definite parellelism between the precipitating potency of the serum and the severity of cutaneous reaction. Massive injections of antigen were shown to result in a reduction of precipitin titre with a subsequent decrease or disappearance of Arthus-type cutaneous hypersensitivity which later reappeared with the rise in the titre.

Skin reactions marked by erythema, induration and occasionally vascular haemorrhage and necrosis were demonstrated by the injection of soluble antigen-antibody complexes intradermally (Cochrane and Weigle, 1958). The lesions were also found to be similar in severity to those resulting from the Arthus reaction, and it was concluded that the presence of antigen-antibody complexes was responsible for the reaction.

Local manifestations of the Arthus reaction were suggested to be due to the accumulation of polymorphonuclear leucocytes in the area of reaction, with intravascular aggregation of platelets and leucocytes leading to thrombosis in the capillaries and veins. In addition, the inflammatory necrotizing reaction failed to develop after the removal of polymorphonuclear leucocytes from the circulation (Stetson, 1951). Similar findings concerning polymorphs were reported by Humphrey (1955a, b).

Cochrane and co-workers (1959) demonstrated that polymorphonuclear leucocytes play an essential role not only in producing the inflammatory vasculitis, but also in ridding the damaged vessel of the antigen, probably by means of proteolytic catabolism at the inflammatory site. In the absence of these cells, antigen and antibody complexes were deposited beneath the intima of small vessels without resulting inflammation. Dixon (1962) reported that antigen-antibody precipitates form by diffusion of antigen and antibody toward each other early in the reaction and that these precipitates are chemotactic for polymorphonuclear leucocytes. Within a few hours these cells infiltrate the involved vessels which undergo necrosis. The cells phagocytose the complex and carry it away from the site of the reaction.

The classical "one shot" serum sickness might be closer to the situation in systemic human disease. Dixon and co-workers (1958) using isotopically labelled antigens demonstrated the sequence of immunological and morphological events occurring subsequent to the injection of a large dose of serum protein antigen in rabbits. Three distinct phases of removal of antigen from the circulation were demonstrated. The equilibration of the intravenously injected foreign protein between the intra- and extra-vascular serum protein pools results in the first and sharpest fall. Next there is a non-immunologically mediated catabolism of circulating free antigen at a rate characteristic for the particular injected protein and the recipient species. Finally there is a phase of rapid loss just preceding the appearance of free circulating antibody. Once the antibody forms, small complexes in an extreme antigen excess capable of remaining in the circulation were formed. Antigen-antibody complexes become larger as increasing amounts of antibody are formed and are progressively removed from the circulation. Accompanying the

presence of complexes, there was a fall in serum complement levels and the appearance of acute inflammatory lesions in the kidneys, heart, arteries and joints resembling the lesions of acute glomerulonephritis, rheumatic fever, lupus erythematosus, polyarteritis nodosa, and rheumatoid arthritis. Endothelial proliferation, increased vascular permeability and infiltration of polymorphonuclear leucocytes were elicited in varying degree in response to antigen-antibody complexes.

Dixon and co-workers (1961) extended the earlier work of McLean and co-workers (1951) by injecting intravenously small amounts of protein daily into rabbits, in order to mimic a chronic clinical situation which might be produced by antigen-antibody complexes. It was shown that the pathological events were of three types depending upon the response. Firstly, there may be the production of a large excess of antibody in the circulation which enables the animal to pass from an antigen excess to an antibody excess environment with the development of sizable amounts of antigen-antibody complexes in the circulation. Acute inflammatory lesions typical of serum sickness developed in the heart, blood vessels and kidneys. Any antigen injected after the formation of a permanent antibody excess was incorporated immediately into insoluble antigen-antibody aggregates and quickly removed from the circulation. Secondly there may be no antibody response in which case the animal has a considerable amount of antigen in the circulation at all times with no evidence of disease. Thirdly those animals making antibody responses too small to cause elimination of antigen but sufficient to be incorporated into circulating complexes were found to develop chronic progressive glomerulonephritis. The disease could be turned off or on by changing the dose of antigen. If the dose was increased to allow a continual antibody excess, the disease did not progress. If the dose

was again changed to a level giving rise to soluble complexes, the disease would progress. Membranous glomerulonephritis was the most common disease and as it progressed, proliferative and scarring reactions developed. Electron microscopy showed a lumpy, dense deposit along the outer aspect of the basement membranes corresponding to the antigen, gammaglobulin, and complement-rich deposits visualized with the fluorescent antibody technique.

Dixon concluded from these experiments that the nature of the disease produced by antigen-antibody interaction depends to a large extent upon the quantitative relationship of the two reactants and little, if at all, upon the absolute amount of antibody or upon the immunological characteristics of the antigen. Where little antibody is produced, for example in response to prolonged exposure to small amounts of endogenous or exogenous antigen, the formation of antigen-antibody complexes in antigen excess could contribute to maintenance of chronic disease. The site of localization of complexes and subsequent injury was determined by the physical character and quantity of complexes, by local anatomical and physiological factors in the tissue, but not by any immunological relationship between complex and tissue. Chronic diseases might result from the prolonged presence of circulating antigen-antibody complexes.

The existence of subclinical levels of complexes in the circulation may act in other situations as adjuncts to other pathogenic mechanisms. Dixon (1962) showed that in animals receiving small clinically ineffective doses of heterologous anti-kidney serum and also injected intravenously with heat aggregated human gammaglobulin or rheumatoid serum in non-pathogenic doses, both anti-kidney antibodies and complexes were localized in the glomeruli and severe glomerulonephritis resulted.

Anti-tissue antibodies or specific toxins of little pathogenic significance and antigen-antibody complexes may participate in the development of disease.

From these experimental observations it has been suggested that complexes alone could not account for all the various pathological features found in a clinical disease. The determination of the existence of and the identification of the pathogenic contribution of immune complexes and the manner in which they are formed might be of great importance. Factors responsible for the focussing of complexes upon specific sites might be present in particular situations and could be responsible for the development of disease in some individuals but not in others.

The mechanism governing the deposition of immune complexes in different tissues has been studied by many workers. Benacerraf and co-workers (1959) and McClusky and Benacerraf (1959) reported from their experiments with mice that antigen-antibody complexes, by releasing substances such as histamine, produce changes in the endothelium of glomeruli and blood vessels which lead to the arrest of complexes at these sites and in turn to tissue damage. Cochrane (1963a, b) showed in guinea-pigs that immune complexes could be made to deposit in the walls of venules by simultaneous infusion of agents that increased vascular permeability. Using particles visible with electron microscopy, Cochrane (1963a) showed that antigen-antibody complexes were localized by being trapped in the vessel wall along the basement membranes. In further experiments, Cochrane and Hawkins (1968) found that only large (greater than 19S) immune complexes became entrapped in vessel walls. Kniker and Cochrane (1968) and Cochrane (1971) demonstrated the presence of an active mechanism whereby immune complexes increase vascular permeability. The administration

of antagonists of vasoactive amines during the time that immune complexes appear in the circulation was found to prevent the deposition of immune complexes in arteries and glomeruli. In addition, when platelets were depleted in rabbits with serum sickness there was a marked suppression of deposition of immune complexes and the subsequent development of arterial and glomerular lesions.

1.2. METHODS USED FOR THE DETECTION OF IMMUNE COMPLEXES

Early attempts to detect immune complexes involved the demonstration of gammaglobulin deposited in tissue lesions associated with various pathological conditions. Utilizing the fluorescent antibody technique, Vazguez and Dixon (1956, 1957) demonstrated the presence of gammaglobulin in amyloid deposits in secondary human amyloidosis and in experimental amyloidosis in rabbits, and in lesions of rheumatic fever, rheumatoid arthritis and systemic lupus erythematosis (SLE).

Mellors and Ortega (1956) found gammaglobulin localized in the site of the glomerular lesions in chronic membranous glomerulonephritis and in other types of glomerulonephritis, as well as in the glomerular and arterial lesions of periarteritis nodosa. Similarly Mellors and co-workers (1957) demonstrated the presence of gammaglobulin in glomerular lesions of renal amyloidosis and lupus erythematosus.

IgG and the C3 component of complement were detected by the immunofluorescent technique in granular intra- and extra-cellular deposits in synovial tissue from patients with rheumatoid arthritis (Fish et al., 1966). Similar results were reported by Brandt and co-workers (1968) in synovial tissue from patients with rheumatoid arthritis, SLE and Reiter's syndorome. Kinsella and co-workers (1969)

detected IgG and C3 in the cytoplasm of most of the phagocytic lining cells of synovia obtained from both sero-negative and sero-positive patients with rheumatoid arthritis. Electron microscopy studies have been used to demonstrate the morphological changes associated with immune complexes. Electron-dense deposits along the outer aspect of the basement membrane of affected glomeruli were detected in kidneys from patients with acute, subacute and chronic glomerulonephritis, and in lupus nephritis (Dixon, 1962).

More direct methods for detecting immune complexes are those demonstrating soluble complexes within the circulation. These methods depend on the biological activity or/and the physical characteristics of the complexes.

Some of those methods which depend on the biological activity of complexes rely on the ability of complexes to fix complement components. The C1q component of complement contains the site through which the first component combines with gammaglobulin (Muller-Eberhard and Calcott, 1966; Muller-Eberhard, 1968). Muller-Eberhard and Kunkel (1961) demonstrated that C1q can be precipitated by soluble gammaglobulin aggregates. Agnello and co-workers suggested the use of C1q to demonstrate immune complexes by precipitation in gel. They used this method to detect gammaglobulin complexes in sera or synovial fluid from patients with various types of connective tissue disease such as SLE and rheumatoid arthritis. Sera from patients with chronic glomerulonephritis and other miscellaneous hypocomplementaemic diseases were also screened by this method (Agnello et al., 1969, 1970, 1971).

Mowbray and co-workers (1973) developed the anti-complementary method for the detection of soluble complexes. This technique was based on the ability of complexes to bind to the C1 component of guinea-pig complement after the destruction of C1 already bound to the

complexes by heat inactivation of the serum. In the presence of complexes, lysis of added sensitized sheep erythrocytes was inhibited. This method was used to detect complexes in sera of patients with dermatitis herpetiformis. The same method was used by Calder and co-workers (1974) to detect complexes in sera of patients with thyroid disorders. Johnson and co-workers (1975) reported the use of this method to detect complexes in sera from patients with disseminated lupus erythematosus, angioedema urticaria, coeliac disease, cutaneous vasculitis, glomerulonephritis with and without fibrinoid necrosis and polyarteritis nodosa.

Creighton and co-workers (1973) demonstrated that antigen-antibody complexes can be precipitated by different concentrations of polyethylene glycol (PEG). Radiolabelled C1q was used to detect complexes after precipitation by PEG (Nydegger et al., 1974). This method was applied to the detection of complexes in sera from patients with SLE and carriers of hepatitis B antigen with or without hepatitis.

This method was modified by Zubler and co-workers who treated the serum with ethylenediaminetetraacetic acid (EDTA), in order to prevent the integration of radiolabelled C1q into the intrinsic C1qrs complex, and applied this modification to the detection of complexes in sera of patients with SLE (Zubler et al., 1976a). Sera and synovial fluid from patients with rheumatoid arthritis were screened using this technique (Zubler et al., 1976b). Sobel and co-workers (1975) developed another method based on the binding of C1q to immune complexes. Radiolabelled C1q was added to serum and sensitized sheep erythrocytes were used to detect unbound C1q. The serum was heat-inactivated in order to free the complexes from bound C1q before addition of the radiolabelled C1q. This assay, the C1q deviation test, was used to detect complexes in sera from patients with Dengue haemorrhagic fever.

Svehag (1975) described a solid-phase radioimmunoassay for C1q-binding immune complexes. This method was based on the absorption of C1q to a fixed inner surface area of polystyrene tube and subsequent incubation with the test serum. After the addition of radiolabelled aggregated IgG, the tubes were washed and counted. Low activity in the tubes was taken to indicate the presence of immune complexes in the test serum. Sera from patients with rheumatoid arthritis, SLE, vasculitis and chronic hepatitis were examined for complexes by this method.

A similar solid-phase radioimmunoassay for C1q-binding complexes has been described by Hay and co-workers (1976). This assay involves the addition of radiolabelled anti-human IgG antibody, and in this case increased activity was indicative of increasing amounts of complexes bound to the C1q on the surface of the tubes. The sera of patients with SLE were tested by this method. Quantitation of the amount of complexes was also reported.

Other assays based on the ability of complexes to bind to different cell receptors - Fc receptors on cytotoxic lymphocytes, macrophages and platelets - have been used to detect immune complexes. Jewell and MacLennan (1973) described a method based on the inhibition of antibody-dependent cell-mediated cytotoxicity, by competition between the soluble complexes under investigation and the antibody-coated target cell for the Fc receptors on the effector cell. This method was based on the finding of MacLennan (1972), that lymphocyte mediated cytotoxicity is susceptible to inhibition by third party immune complexes which compete with target cell bound antibody for immunoglobulin receptors on the cytotoxic lymphocytes. Using rat spleen lymphocytes as effector cells and Cr^{51} labelled Chang liver cells as target cells, complexes could be detected by counting the

activity released by effector cells previously incubated with serum containing complexes. Complexes were detected in inflammatory bowel diseases (Crohn's disease and ulcerative colitis) using this method (Jewell and MacLennan, 1973). A modification of this method was used to detect complexes in sera from patients with bronchogenic carcinoma and thyroid disorders (Barkas et al., 1976).

Onyewotu and co-workers (1974) reported the use of guinea-pig peritoneal exudate cells to detect immune complexes. It was based on the competitive inhibition of the ability of these cells to ingest soluble immune complexes. Guinea-pig peritoneal macrophages were treated with human IgG, and heat-inactivated test sera and radiolabelled aggregates were added simultaneously to the washed cells. In the presence of complexes in test serum less activity could be detected in the cells. Sera from patients with SLE were tested by this method.

Mohammed and co-workers (1977) described a modification of this method. The plastic tubes used for the assay were coated with guinea-pig serum to reduce the non-specific uptake of radioactivity on the wall of the tube. This modified method was used to detect complexes in sera from patients with SLE and rheumatoid arthritis.

Human platelets were found to aggregate in the presence of soluble immune complexes (Penttinen amd Myllyla, 1968; Penttinen et al., 1969). This property has been used by many workers to detect soluble complexes. The binding of immune complexes to platelets was found to be through Fc receptors. Sera from patients with fever of unknown aetiology, hepatitis, rheumatoid arthritis, SLE, leprosy, mycoplasma pneumonia, sarcoidosis, kidney transplantation and nitrofurantoin treatment were tested by this method (reviewed by Penttinen, 1977).

C3 complement receptors on lymphocytes derived from adenoid tissue were used by Ezer and Hayward (1974) for the detection of soluble immune complexes. The method was based on the inhibition of complement-dependent rosette formation by complexes. Washed lymphocytes were incubated with test sera and then with erythrocytes coated with antibody and complement. In the presence of complexes rosette formation was inhibited. Sera from patients with Crohn's disease, leprosy and membranous glomerulonephritis were tested by this method.

Theofilopoulos and co-workers (1976) developed a radioimmunoassay based on the binding of complexes to complement receptors on a human lymphoblastoid cell line (Raji cells). The amount of complexes bound to the cells was determined by estimating the amount of radiolabelled anti-human IgG bound to the complexes and referring to a standard curve of radiolabelled uptake by cells previously incubated with various amounts of aggregated human IgG. This method was used to detect and quantitate complexes in sera from patients with SLE, malignancies, serum hepatitis, various types of vasculitis and subacute sclerosing panencephalitis.

The ability of bovine conglutinin to bind fixed complement was used to detect immune complexes through binding to C3 fixed to complexes (Eisenberg et al., 1977). A solid state radioimmunoassay was used in which the conglutinin was adsorbed to plastic tubes. Sera from patients with SLE, subacute bacterial endocarditis, vasculitis, glomerulonephritis, multiple sclerosis and cancer were tested for complexes by this method. A similar method was used by Casali and co-workers (1977) to detect complexes in the sera of patients with SLE, vasculitis, leprosy and leukaemia.

The ability of rheumatoid factor to react with IgG containing complexes has been used by many workers to detect immune complexes. Kunkel (1961) described the inhibition by certain human sera of the capacity of rheumatoid factor to agglutinate erythrocytes coated with antibodies. Inhibition of agglutination of IgG-coated polystyrene particles (latex) was applied for the detection of IgG complexes in eluates from rheumatoid tissue (Munthe and Natvig, 1971). IgM rheumatoid factor was mixed with the test sample and then with the latex reagent, and in the presence of complexes no agglutination could be detected. Cowdery and co-workers (1975) described a radioimmunoassay which measured the competitive binding of immune complexes to rheumatoid factor. It was based on the inhibitory activity of immune complexes in the test sera on the binding of rheumatoid factor to radiolabelled aggregated IgG. Sera from patients with SLE, rheumatoid arthritis, polymyositis, hepatitis, melanoma and lymphocytic leukaemia were tested for complexes by this method. Inhibition of agglutination of IgG-coated particles by rheumatoid factor and C1q was used by Lurhuma and co-workers (1976) to detect complexes in sera from patients with Crohn's disease, liver disease, idiopathic thrombocytopenia, glomerulonephritis, rheumatoid arthritis, mylomatosis, different types of leukaemia, lymphosarcoma, SLE, ankylosing spondylitis, scleroderma, autoimmune haemolytic anaemia and chronic urticaria. Cambiaso and co-workers (1977) have described the use of an automated version of this method, while Levinsky and Soothill (1977) have reported the use of low affinity IgM antibodies to human immunoglobulins instead of rheumatoid factor.

Radiolabelled goat anti-human IgG monovalent (Fab) antibody fragments were used to detect immune complexes by Ludwig and Cusumano (1974). The method was based on the incubation of test sera with the

radiolabelled monovalent antibody and the subsequent appearance of a radiopeak after sucrose gradient velocity sedimentation. This method was used to detect complexes in patients with cancer or collagen disease.

Indirect markers of circulating immune complexes have been used to show the presence of complexes. C3 bound to complexes was detected after separation from free C3 by chromotography (Williams and Slaney, 1977). The test sera were fractionated on Sephadex G-200 and the eluted factions were tested for the presence of C3. Free C3 usually appeared in the second peak, while C3 bound to immune complexes appear in the first peak.

1.3. IMMUNE COMPLEXES AND THYROID DISORDERS

The possible role of humoral antibodies and cell mediated hypersensitivity in the pathogenesis of thyroid disease has received much attention.

In the late 1950s and early 1960s interest in the immunological aspects of thyroid disease was centred around the identification and possible pathogenic significance of thyroid-specific autoantibodies present in the serum of patients with autoimmune thyroid disease. Evidence for and against the direct mediation of tissue damage by circulating antibodies has been obtained from a number of studies involving both passive and active immunization. High-titred antiserum from autoimmunized rats or rabbits has been injected repeatedly into normal recipients without evoking any specific lesions in their thyroid glands (Anigstein, 1957; Rose et al., 1962). Nakamura and Weigle (1969) reported that thyroid lesions developed in rabbits receiving pooled sera from thyroidectomized donors, but only if the serum was

taken within 15 days after thyroidectomy. Immunofluorescent studies of recipient thyroid glands showed focal fixation of rabbit IgG and C3 complement components in the thyroid follicles. There were no thyroid lesions in recipient animals injected with late antiserum or with antiserum from non-thyroidectomized donors.

Other workers have reported the successful passive transfer of thyroiditis using antisera prepared in foreign species (Lilien, 1954). Sharp and co-workers (1967) injected guinea-pigs with antiserum prepared by immunizing rabbits with guinea-pig thyroglobulin and observed intense eosinophilic infiltration in the interstitial areas of the gland. They presumed that the formation of thyroglobulin-antibody complexes attracted the eosiniphils. The lesion was resolved in 2 to 4 weeks without evidence of typical lymphocytic thyroiditis. On the other hand, Roitt and Doniach (1958) demonstrated that the transfusion of large quantities of Hashimoto serum to Rhesus monkeys produced no changes in the thyroid gland. These observations, together with the fact that thyroiditis is rarely observed in babies of affected mothers although anti-thyroglobulin antibodies in high titre can cross the placenta (Parker and Beierwaters, 1961), have shown that antibody cannot be solely responsible for lesions observed in the thyroid gland.

The role of cell mediated immunity in the pathogenesis of thyroid disease has been studied by many workers using the leucocyte migration inhibition test. Soberg and Hallberg (1968) first demonstrated that the migration of leucocytes from 80 per cent of patients with Hashimoto thyroiditis was significantly inhibited when an homogenate of human thyroid gland was added to the culture medium. Subsequently others have reported similar findings using not only thyroid gland homogenate but also thyroglobulin and microsomal antigens (Brostoff, 1970; Calder et al., 1972; Lamki et al., 1973; Wartenberg et al., 1973a, b). The

inhibitory effect of thyroid antigens on the migration of leucocytes from patients with Hashimoto thyroiditis or thyrotoxicosis was suggested to be due to the release of migration inhibition factor from T lymphocytes specifically sensitized to thyroid autoantigens, although this phenomenon could also be independent or only partially dependent upon the presence of autoreactive T cells (Calder and Irvine, 1975).

The antigen-induced transformation of small lymphocytes to large "blast like" forms has also been used as an indication of cellular hypersensitivity, with conflicting results. Although some workers were able to demonstrate the transformation of lymphocytes from patients with thyroiditis in response to thyroglobulin (Ehrenfeld et al., 1971; Delespesse et al., 1972), others have been unsuccessful (Groot and Jaksina, 1969; Calder and Irvine, 1975).

Evidence that immune complexes may be involved in autoimmune thyroid disorders has been presented in the last few years. Werner and co-workers (1972), using immunofluorescent techniques, demonstrated the presence of human IgG, IgM, IgE and complement components C1q and C3 in focal areas of the stroma as well as in the follicular basement membrane of thyroid glands from patients with Graves' disease. Kalderson and co-workers (1973) demonstrated the presence of distinct electron-dense deposits in the follicular basement membrane of some follicles in the thyroid gland of eight patients with Hashimoto thyroiditis which closely resembled the antigen-antibody deposits of immune complexes nephropathies. Podleski (1972) reported the cytotoxic effect of lymphocytes from patients with Hashimoto thyroiditis on target cells coated with either thyroglobulin or the microsomal fraction of human thyrotoxic gland. Calder and co-workers (1973b) obtained similar results using lymphocytes from patients with Hashimoto thyroiditis and thyroglobulin-coated chicken red blood cells.

Normal human lymphocytes were found to be cytotoxic to thyroglobulin-coated chicken red blood cells which had been pre-incubated with decomplemented sera (Calder et al., 1973a, 1975). The cytotoxic activity was found to correlate with the titre of thyroglobulin tanned cell haemagglutinating antibody. Wasserman and co-workers (1974) reported similar findings, but found no correlation between cytotoxic activity and antibody titre. The cytotoxic factor in Hashimoto serum was found to be an antibody of the IgG class, detectable in both the 19S and 7S G-200 Sephadex fractions of serum (Calder et al., 1973c). It was suggested that the cytotoxic activity associated with higher molecular weight was due to the presence of antigen-antibody complexes, and that these complexes in antibody excess might be responsible for the arming of killer cells, giving them the specificity to destroy appropriate antigen-labelled target cells. Lymphoid cells from normal donors were incubated with Hashimoto serum known to contain 19S-localized cytotoxic activity and then washed thoroughly. These cells were able to destroy target cells coated with thyroglobulin, but not uncoated target cells (Calder et al., 1973a).

Circulating soluble immune complexes were demonstrated using the anti-complementary method in the sera of patients with thyroid disorders (Calder et al., 1974; Calder and Irvine, 1975). Anti-complementary activity was found to be in a higher precentage of sera from patients with Hashimoto thyroiditis than those from patients with primary hypothyroidism or thyrotoxicosis. This activity of serum samples from patients with Hashimoto thyroiditis was localized predominantly in the ascending peak of the IgG distribution profile. It was suggested that this localization was due to the presence of immune complexes and that the antigen involved might be thyroglobulin or thyroid microsomal antigen.

In the present study the detection and quantitation of soluble immune complexes in the sera of patients with thyroid disorders was carried out. The correlation between the presence and quantity of complexes and the clinical features of the patients was also studied.

1.4. IMMUNE COMPLEXES AND DIABETES MELLITUS

Considerable interest has lately been shown in the possibility that immunological processes may have an aetiological role in the pathogenesis of diabetes mellitus, and in particular the insulin dependent type which is often associated clinically and serologically with autoimmune diseases of the thyroid, the gastric mucosa and the adrenal cortex (Irvine et al., 1970; Nerup and Binder, 1973).

Pancreatic islet-cell antibodies were first described in the sera of patients with polyendocrine disease associated with insulin-treated diabetes (Bottazzo et al., 1974; MacCuish et al., 1974a). Subsequently, Lendrum and co-workers (1975) reported the presence of antibodies in newly diagnosed diabetics. These antibodies were found to be more frequently detected in newly diagnosed insulin dependent diabetics than in those studies one year after diagnosis (Lendrum et al., 1976; Irvine et al., 1977). Cell mediated hypersensitivity to pancreatic antigens has been demonstrated predominantly in patients with juvenile diabetes of short duration (Nerup et al., 1971, 1974; MacCuish et al., 1974b; Richens et al., 1976). Lymphocytic infiltration in and around the islets of Langerhans is characterisitic of this type of diabetes (Warren, 1927; LeCompte, 1958; Gepts, 1965).

The possible pathogenic significance of insulin-anti-insulin antibody complexes has been indicated by the observations of a number of investigators. The presence of anti-insulin antibody in sera of

diabetics was detected as early as 1944 when Lowell reported the presence of insulin neutralizing factor in sera of insulin-dependent resistant diabetics. Subsequently, Berson and co-workers (1956) demonstrated the presence of "insulin-transporting globulin" complexed with insulin in the blood of insulin treated subjects. The presence of anti-insulin antibodies has been confirmed by other investigators (Kalant et al., 1958; Skom and Talmage, 1958; Pav et al., 1963; Ortved Andersen, 1972). Quantitative studies of the interaction between insulin and insulin binding antibodies showed the existence of high and low affinity insulin antibodies in sera of insulin treated subjects (Berson and Yallow, 1959).

Freedman and co-workers (1960) using a fluorescence technique were able to demonstrate gammaglobulin in nodules of diabetic glomerulosclerosis. Similarly Blumenthal and co-workers (1964) demonstrated the presence of insulin and complement in the affected glomerular tissue in diabetics.

Diabetes has been produced in animals by the infusion of serum obtained from guinea-pigs immunized with insulin (Wright, 1961). Grieble (1960), Blumenthal (1964) and Ditscherlein and co-workers (1967) induced glomerulosclerosis in rabbits immunized with insulin. Similar findings were reported by Zampa and Mancini (1965) and Andreev and co-workers (1970) in guinea-pigs after immunisation with insulin.

The binding of radiolabelled insulin to vessels from subcutaneous and muscular tissues of insulin-treated diabetics has been reported (Poulsen and Werner, 1969). Werner and Larsen (1969) demonstrated the binding of fluorescein-conjugated insulin and IgG from diabetics to ocular structures from insulin-treated diabetics. Osterby (1972) demonstrated by electron-microscopy a thickening of the glomerular basement membrane 2-3 years after the beginning of insulin treatment.

Ortved Andersen (1976) reported that patients with diabetic complications, especially those with early onset of proliferative retinopathy or nephropathy, had a slightly elevated plasma insulin-binding capacity and suggested that insulin-anti-insulin antibody complexes may aggravate the vascular complications.

Soluble antigen-antibody complexes were detected only indirectly in sera from patients with insulin-resistant juvenile diabetes (Faulk et al., 1970), and in insulin treated diabetics (Jayarao et al., 1973) by measuring insulin antibody before and after acid dissociation and removal of insulin by charcoal-adsorption. The increase in antibody titre after dissociation was considered to reflect the presence of immune complexes.

Folling (1976) demonstrated the existence of different types of insulin-anti-insulin complexes with different biological properties. It was suggested that complexes which adhere to vascular walls bind and activate complement and thereby elicit inflammation.

In the present study detection and quantitation of soluble immune complexes in sera of patients with different types of diabetes was carried out. The correlation between the presence and quantity of complexes and the presence of different types of antibodies and complications was also studied.

1.5. AIM OF INVESTIGATION

The aims of this study were to investigate the possible presence of immune complexes in sera of patients with thyroid diseases or diabetes mellitus, to correlate the presence of immune complexes with the severity of disease and with the occurrence of serum autoantibodies, and to define the role of immune complexes in the development of these diseases.

In addition, attempts were made to characterize the detected complexes in terms of their antigen components and to develop a more sensitive assay for the detection of immune complexes.

1.6. METHODS USED IN THIS STUDY

When the present study was initiated there was a limited number of relatively insensitive methods available for the detection of circulating immune complexes in human sera. Of these, the most useful appeared to be the ADCC inhibition assay of Jewell and MacLennan (1973) which was based on the competitive binding of immune complexes and antibody-coated target cells (Chang liver cells) to the Fc receptors on effector cells (human peripheral blood lymphocytes).

In this laboratory, experiments were being carried out to modify and improve the sensitivity of this assay by using chicken red blood cells as target cells and human peripheral blood lymphocytes as effector cells. However, difficulties were encountered with this modification due to the variability of the peripheral blood lymphocytes obtained from different donors, and the technique was again modified by using chicken red blood cells as targets and rat spleen lymphocytes as effector cells. This assay was found to be sensitive and reproducible and was used in the first part of this study.

During the progress of the present study, two new methods appeared in the literature. In 1975, Sobel and co-workers described a C1q deviation test which was based on the competition between immune complexes and antibody-coated sheep erythrocytes for the binding of radiolabelled C1q. In the following year, Theofilopolous and co-workers described the Raji cell radioimmunoassay. This was based on the

binding of immune complexes to the complement receptors on human lymphoblastoid cells and the subsequent detection of these complexes by radiolabelled anti-IgG antibodies.

Both methods were reported to be more sensitive than the ADCC inhibition assay and experiments were carried out to apply these techniques in this study. The Raji cell radioimmunoassay differed from the ADCC inhibition and C1q deviation assays in that heat-inactivation of serum was not required, thus eliminating the possibility of aggregate formation. In addition, it also permitted the quantitation of complexes present in the sera under test. A modification of the assay was used in attempts to characterize the immune complexes detected in sera of patients with thyroid disease and to eliminate the possible influence on the sensitivity of the assay of monomeric IgG present in serum samples with high levels of IgG.

Finally, a novel sensitive and reproducible assay was developed using the binding of immune complexes to the Fc receptors on rat spleen leucocytes and the subsequent detection of these complexes by the addition of radiolabelled anti-IgG antibody. Immune complexes were also quantitated by this method.

2. MATERIALS AND METHODS

2.1. INHIBITION OF ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY (ADCC)

2.1.1. Reagents

Eagle's medium (1x strength, without glutamine) with added antibiotics (1000μ/ml penicillin and 500ng/ml streptomycin) was used as a basic diluent throughout this work. For the assay procedure, this medium was further supplemented with 10% foetal calf serum (FCS) and 2mM glutamine. The FCS was heated in a water bath at 56°C for 30 min before use in order to inactivate the complement.

2.1.2. Preparation of effector cells

Preliminary studies in this laboratory have shown that in different inbred strains of rat there may be considerable variation in the antibody-dependent cell-mediated cytotoxicity exerted by spleen cells. One of the strains, namely Liverpool Hooded (Ag-B^5 Genotype), whose spleen cells were among the more effective, was chosen for this work. The animals were obtained from a colony maintained within the animal house of the Unit of Reproductive Biology, Forrest Road, Edinburgh.

9-15 week-old male rats were killed by asphyxiation in a carbon dioxide atmosphere. The spleens were removed and gently hand-homogenized in a few ml of Eagle's medium using a glass homogenizer. The cell suspension was decanted and centrifuged at 400g for 5 min. The cells were resuspended in 10ml Eagle's medium + supplements, and incubated at 37°C for two hours in a flat-sided glass bottle to allow adherence of mononuclear phagocytes. The non-adherent cells were then removed, centrifuged and resuspended in 20ml Eagle's medium plus supplements. The leucocytes were counted, and the suspension diluted to the required concentration.

The viability of the cells was assessed by staining with Trypan Blue. Equal volumes of cells in Eagle's medium ($10x10^6$/ml) and 1% Trypan Blue in phosphate buffered saline (PBS, 0.1M pH7.2) (Appendix II) were mixed for 5 min. at room temperature. Cell viability, estimated using a haemocytometer, was generally 90-95%.

2.1.3. Preparation of target cells

Blood was obtained from the wing vein of White Leghorn chickens aged between 6 to 12 weeks. 2 to 5ml were collected on the day of use into a tube containing sufficient heparin to give a final concentration of 10u/ml. The suspension was centrifuged at 200g for 10 minutes, the plasma and buffy coat discarded, and the cells washed twice in Eagle's medium and once in PBS.

50µl of an 8% suspension of the chicken red blood cells (CRBC) in Eagle's medium were incubated with 20µl (200µCi) ^{51}Cr-labelled sodium chromate at 37°C for 2 hours. The radiolabelled cells were then washed three times with Eagle's medium, made up to 2ml in Eagle's medium plus supplements, counted, and diluted to $2x10^6$/ml.

2.1.4. Preparation of antiserum to CRBC

Antisera were raised in rabbits by a course of two intramuscular injections separated by an interval of 10 days. Each injection consisted of 1ml 10% suspension of CRBC in PBS emulsified with an equal volume of Freund's complete adjuvant. The rabbits were boosted with a further injection of CRBC in adjuvant 5 months later. They were bled from the ear vein 10 days after the second injection and again 10 days after the booster injection and the sera were stored in 100µl aliquots at -20°C. All sera were heat inactivated at 56°C for 30 minutes prior to use and then tested for potency in the antibody-dependent cell-mediated cytotoxicity assay.

2.1.5. Preparation of aggregated human IgG and formed antigen-antibody complexes

The sensitivity of the ADCC inhibition assay for the detection of complexes was investigated using aggregated human IgG and formed antigen-antibody complexes.

2.1.5.1. Aggregated human IgG

As a source of human IgG, normal human immunoglobulin was obtained from the Blood Transfusion Service, Edinburgh. This material was prepared for therapeutic use by ethanol fractionation. Its stated purity was 93% IgG. No further tests on its purity were carried out. The concentration was adjusted to 40mg/ml in PBS, and the solution heated at 63°C for 15 minutes. The suspension of aggregated IgG was used without further treatment.

2.1.5.2. Ovalbumin-antiovalbumin complexes

Varying concentrations of ovalbumin were added to neat antiovalbumin antiserum in a microprecipitation test in order to find the optimal concentration for the formation of precipitates. After precipitation the supernatants were tested for the presence of free antigens and antibody using Ouchterlony immunodiffusion. The optimal concentration of ovalbumin for the formation of a precipitate with neat antiserum was found to be 312µg/ml.

The precipitate was suspended in 0.2ml PBS; this initial concentration of formed complexes as determined by spectrophotometery was 600µg/ml. The suspension was further diluted to 60µg/ml using 1/10 heat inactivated normal human serum in Eagle's medium. Subsequent dilutions were made using Eagle's medium only.

2.1.6. Serum samples

Serum samples were obtained from patients with thyroid disorders attending the Endocrine Unit, Royal Infirmary of Edinburgh (RIE) for diagnostic investigation before treatment, or for follow-up within 5 years of diagnosis. The patients were selected after clinical examination and according to certain criteria after investigations carried out by the Endocrine Unit (radio-iodine uptake, anti-thyroid antibody titres), the clinical chemistry laboratory, RIE (total plasma thyroxine and triiodothyronine, effective thyroxin ratio, protein bound iodine (PBI)) and the Regional Hormone Laboratory, Forrest Road, Edinburgh (thyroid stimulating hormone (TSH) measurement with or without thyroid releasing hormone (TRH) stimulation as appropriate).

2.1.6.1. Hashimoto thyroiditis

Twenty-five female goiterous patients (Age 42-71, mean 56.5) were studied. Of these, one was newly diagnosed and the remainder had been diagnosed up to 10 years previously (mean 32.4 months). Nineteen had significant titres of thyroid antibodies (tanned red cell haemagglutination (TCH) titre $> 1:2500$ and/or a complement fixation (CFT) titre $> 1:32$). Three were confirmed histologically by needle or open biopsy, while the remaining three patients were diagnosed on the concurrence of goitre, hypothyroidism and low titres of anti-thyroid antibodies.

One patient was hypothyroid and another was mildly hypothyroid at the time of the study, while the remainder were euthyroid. All the patients except three were taking thyroxine for either hypothyroidism or impaired thyroid reserve or to reduce the goitre size.

2.1.6.2. Primary hypothyroidism

Twenty-nine patients (F22, M7, Age 15-83, mean 60.9) were studied. Of these, 10 were newly diagnosed and the remainder had been diagnosed up to 5 years previously (mean 19.7 months). Initially all were clinically and on investigation unequivocally hypothyroid (low level of total serum thyroxin or PBI and elevated level of TSH or no response to exogenous TSH stimulation), with no palpable goitres. All except the newly diagnosed patients were on thyroxine at the time of the study and were euthyroid apart from one who remained hypothyroid 6 months after treatment.

2.1.6.3. Thyrotoxicosis

Twenty-six patients (F23, M3, Age 14-66, mean 37) who were clinically and on investigation unequivocally thyrotoxic (elevated total serum thyroxine and/or triiodothyronine and suppressed TRH response) were studied prior to any definitive treatment. Four of these were on propranolol. Two had previously been treated with carbimazole and had subsequently relapsed, but none had undergone surgery or radio-iodine treatment. Goitres were palpable in all patients with none showing evidence of nodularity clinically or on scanning.

2.1.6.4. Treated thyrotoxicosis

Twenty-six patients (F19, M7, Age 40-81, mean 52.7) had been treated with 4-15 mCi ^{131}I for unequivocal thyrotoxicosis at least one year before study. Of these, two had required a second treatment. None were thyrotoxic at the time of sampling. One was hypothyroid and two were mildly hypothyroid and were on thyroxine treatment. The rest were euthyroid and on no treatment.

2.1.6.5. Controls

Serum samples from age- and sex-matched controls were obtained for each patient on the day of sampling. The controls were unrelated friends of patients, hospital employees, voluntary workers and hospital patients with illness not considered to be associated with circulating immune complexes. None had a history of thyroid or other autoimmune disease.

The sera were stored in 250µl aliquots at -20°C.

2.1.7. Serology

2.1.7.1. Autoantibodies

All sera were routinely tested in the Endocrine Unit for anti-thyroid antibodies by indirect immunofluorescence, using rabbit anti-human immunoglobulin conjugated with fluorescein isothiocyanate and frozen sections of human thyroid gland, and for anti-thyroglobulin antibodies by the tanned red cell agglutination test (TCH), using purified thyroglobulin. All sera found positive for anti-thyroid antibodies were also tested for the presence of anti-microsomal antibodies by the complement fixation test (CFT), using thyroid extract. In addition, all sera were examined for the presence of anti-gastric parietal cell antibodies by indirect immunofluorescence, using frozen sections of rat stomach.

2.1.7.2. Estimation of serum IgG levels

Serum concentrations of IgG were determined in all thyroid patients and controls using commercial radial immunodiffusion plates. The tested sera and the known control serum were diluted 1 in 5 with physiological saline. For each plate the well positions of the three

reference sera, the known control serum and each test sample were recorded. 5µl of each sample was applied to the appropriate well using a disposable glass capillary tube. The plates were left undisturbed at room temperature but covered until the serum had diffused into the gel, and then incubated upside down at room temperature for 20 hours. The diameter of precipitate formed around each well was measured using a scale rule.

Data for the three reference sera were plotted on semilog graph paper against concentration of IgG in mg/100ml and the test sera and the known control serum were quantitated by relating the ring diameter measurements to IgG concentration on the curve constructed from the reference sera data. 1/10 instead of 1/5 dilution was used for sera giving diameters greater than the highest reference sample.

2.1.8. Fractionation of serum samples

Eight serum samples (3 Hashimoto, 1 hypothyroid and their matched controls) were fractionated on Sephadex G-200.

2.1.8.1. Preparation of the gel

30gm of Sephadex G-200 was added to 400ml PBS, and the gel slurry maintained in a boiling water bath for 5 hours. The gel was deaerated by water vacuum and poured into a 2.5x82cm column, and the gel bed was packed by passing buffer through the column.

2.1.8.2. Sample preparation and application

Serum samples were dialysed against two changes of PBS at $4^{o}C$. 2ml of dialysed serum was applied to the top of the gel. When the sample had entered the gel, buffer was pumped through the column at a flow rate of 20ml/hour using a peristaltic pump. The eluate was

collected in 10ml volumes using an Ultrarac fraction collector. The optical density of the column effluent was estimated using a Uvicord spectrophotometer reading at 254nm in order to locate the protein peaks.

2.1.9. Assay procedure

The ADCC inhibition assay used was a modification of that of MacLennan (1972). Incubations were set up in triplicate in LP3 tubes and the mean of the results taken.

Effector cells were adjusted to $10x10^6$/ml in supplemented Eagle's medium, giving an effector to target cell ratio of 50:1. 200µl of each serum sample were heat inactivated for 30 minutes at 56°C, and medium added to make 1/10, 1/20, 1/40 and 1/80 dilutions. For each dilution of serum tested, two sets of tubes containing 200µl of the spleen cell suspension and 100µl of diluted serum were set up. In addition, two sets of tubes containing 200µl of the spleen cell suspension and 100µl of medium were prepared. All tubes were incubated at 37°C for 2 hours in an air-5% CO_2 atmosphere.

At the end of this period, 20µl of radiolabelled CRBC were added to each tube. 100µl of appropriately diluted anti-CRBC antiserum were then added to one set of tubes, while the remaining tubes received 100µl of medium, and all tubes were incubated at 37°C for 16 hours in an air-5% CO_2 atmosphere.

The tubes were then centrifuged at 200g for 10 minutes. 200µl of supernatant were removed and the radioactivity estimated using a well-type automatic gamma-counter. Total activity was estimated by counting aliquots of labelled cells. In a few cases where release in the presence of serum and absence of antibody exceeded that in the absence of serum and absence of antibody, indicating natural anti-CRBC

antibodies, the sample and control sera were absorbed with an equal volume of packed CRBC for 1 hour at $4^{o}C$, and reassayed.

The percentage inhibition was expressed according to the following calculations:

$$\text{i) \% release} = \frac{\text{release by effector cells x 2}}{\text{total activity}} \times 100$$

$$\text{ii) \% inhibition} = \frac{\text{(\% release in absence of serum sample) - (\% release in presence of serum sample)}}{\text{\% release in absence of serum sample}} \times 100$$

Release of label in the absence of antibody was less than 5%.

2.2. RADIOIMMUNOASSAYS

2.2.1. Reagents

Eagle's medium (1x strength, without glutamine) with added antibiotics (1000μ/ml penicillin and 500ng/ml streptomycin) was used as a diluent in all radioimmunoassays. For the maintenance of Raji cell cultures, this medium was supplemented with 20% heat-inactivated FCS, 4mM glutamine and 10% tryptose phosphate broth.

2.2.2. Preparation of antisera

Goat antiserum to human IgG was obtained commercially. Rabbit antiserum to human thyroglobulin was prepared by repeated subcutaneous and intramuscular injections of 1ml of human thyroglobulin (10mg/ml) in PBS emulsified with an equal volume of Freund's complete adjuvant. The rabbits were bled 10 days after the second injection and the sera tested for antithyroglobulin antibodies by Ouchterlony immunodiffusion.

Immunoglobulin containing fractions from both antisera were prepared by precipitation with 50% saturated ammonium sulphate solution.

The precipitates were dissolved in normal saline, and the solutions dialysed against several changes of phosphate buffer (pH7, 0.1M). The protein concentration was adjusted to 1mg/ml using phosphate buffer.

2.2.3. Fractionation of goat anti-human IgG antiserum on DEAE cellulose

2.2.3.1. Preparation of the column

DE52 cellulose was washed with 0.5M HCl for 1 hour, several changes of distilled water, 0.5M NaOH for 1 hour, several changes of distilled water, and finally several changes of phosphate buffer (0.5M, pH8). The gel was then equilibrated in phosphate buffer (0.01M, pH8) and the slurry poured into a 4x30cm column.

2.2.3.2. Preparation of antiserum

2ml of goat anti-human IgG antiserum was dialysed overnight at $4^{o}C$ against several changes of phosphate buffer (0.01M, pH8). The dialysed serum was applied to the top of the column which was then connected to an ionic gradient generator.

2.2.3.3. Ionic gradient separation of IgG

IgG immunoglobulins were separated from other immunoglobulins and from other serum proteins using an ionic strength gradient. A simple exponential gradient was prepared (Fahey & Terry, 1973) using 0.5M NaCl in phosphate buffer (0.01M, pH8) in the reservoir, and buffer only in the mixing chamber. The effluent from the mixing chamber was pumped to the top of the column. Serum and buffer were then passed through the column at a flow rate of 4ml/hour by the action of a peristaltic pump, and the eluent collected in 2ml volumes using an

Ultrarac fraction collector. The optical density of the column effluent was estimated using a Uvicord spectrophotometer reading at 254nm in order to locate the protein peaks. The anti-human IgG activity of each fraction was estimated by Ouchterlony immunodiffusion. The fractions in each protein peak were bulked and then concentrated using an Amicon model 12 cell and Diaflo ultrafilters UM 20E (10,000-20,000 MW). Each concentrate was then again tested for anti IgG activity as above.

2.2.4. Aggregation of IgG

Purified human IgG was aggregated as described in 2.1.5.1.

2.2.5. Radioiodination of proteins

Iodination of C1q and of the immunoglobulin fractions of goat anti-human IgG and rabbit anti-human thyroglobulin was carried out using a modification of the method of McConahy and Dixon (1966).

2.2.5.1. Preparation of column

One gm Sephadex G-50 was added to 40ml of phosphate buffer (0.1M, pH7), and the gel slurry maintained in a boiling water bath for 3 hours. The gel was poured into a $\frac{1}{2}$x30cm column and the gel bed was packed by passing buffer through the column. 30ml of 1% bovine serum albumin (BSA) in phosphate buffer were allowed to run through the column in order to reduce non specific binding of iodinated protein to the gel.

2.2.5.2. Iodination procedure

One mg protein in 1ml phosphate buffer was labelled with 1mCi ^{125}I. After adding 100µl of 200µg/ml chloramine T, the solution

was mixed on a magnetic stirrer for 5 minutes at room temperature. The reaction was stopped by the addition of 100µl of 200µg/ml sodium metabisulphite.

The solution was applied to the top of the column by pasteur pipette, and 1ml fractions were collected by adding more 1% BSA in phosphate buffer to the top of the column. The fractions containing the radioactivity were identified using a Scaler-Ratemeter SR3. Labelled proteins usually appeared in the fifth and sixth fractions. Free iodine appeared mostly in the ninth and tenth fractions. The labelled proteins were stored in 200µl aliquots at -20°.

2.2.6. Collection and preparation of serum samples

2.2.6.1. Serum samples from patients with thyroid disorders

Sera from patients with thyroid disorders were collected according to the criteria used in 2.1.6.

Twenty three female goiterous patients (Age 18-76, mean 55 years) with Hashimoto thyroiditis were studied. Of these, two were newly diagnosed and the remainder had been diagnosed up to 15 years previously (mean 51 months). Thirteen had significant titres of thyroid antibodies. Eight were confirmed histologically by needle or open biopsy, while the remaining two were disgnosed on the concurrence of goitre, hypothyroidism and low titres of anti-thyroid antibodies.

Three patients were clinically hypothyroid at the time of study; two of these were newly diagnosed and had not yet been started on treatment while the other had disabling angina and thyroxine was discontinued. The remaining patients were euthyroid and all except one were taking thyroxine for either hypothyroidism or impaired thyroid reserve or to reduce the goitre size.

Twenty-eight patients (F26, M2, Age 37-78, mean 61.7 years) with primary hypothyroidism were studied. Of these, 6 were newly diagnosed and the remainder had been diagnosed up to 8 years previously (mean 41.4 months). Initially all were clinically and on investigation unequivocally hypothyroid with no palpable goitres. All except the newly diagnosed patients were on thyroxine at the time of study and were euthyroid except one who remained hypothyroid 3 months after treatment.

Twenty patients (F16, M4, Age 21-68, mean 40.1 years) who were clinically and biochemically and on investigation unequivocally thyrotoxic were studied prior to any definitive treatment. Ten were taking propranolol at the time of study. Nine had recurrent thyrotoxicosis after carbimazole treatment 1 to 11 years previously. Goitres were palpable in 18 patients with none showing evidence of nodularity clinically or on scanning.

The patients used in this study were not the patients used in the previous study (ADCC inhibition assay).

2.2.6.2. Serum samples from diabetics

Serum samples were collected from patients attending the diabetic outpatients department, Royal Infirmary, Edinburgh.

Thirteen newly diagnosed juvenile insulin dependent diabetics (F4, M9, Age 13-30, mean 20.8 years) were studied. These had not been treated with insulin or had been receiving insulin for only a short period (from a few hours to a few days).

Thirty-two patients (F15, M17, Age 16-83, mean 43 years) with insulin dependent diabetes who had been treated with insulin for periods ranging from 1 month to 30 years were studied. Thirty-one diabetics (F21, M10, Age 15-80, mean 59.9 years) treated with oral hypoglycemic agents (OHA) who were either newly diagnosed or had been

receiving treatment for periods ranging from a few days to 21 years, and twenty one diabetics (F11, M10, Age 37-82, mean 60.5 years) receiving no treatment other than restrictions on diet and who were either newly diagnosed or had been diagnosed up to 25 years previously were also studied.

Serum samples were obtained from age- and sex-matched controls as described in 2.1.6. All sera were stored in 500µl aliquots at -40°C.

2.2.7. Serology

2.2.7.1. Sera from patients with thyroid disorders

All sera obtained from patients with thyroid disorders were tested as described in 2.1.7.1.

2.2.7.2. Diabetic sera

i. Anti-islet cell antibodies

All sera were tested by the Endocrine Unit, RIE, for anti-islet cell antibodies by indirect immunofluorescence, using fresh post mortem snap-frozen pancreatic tissue (blood group 0). Sera that were positive when tested neat were titrated using doubling dilutions.

ii. Anti-viral antibodies

The sera of the thirteen newly diagnosed insulin-dependent diabetics and their age- and sex-matched controls were screened and titrated for antibodies to Coxsackie viruses B1-5 by a metabolic inhibition test, to Epstein Barr virus by an indirect immunofluorescence test, and to Rubella virus using a haemagglutination inhibition test. Sera were screened and titrated for complement fixing antibody to the following antigens - Influenza A, B and C, Parainfluenza type 1, Measles, Mumps S and V, Respiratory Syncytial virus, Adenovirus, Cytomegalovirus,

Varicella zoster, Herpes simplex, Coxiella burneti phase 2, Chlymydia Group B and Mycoplasma pneumoniae. These tests were all carried out by the Virology Department, City Hospital, Edinburgh.

iii. Anti-insulin antibodies

Antibodies to insulin were estimated in all diabetic sera by a minor modification of the method of Ortved Andersen and colleagues (1972), according to Mustaffa and co-workers (1977). Insulin binding of 10μU/ml serum was regarded as indicative of the presence of anti-insulin antibodies. Insulin binding between 10-300μU/ml serum was regarded as indicating moderate anti-insulin antibody titre, while binding of > 300μU/ml was interpreted as binding due to high titres of anti-insulin antibodies. This investigation was carried out in the Middlesex Hospital, London.

2.2.8. C1q deviation test (modified from Sobel et al., 1975)

2.2.8.1. Preparation of sensitized sheep erythrocytes (SRBC)

Blood was collected from the jugular vein of a male sheep, heparinised (Evan's 10u/ml) and centrifuged at 200g for 10 minutes. The plasma and buffy coat were discarded and the cells washed 3 times in PBS. The cell concentration was adjusted to 20×10^9 per ml in PBS. 1/100 rabbit anti-SRBC antiserum in PBS was added in equal volume to SRBC in PBS, and incubated at 37°C for 30 minutes. After washing in Eagle's medium, the sensitized cells were resuspended in Eagle's medium.

2.2.8.2. Preparation of radiolabelled C1q

C1q was kindly donated by Dr. K. Reid, Biochemistry Department, Oxford University. The concentration was adjusted to 1mg/ml in phosphate buffer (o.1M, pH7) and the C1q radiolabelled with ^{125}I as described in 2.2.5.

2.2.8.3. Sensitivity of C1q deviation test

Aggregated human IgG was added at various concentrations (2µg/ml-2mg/ml) to fresh normal human serum. 50µl of serum alone or serum containing aggregates were added to 100µl saline and 100µl sucrose veronal buffer and mixed in LP3 tubes. The mixture was heat inactivated at 56°C for 30 minutes. 10µl of 1% BSA in phosphate buffer (0.1M, pH7) containing 1µg radiolabelled C1q was added, and the mixture was incubated for 15 minutes at room temperature. Sensitized SRBC ($4x10^8$) were added and the suspension incubated for 15 minutes at room temperature. After centrifugation at 400g for 5 minutes the radioactivity in the supernatents and in the pellets was determined using an automatic gamma counter (Wallac).

The percentage of inhibition of binding of radiolabelled C1q to the pellet was calculated as follows:

$$\text{i Calculation of uptake} = \frac{\text{cpm pellet}}{\text{cpm pellet + cpm supernate}} \times 100$$

$$\text{ii Calculation of inhibition} = \frac{\text{\% uptake (serum alone) - \% uptake (serum + aggregates)}}{\text{\% uptake (serum alone)}} \times 100$$

2.2.9. Raji cell radioimmunoassay

2.2.9.1. Preparation of cells

The first sample of cultured Raji cells was kindly donated by Miss Judith Evans from Dr. C.M. Steel's laboratories in the MRC Clinical & Population Cytogenetics Unit, Western General Hospital, Edinburgh.

Cells were cultured at 37°C in glass bottles in supplemented Eagle's medium (see 2.2.1.). The cells were subcultured at 2-7 day intervals by dividing the contents of each glass bottle into two and reconstituting to the original volume with culture medium.

2.2.9.2. Assay procedure

All sera were tested in duplicate. $2x10^6$ Raji cells in 50µl Eagle's medium were incubated in LP3 tubes with 25µl of a 1:4 dilution of the test serum in saline.

After 45 minutes incubation at 37°C, the cells were washed 3 times in Eagle's medium and incubated at 4°C for 30 minutes with approximately 15µg of ^{125}I goat anti-human IgG in 30µl 1% BSA in phosphate buffer (0.1M, pH7). Unbound ^{125}I-antiserum was removed by 3 washes with 1% BSA in PBS, and the radioactivity in the cell pellet was counted in an automatic Gamma-counter.

The amount of uptake was referred to a standard curve of radioactive antibody uptake by cells incubated with 25µl of 1:4 dilution of pooled normal human serum, to which varying amounts (from 50ng to 25µg) of aggregated IgG had been added. The amount of complexes in each serum tested was expressed as µg aggregated IgG equivalents per ml of serum (µg eq.AggIgG).

2.2.9.3. Detection of immune complexes after blocking Fc receptors on Raji cells

$2x10^6$ Raji cells were incubated with 50µl of normal rabbit serum (NRS) at 37°C for 1 hour. The cells were washed with Eagle's medium 3 times and incubated at 37°C for 45 minutes with 25µl of 1:4 dilutions of Hashimoto and control sera. The cells were then washed with Eagle's medium and incubated with 15µg of ^{125}I goat anti-human

IgG antibody in 30µl 1% BSA in PBS in phosphate buffer (0.1M, pH7). The cells were washed with 1% BSA in PBS and the amount of radiolabelled antibody bound to the cells was counted. The amount of complexes in each serum sample was estimated by referring to a standard curve of radiolabel uptake by cells preincubated with NRS and incubated with varying amounts of aggregated IgG.

2.2.9.4. Characterization of immune complexes

25µl of 1:4 dilution of sera from thyroid patients which were found positive for the presence of immune complexes and their controls were incubated with $2x10^6$ Raji cells at 37^oC for 45 minutes. The cells were washed 3 times with Eagle's medium and incubated with 15µg of ^{125}I rabbit anti-human thyroglobulin antibody in 30µl 1% BSA in phosphate buffer (0.1M, pH7) at 4^oC for 30 minutes. The cells were washed 3 times with 1% BSA in PBS and the label bound to the cell pellet was counted. The difference in the count between patients' sera and their controls was estimated.

2.2.10. Rat spleen leucocyte radioimmunoassay

2.2.10.1. Preparation of rat spleen leucocytes (RSL)

9-15 week old male rats (Liverpool Hooded) were killed by asphyxiation in a carbon dioxide atmosphere. The spleens were removed and gently hand-homogenised in a small volume of Eagle's medium.

The cell suspension was decanted and centrifuged at 400g for 5 minutes. The cells were resuspended in Eagle's medium and left undisturbed for 10 minutes to allow coarse debris to settle. The supernatatant was centrifuged at 400g for 5 minutes and the cells were

resuspended in 10ml Eagle's medium. The leucocytes were counted and the suspension adjusted to the required concentration.

2.2.10.2. Preliminary observations

In order to detect and quantitate immune complexes in serum using the RSL radioimmunoassay, it was necessary to construct a standard curve of radiolabelled uptake by RSL incubated with varying amounts of aggregated human IgG. $4x10^6$ RSL in 100µl Eagle's medium were incubated for 1 hour at 37°C with 50µl NHS or medium to which varying amounts (2µg-4mg/ml) of aggregated IgG had been added. The cells were washed 3 times with Eagle's medium and incubated for 30 minutes at 4°C with 30µl 1% BSA in phosphate buffer (0.1M, pH7) containing approximately 15µg ^{125}I anti-human IgG antiserum. The cells were then washed with 1% BSA in PBS and the radioactivity bound to cell pellet was determined.

When RSL were incubated with aggregated IgG in fresh normal human serum, the degree of binding was found to be extremely low. Dilution of the serum resulted in an increase in binding for a given amount of aggregated IgG. The possibility that complement activity was responsible for these observations was investigated using chelating agents to inactivate the complement by complexing with Ca^{++} and/or Mg^{++} ions. The classical pathway of complement requires the presence of both ion species, while the alternative pathway requires only Mg^{++} ions.

Ethylenediamine tetra-acetic acid (EDTA), which complexes with Mg^{++} and Ca^{++}, was used to inactivate complement by interfering with both the classical and alternative pathways, while ethyleneglycol bis-(aminoethyl) tetra-acetic acid (EGTA), which binds to Ca^{++} and not to Mg^{++}, was used to block the classical pathway only.

Serum samples from 2 normal subjects were dialysed at 4°C against 2 changes of complement fixing diluent buffer (CFD) (appendix II), CFD containing 20mM EDTA and CFD containing 20mM EGTA. Aggregated IgG was added to the dialysed sera to a concentration of 256 or 64µg/ml, and assayed for binding to RSL.

In addition, the incubation of RSL with NHS resulted in agglutination of erythrocytes present in the spleen cell preparation. Accordingly, experiments were carried out to detect the presence of anti-rat erythrocyte antibodies in human serum and to determine the effect of these antibodies on the RSL radioimmunoassay. Serum samples were obtained from 40 normal subjects, of whom 20 were blood donors attending the Blood Donor Centre, Royal Infirmary, Edinburgh. The remainder were hospital employees and laboratory workers. 5ml of blood was aspirated from the vena cava of a rat, heparinized (10u/ml) and centrifuged at 300g for 10 minutes. The plasma and buffy coat were discarded and the cells were washed 3 times in PBS. Each serum sample was tested for the presence of anti-rat erythrocyte antibodies by incubating 20µl neat or serially diluted (1:2 to 1:256) serum with 20µl 2% rat erythrocytes in PBS at room temperature overnight. Known amounts of aggregated IgG were added to NHS samples before and after absorption of the sera with an equal volume of packed rat erythrocytes and the sera tested in the RSL assay.

The results of these preliminary experiments indicated the necessity of removing complement activity and anti-rat erythrocyte antibodies before testing sera for the presence of complexes. Accordingly all sera tested in the RSL radioimmunoassay were treated with EDTA and absorbed with packed rat erythrocytes before use.

2.2.10.3. Assay procedure for the detection of soluble complexes

$4x10^6$ RSL in 100µl Eagle's medium were incubated for 1 hour at 37°C with 50µl of test serum previously treated by the addition of 1 volume 100mM EDTA to 9 volumes serum and absorbed with packed rat erythrocytes.

The cells were washed 3 times in Eagle's medium and incubated for 30 min at 4°C with 30µl 1% BSA in phosphate buffer containing approximately 15µg ^{125}I anti-human IgG antiserum. The radioactivity bound to the cell pellet was referred to a standard curve of radioactive antibody uptake by RSL incubated with 50µl inactivated and absorbed pooled NHS to which varying amounts (from 2µg-4mg/ml) of aggregated IgG had been added.

2.2.10.4. Detection of anti-rat antibody in human sera absorbed with rat erythrocytes

In order to determine whether one absorption of human sera with rat erythrocytes was sufficient to remove anti-rat antibody activity, the sera were tested for binding to RSL and to thymocytes after one or two absorptions with rat erythrocytes or one absorption with rat erythrocytes and one with thymocytes. Rat thymocytes were prepared by hand homogenising the thymus in a few ml of Eagle's medium using a glass homogenizer.

After absorption with equal volumes of rat erythrocytes and/or thymocytes, the sera were assayed for binding to RSL or thymocytes. 50µl of serum were incubated for 1 hour at 37°C with $4x10^6$ RSL or $4x10^6$ thymocytes. The cells were then washed three times with Eagle's medium and incubated for 30 min at 4°C with 15µg of radiolabelled anti-human IgG antibody in 30µl of 1% BSA in phosphate buffer (0.1M, pH7). The cells were washed 3 times with 1% BSA in PBS and the activity of each cell pellet was determined.

In a further experiment to confirm that the binding of absorbed positive test sera to RSL was due to the presence of complexes and not to residual anti-rat antibodies, the RSL were pretreated with aggregated rat IgG in order to block the Fc receptors. The cells were incubated for 1 hour at 37°C with 50µl of rat serum which had been aggregated by heating at 63°C for 15 minutes, and then washed 3 times with Eagle's medium. 50µl volumes of sera found positive for complexes in the RSL assay and of their corresponding controls were incubated for 1 hour at 37°C with $4x10^6$ of the pretreated cells, or with $4x10^6$ untreated cells. After incubating with radiolabelled anti-human IgG antibody the activity of the cells were determined as described above.

2.2.10.5. Effect of heat inactivation

The amount of aggregate formed by heat inactivation of human serum was assayed using serum samples obtained from 20 normal donors (F11, M9, Age 21-59, mean 37.5). Each serum sample was treated with 10mM EDTA and absorbed with rat erythrocytes and divided into 2 aliquots, one of which was heated for 30 minutes at 56°C. Each aliquot was then assayed in duplicate for the presence of complexes as described in 2.2.10.3.

2.3. STATISTICAL ANALYSES

For the analysis of the data obtained using the ADCC inhibition assay (percentage inhibition, IgG levels), Student's t test was used. The incidences of immune complexes detected in sera from patients and controls using the Raji cell radioimmunoassay were compared using the Chi-squared test. The association between the presence of immune complexes and anti-islet cell antibodies or anti-insulin antibodies in

the sera of diabetics was determined using Fisher's exact test and the Chi-squared test respectively. The Chi-squared test was also used to compare the incidence of late diabetic complications in insulin-treated diabetics found positive or negative for the presence of circulating immune complexes. The prevalence of anti-Coxsackie B4 antibodies in patients and controls were compared using the McNemar Test. Differences among the counts obtained in the RSL assay using serum absorbed once or twice with rat erythrocytes and/or rat thymocytes were compared using Student's t test. The Wilcoxon Rank Sum Test was used to compare the quantity of complexes detected in Hashimoto sera using Raji cells untreated or pre-treated with normal rabbit serum.


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3. RESULTS

3.1. INHIBITION OF ADCC

3.1.1. ADCC mediated by rat spleen cells

Rat spleen cells were shown to have considerable ADCC activity. The spleens from each of 6 rats were homogenized and the cells incubated on glass at $37^{o}C$ for 2 hours. The concentration of cells in each suspension was adjusted to $20x10^{6}$/ml. 20µl of $2x10^{6}$/ml Cr^{51}-labelled CRBC were incubated with 200µl rat spleen cell suspension at five different dilutions in order to obtain effector cell/target cell ratios of 6.25:1, 12.5:1, 25:1, 50:1 and 100:1. Tubes were set up in two sets of triplicates.

100µl anti-CRBC antiserum at concentrations of $2x10^{-3}$ and $4x10^{-3}$ were added to each set of triplicates. The cytotoxicity mediated by the effector cells in each tube was calculated according to the following formula:

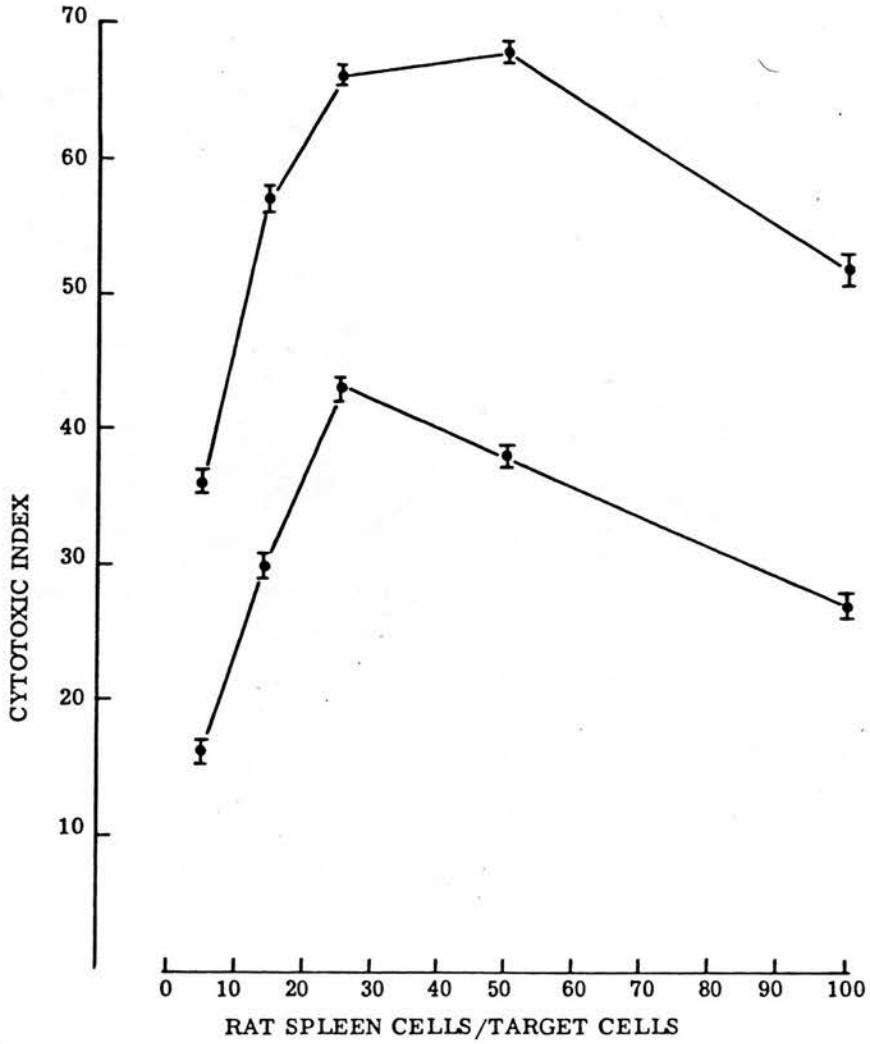
$$\text{cytotoxic index} = \frac{Cr^{51} \text{ release by effector cells}}{\text{maximum release}} \text{ X } 100$$

The maximum release of Cr^{51} from labelled target cells was obtained by lysis with distilled water. The cytotoxic index for each effector cell/target cell ratio at each of the two concentrations of anti-CRBC antiserum is shown in Figure 1. With increasing numbers of effector cells, the degree of cytolysis increased, reached a maximum and then declined. In further studies, effector/target cell ratios of 20:1 and 50:1 were used.

3.1.2. The effect of antibody concentration on cytotoxicity

Rat spleen cells and target cells at ratios of 20:1 and 50:1 were incubated with various dilutions of anti-CRBC antiserum. The

Fig. 1 The cytotoxic indices at various effector/target cell ratios using two different concentrations of anti-CRBC antiserum. Each point represents the mean and standard error obtained using spleen cells from six individual rats. Upper line: antibody concentration of 4×10^{-3}; lower line: antibody concentration of 2×10^{-3}.



cytotoxic indices obtained are shown in Figure 2. At low antiserum concentrations (10^{-4}, $2x10^{-4}$) the degree of cytotoxicity was negligible; increasing the antiserum concentration resulted in a linear increase in the cytotoxic index to a maximum of 75% (50:1) and 65% (20:1) at a concentration of 10^{-2}.

On the basis of these results, the conditions initially chosen for the inhibition of ADCC were as follows: 20:1 effector/target cell ratio, $2x10^{-3}$ antibody concentration. This gave a cytotoxic index of 45%.

3.1.3. Sensitivity of the inhibition assay

Aggregated and preformed complexes were used to investigate the sensitivity of the ADCC inhibition assay.

3.1.3.1. Sensitivity of the assay to ovalbumin-antiovalbumin complexes

The precipitate formed by the interaction of 312µg/ml ovalbumin and neat antiserum was dissolved in 0.2ml PBS and 50µl of this solution was diluted by PBS to 2ml. The protein concentration of the solution was estimated using a Zeiss spectrophotometer at 280nm. The solution was diluted to 600µg/ml using 1/10 heat inactivated serum. 100µl of this solution and of 1/2, 1/4, 1/8 dilutions of this solution were added in triplicate to 200µl of $10x10^6$ rat spleen cells and the suspensions incubated at 37°C for 2 hours in an atmosphere of 5% CO_2 in air. After incubation with 100µl $4x10^{-3}$ anti-CRBC antiserum and 20µl $2x10^6$ Cr^{51}-labelled target cells for 18 hours, the degree of inhibition of isotope release was calculated as described in 2.1.9. As shown in Figure 3, there was a linear increase in the percentage inhibition with increasing amounts of antigen-antibody complexes.

Fig. 2 The cytotoxic indices at two effector/target cell ratios using various dilutions of anti-CRBC antiserum. Each point represents the mean of two experiments.

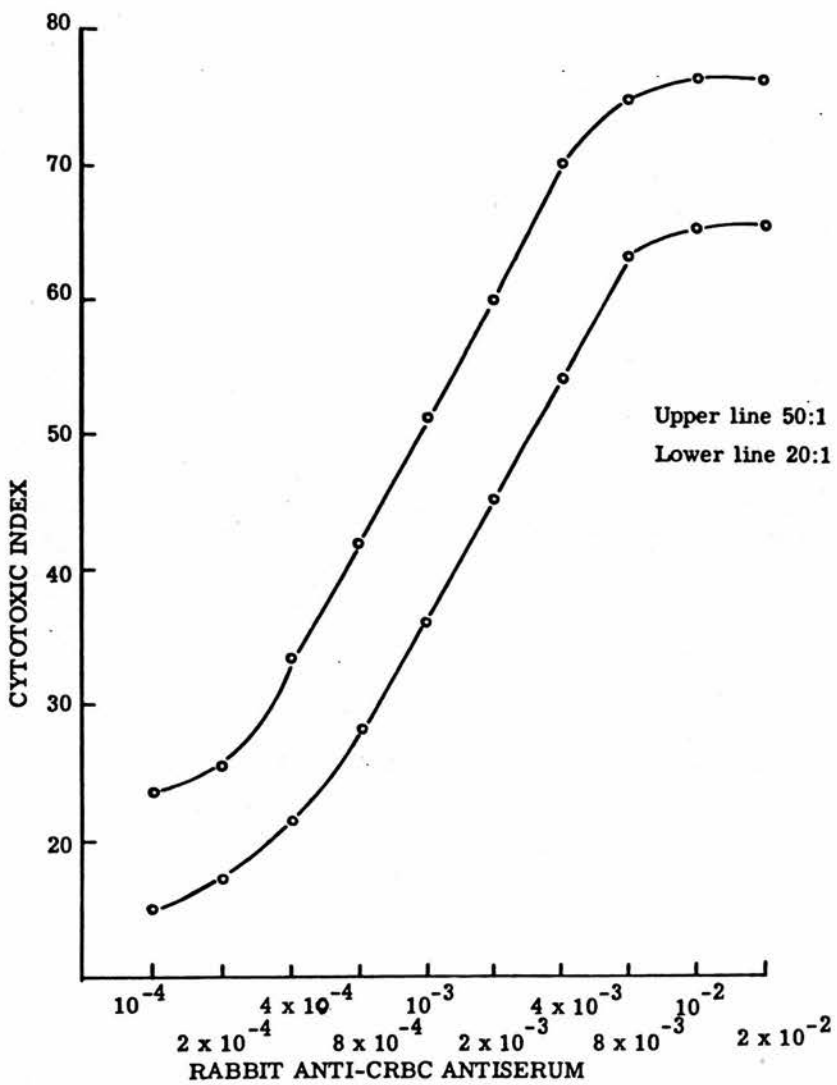
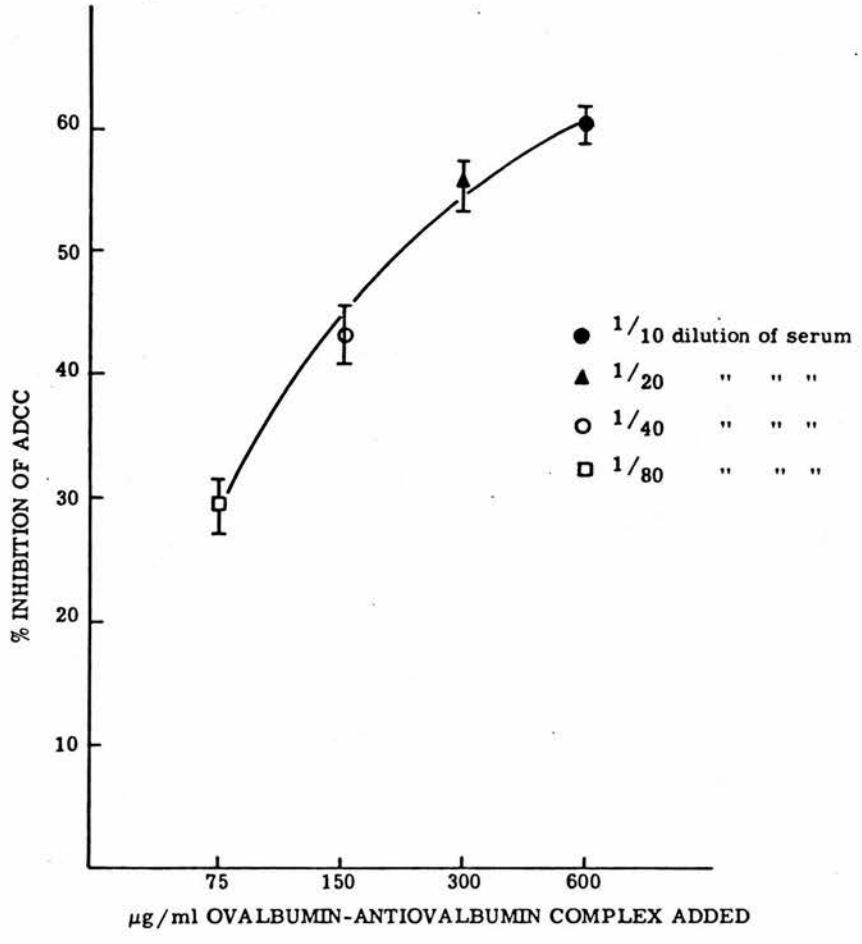


Fig. 3 Percentage inhibition of ADCC by ovalbumin -antiovalbumin complexes. Each point represents the mean and standard error of five experiments using 600µg/ml added complexes in 1/10 heat-inactivated normal human serum and serial dilutions in medium.



3.1.3.2. Sensitivity of the assay to aggregated IgG

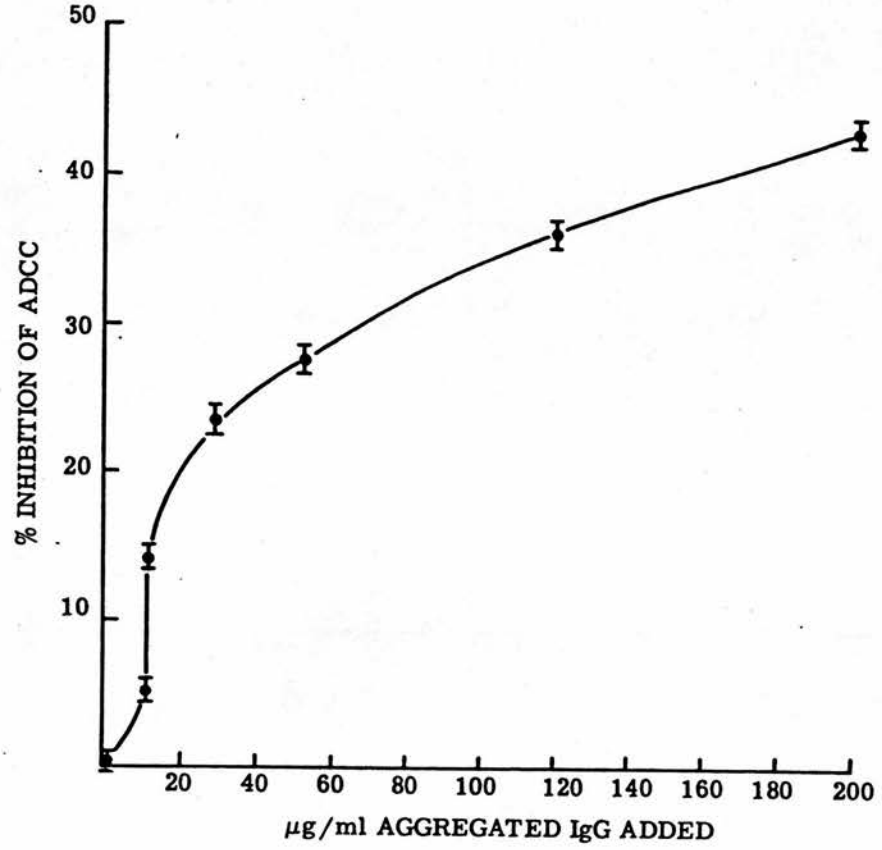
Heat-inactivated normal human serum was diluted with Eagle's medium to 1/10, 1/20, 1/40 and 1/80. Aggregated IgG was added to each of these serum dilutions and to medium alone, to give concentrations of 100, 50, 25, 12.5, 6.25 and 3.125µg/ml. 100µl of medium and serum containing aggregates were added in triplicate to 200µl $10x10^6$/ml rat spleen cells. After incubation at 37°C for 2 hours in an atmosphere of 5% CO_2 in air, anti-CRBC antiserum and target cells were added as described in 3.1.3.1.

The mean percentage inhibition in each triplicate was calculated and the values obtained in 6 experiments using various concentrations of aggregated IgG in medium alone are shown in Figure 4. With increasing amounts of aggregated IgG there was a linear increase in the degree of inhibition of ADCC. The mean percentage inhibition obtained in 6 experiments using various concentrations of aggregated IgG in different dilutions of heat-inactivated normal human serum is shown in Figure 5. The degree of inhibition increased with increasing amounts of aggregated IgG at all dilutions of serum. The limit of detection was approximately 25µg/ml of added aggregated IgG, or a final concentration of 6.25µg/ml. The degree of inhibition of ADCC at low concentrations of serum was higher than with other dilutions of serum. This appears to be due to the fact that a 1/10 dilution of normal serum itself significantly inhibited ADCC. This inhibition was greatest when using a 20:1 cell ratio and antiserum dilution of $2x10^{-3}$ (Figure 6). The sensitivity of the assay was therefore approximately 250 µg/ml in neat serum.

3.1.4. Inhibition of ADCC by sera from patients with thyroid disorders

Sera from twenty five patients with Hashimoto thyroiditis, twenty nine patients with primary hypothyroidism, twenty six patients with

Fig. 4 Percentage inhibition of ADCC by various concentrations of aggregated IgG. Each point represents the mean and standard error for six experiments. The effector/target cell ratio was 50:1, and the antibody concentration was 4×10^{-3}.


% INHIBITION OF ADCC
50
40
30
20
10
20
40
60
80
100
120
140
160
180
200
μg/ml AGGREGATED IgG ADDED

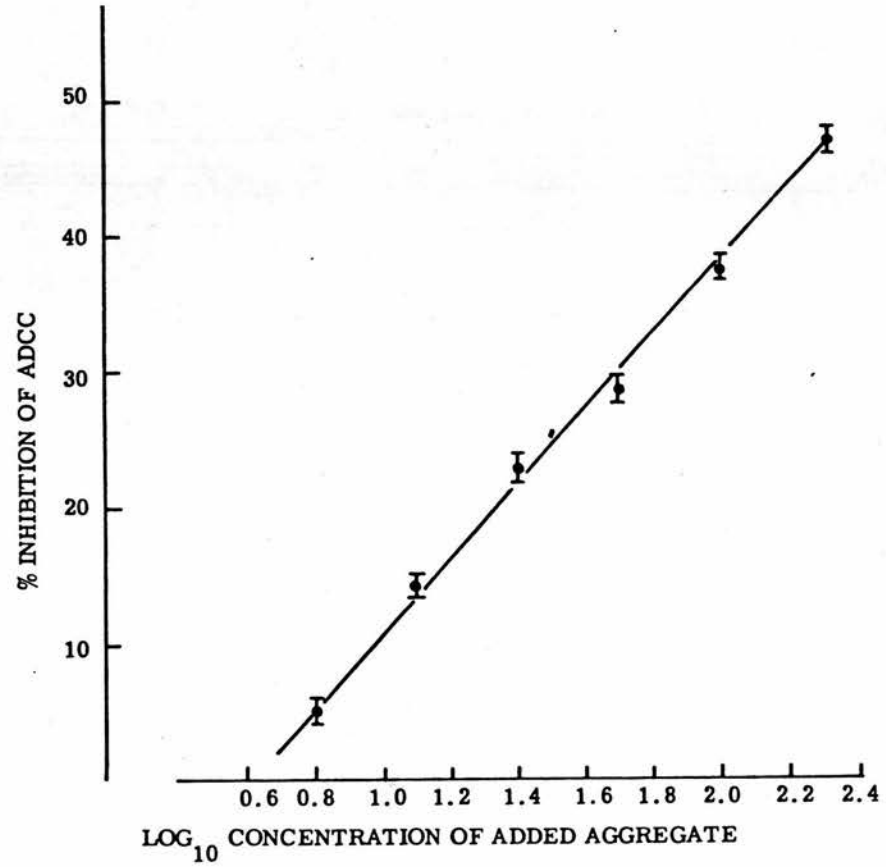

% INHIBITION OF ADCC
50
40
30
20
10
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
LOG10 CONCENTRATION OF ADDED AGGREGATE

Fig. 5 Percentage inhibition of ADCC by various concentrations of aggregated IgG in different dilutions of serum. Aggregates were added to 1/10, 1/20, 1/40 and 1/80 dilutions of heat-inactivated normal human serum.

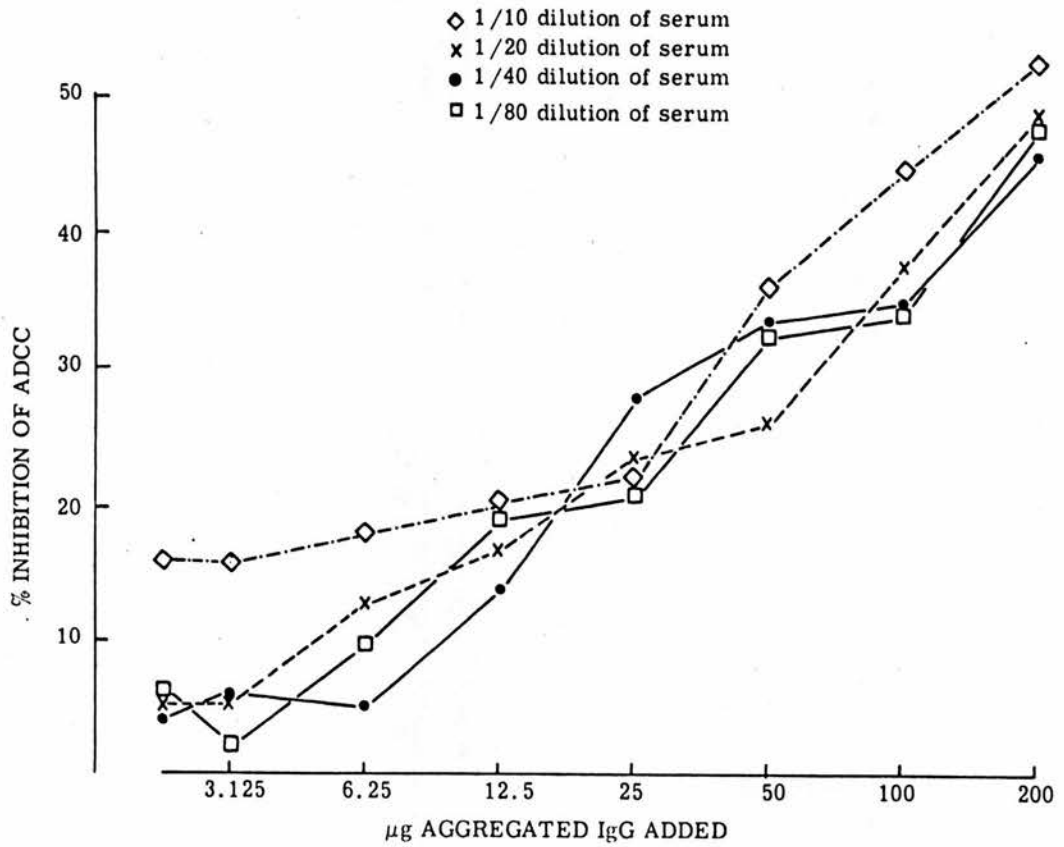
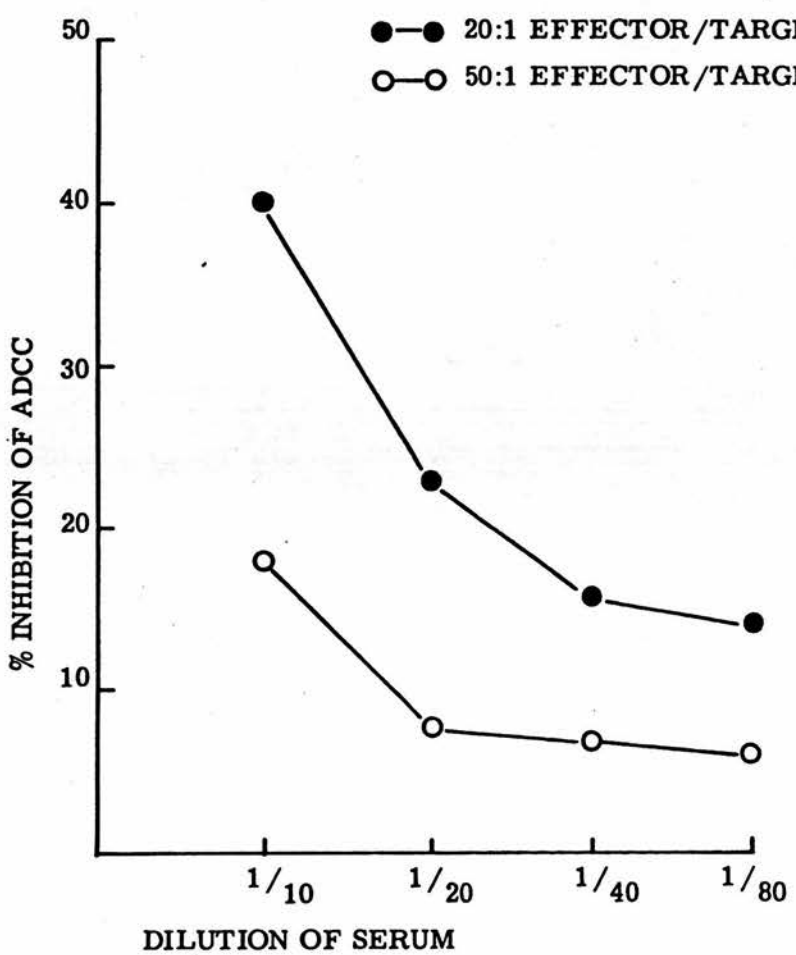


Fig. 6 Percentage inhibition of ADCC by serial dilutions of heat inactivated normal human serum using two effector/target cell ratios.



untreated thyrotoxicosis and twenty seven patients with treated thyrotoxicosis were tested in triplicate for inhibition of ADCC, using 1/10, 1/20, 1/40 and 1/80 dilutions of each serum. The results are shown in Table 1. Significantly, increased inhibitions were obtained with all dilutions of the sera of patients with Hashimoto thyroiditis or hypothyroidism, as compared with age- and sex-matched controls, whereas no significant inhibition was found with the thyrotoxic group, except at the two lowest dilutions of sera. To produce similar degrees of inhibition, control sera had to be used at concentrations of more than twice that of Hashimoto thyroiditis or hypothyroid sera.

3.1.5. Determination of serum IgG levels

Increased inhibition of ADCC by patients' sera could result from factors other than the presence of immune complexes, including increased levels of IgG. To investigate this possibility, determinations of IgG levels were carried out on the sera used in the inhibition assay. The results are shown in Table 2. Significant elevations of IgG levels were found in two groups, the greatest elevation being in the Hashimoto thyroiditis patients. A smaller increase was found in the untreated thyrotoxic patients, while patients with primary hypothyroidism or with treated thyrotoxicosis had serum IgG levels similar to those of their age- and sex-matched controls.

3.1.6. Correlation of inhibition with IgG levels in the test sera

In order to determine whether the inhibition observed was related to the level of IgG in the test sera, the percentage inhibition was compared with serum IgG concentration. The results of this comparison are shown in Figures 7 and 8. It will be observed that there was no correlation between percentage inhibition and IgG levels.

Table 1 Percentage inhibition (mean ± SE) of ADCC by sera from patients with thyroid disorders

Sample	No. of samples	Dilution of serum							
		10		20		40		80	
Hashimoto thyroiditis	25	35.4±3.54	P< 0.001	24.0±2.84	P< 0.001	15.0±1.92	P< 0.001	11.7±1.70	P< 0.01
controls	25	20.5±1.99		10.2±1.42		5.40±0.87		1.56±0.48	
primary hypo-thyroidism	29	29.6±3.09	P< 0.001	19.9±2.23	P< 0.001	11.5±1.95	P< 0.01	7.03±1.58	P< 0.025
controls	29	16.5±1.95		10.3±1.45		5.90±0.91		3.00±0.78	
untreated thyrotoxicosis	26	24.0±2.12	N.S.	15.5±2.00	P< 0.05	7.16±1.31	N.S.	3.08±1.01	N.S.
controls	26	17.0±2.63		9.95±2.04		6.50±1.51		3.16±0.99	
treated thyrotoxicosis	27	26.4±3.31	N.S.	17.2±2.46	N.S.	9.00±1.58	N.S.	4.50±0.98	N.S.
controls	27	19.3±1.92		10.8±1.50		5.80±1.23		3.18±0.84	

N.S. = not significant

Table 2 Serum IgG levels (mean ± SE) of patients with thyroid disorders

Sample	No. of samples	Serum IgG level (mg/100ml)	P
Hashimoto thyroiditis	25	1642±84.4	< 0.001
controls	25	1189±53.6	
primary hypothyroidism	29	1256±44.9	> 0.1
controls	29	1133±60.7	
untreated thyrotoxicosis	26	1329±61.8	< 0.05
controls	26	1088±43.7	
treated thyrotoxicosis	27	1216±47.2	> 0.1
controls	27	1105±61.6	

Fig. 7 Correlation of percentage inhibition of ADCC by sera from patients with Hashimoto thyroiditis and hypothyroidism with the IgG levels of these sera. Standard errors of the means are shown by horizontal and vertical bars.

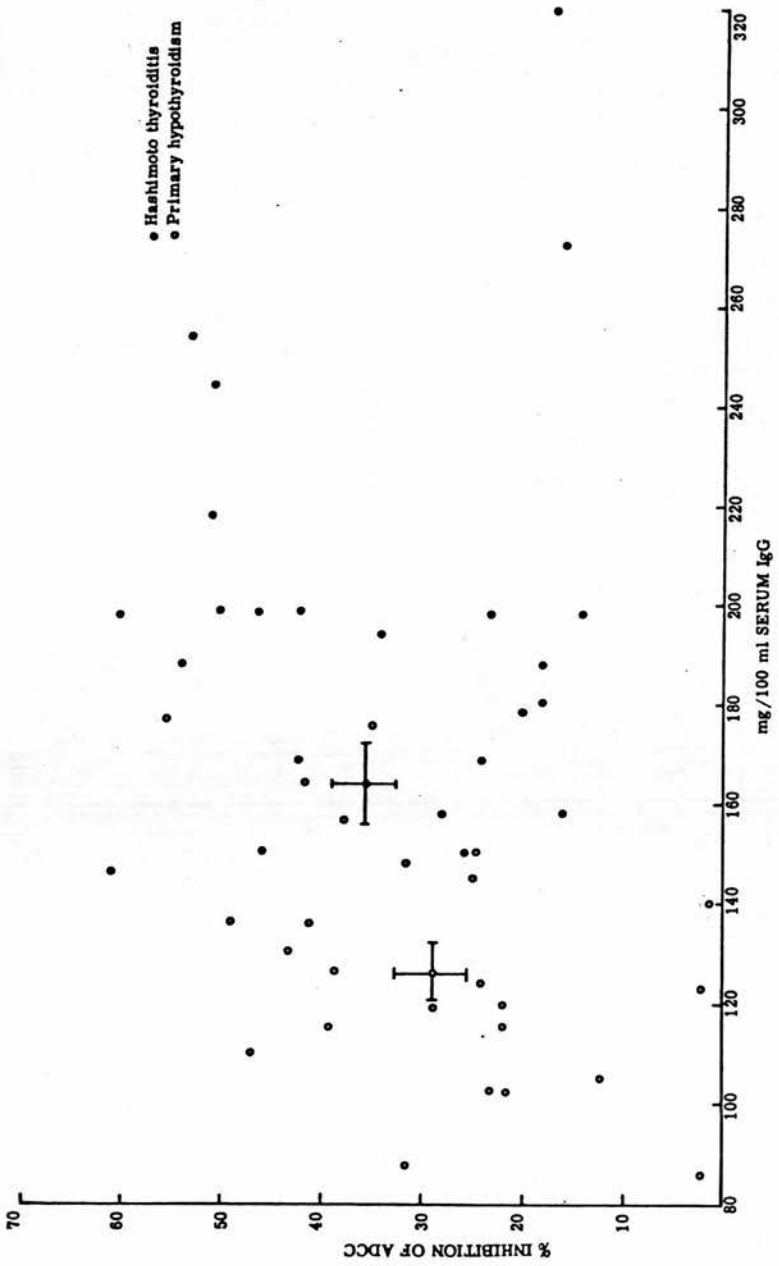
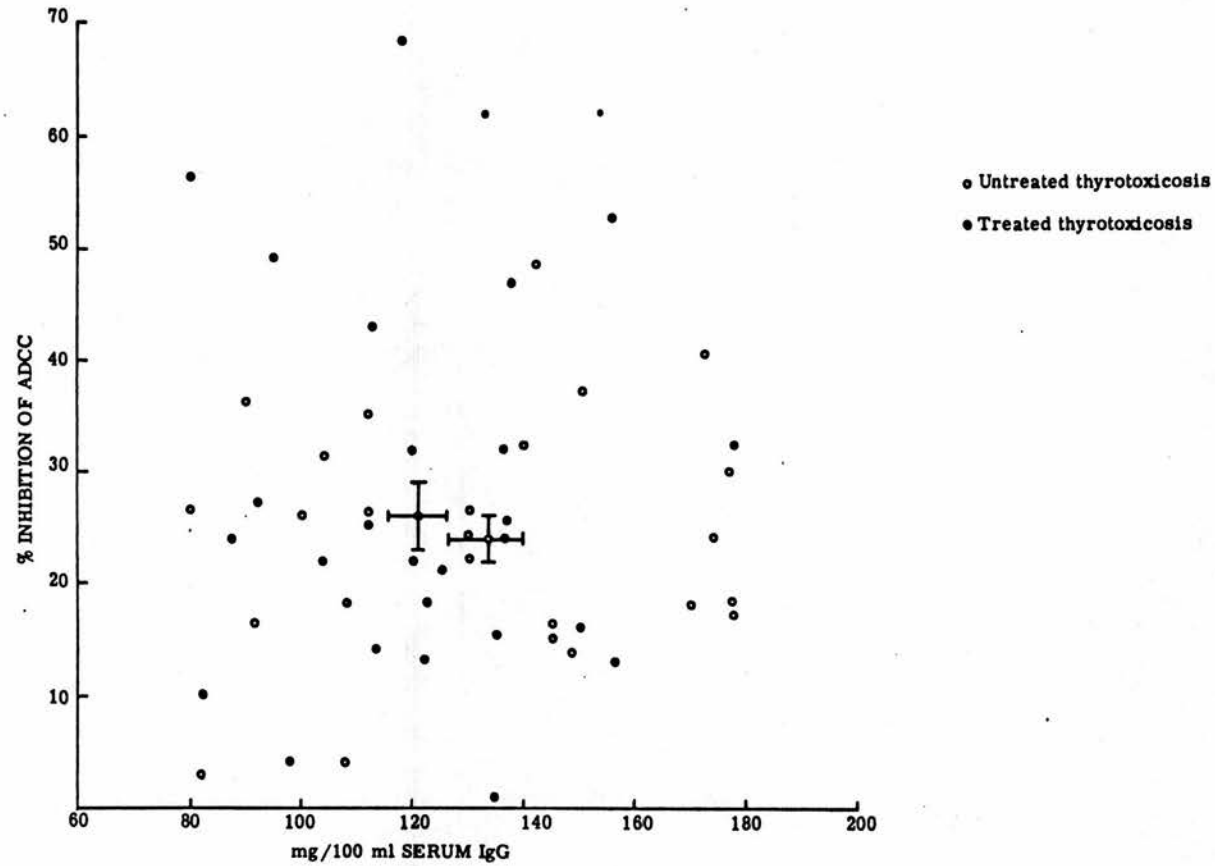


Fig. 8 Correlation of percentage inhibition of ADCC by sera from patients with thyrotoxicosis with the IgG levels of these sera. Standard errors of the means are shown by horizontal and vertical bars.



In certain instances sera which were highly inhibitory contained low levels of IgG, while in other cases sera with high levels of IgG gave a low percentage of inhibition.

3.1.7. Correlation of inhibition with the IgG levels of both test and control sera

The percentage inhibition and IgG levels at each of the four dilutions of test serum were compared with those of the controls.

Figure 9 shows the mean percentage inhibition and the mean IgG levels in Hashimoto and control sera at the four dilutions of serum used. Figures 10 - 12 show the corresponding data for primary hypothyroidism, untreated thyrotoxicosis and treated thyrotoxicosis respectively together with their controls. It is apparent that, at the same level of IgG, sera of patients with Hashimoto thyroiditis or hypothyroidism are markedly more inhibitory than those of the controls. On the other hand, differences between the sera of thyrotoxicosis patients and their controls are only slight.

3.1.8. Inhibition of ADCC by serum fractions

Serum fractions obtained by passing serum samples through Sephadex G-200 were assayed for inhibitory activity.

3.1.8.1. Hashimoto sera

Three Hashimoto sera and their controls were fractionated and assayed for inhibitory activity. The results using fractions from one Hashimoto serum are shown in Figure 13. These indicate that the greatest inhibition was found in fractions located in the ascending limb of second elution peak. Negligible inhibitory activity was

Fig. 9 Correlation of inhibition of ADCC by sera from patients with Hashimoto thyroiditis and their controls with IgG levels. Each serum was tested at dilution of 1/10, 1/20, 1/40 and 1/80 for inhibition of ADCC. Each point represents the mean and standard error for 25 individuals. Upper line: patients; lower line: controls. (Data from tables I and 2)

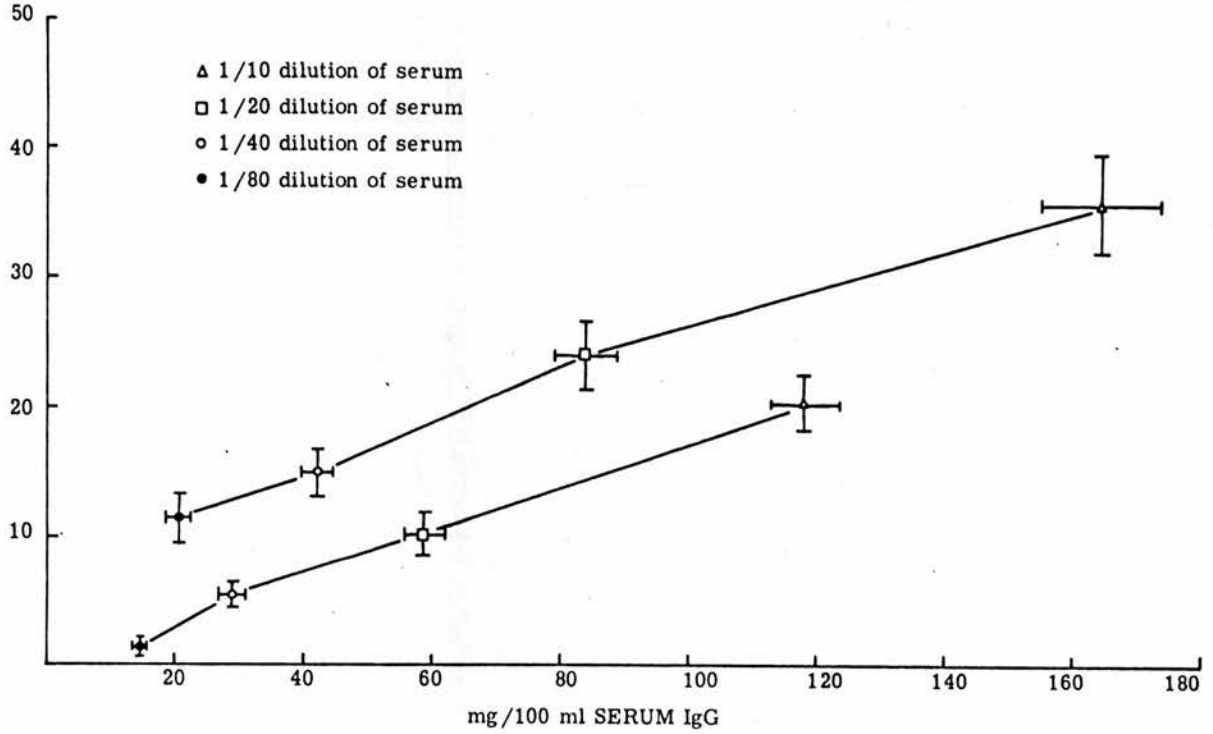


Fig. 10 Correlation of inhibition of ADCC by sera from patients with primary hypothyroidism and their controls with IgG levels. Each serum was tested at dilution of 1/10, 1/20, 1/40 and 1/80 for inhibition of ADCC. Each point represents the mean and standard error for 29 individuals. Upper line: patients; lower line: controls.

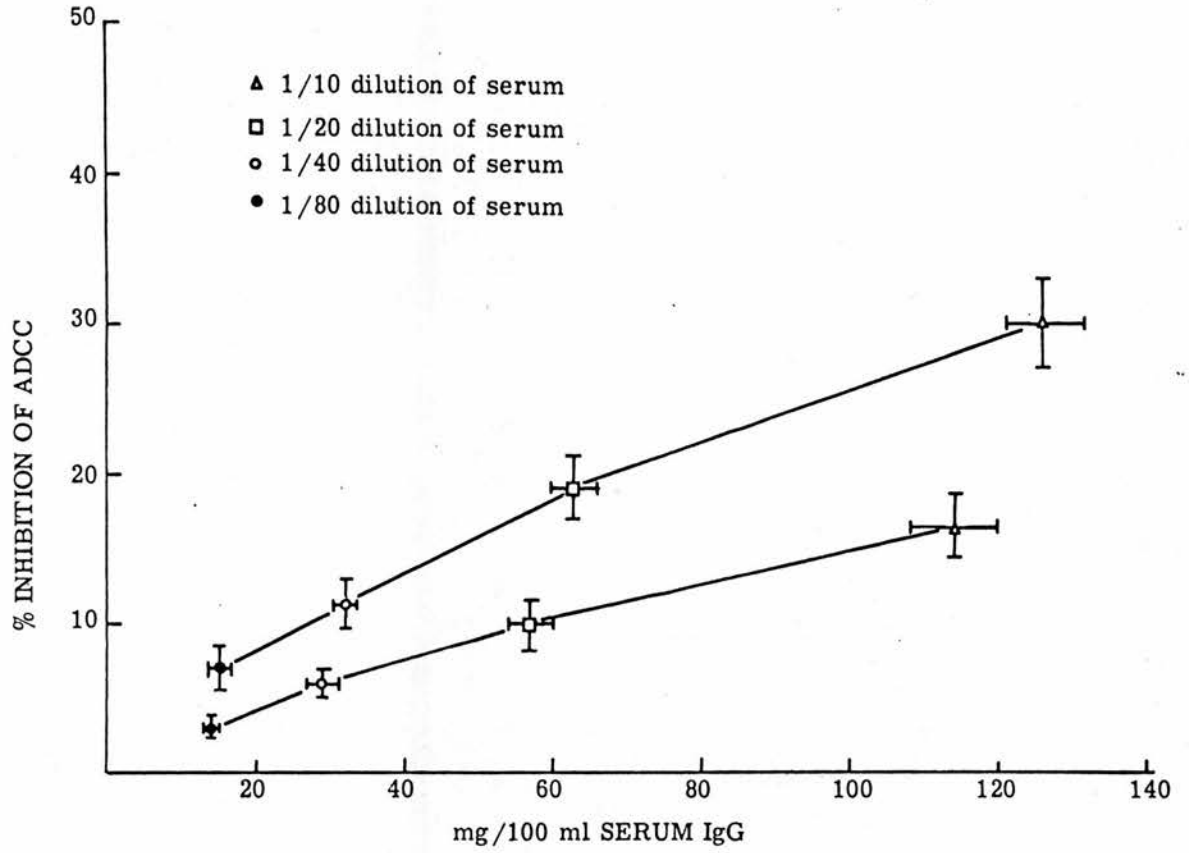


Fig. 11 Correlation of inhibition of ADCC by sera from patients with untreated thyrotoxicosis and their controls with IgG levels. Each serum was tested at dilution of 1/10, 1/20, 1/40 and 1/80 for inhibition of ADCC. Each point represents the mean and standard error for 26 individuals. Upper line: patients; lower line: controls.

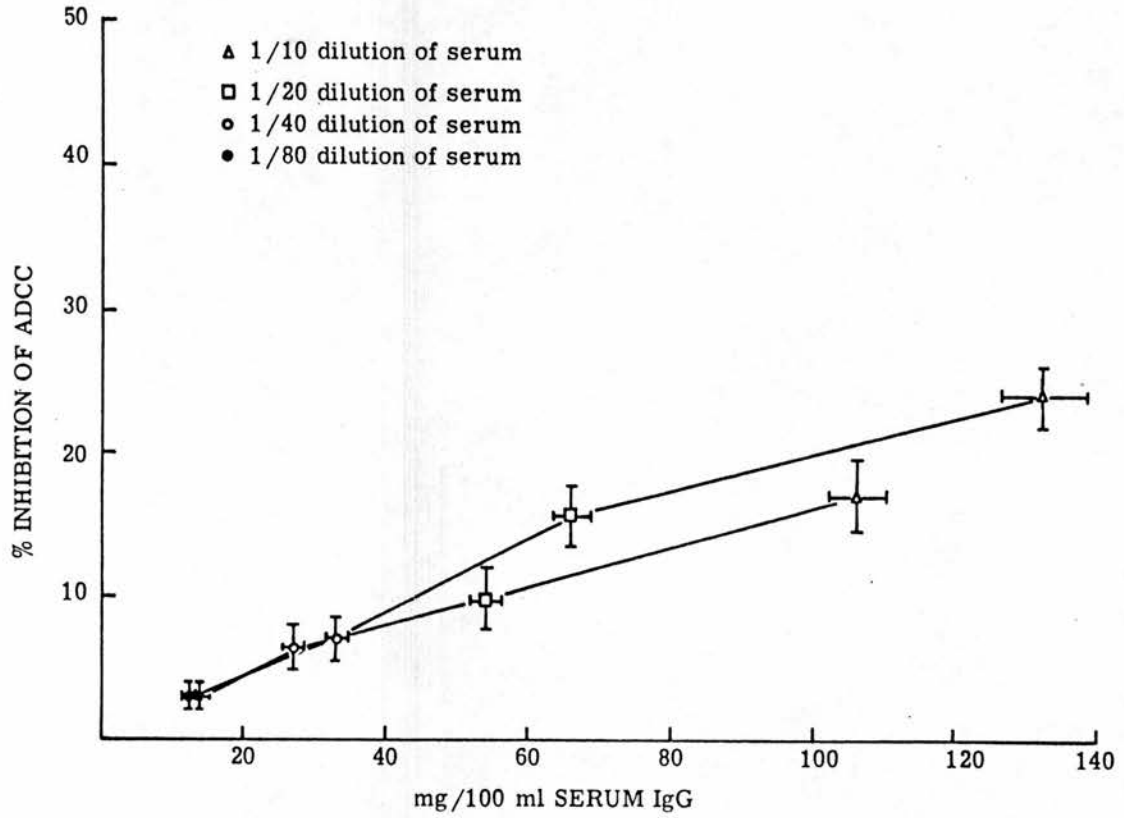


Fig. 12 Correlation of inhibition of ADCC by sera from patients with treated thyrotoxicosis and their controls with IgG levels. Each serum was tested at dilution of 1/10, 1/20, 1/40 and 1/80 for inhibition of ADCC. Each point represents the mean and standard error for 27 individuals. Upper line: patients; lower line: controls.

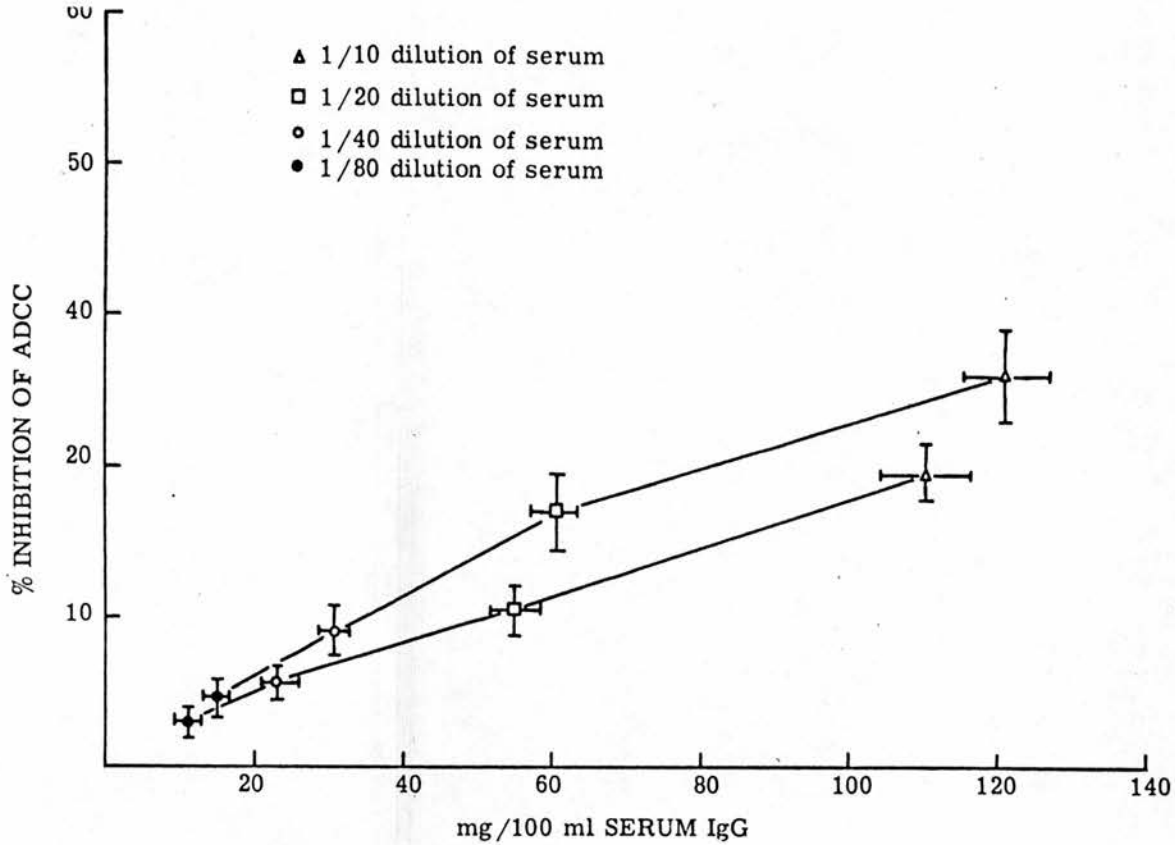
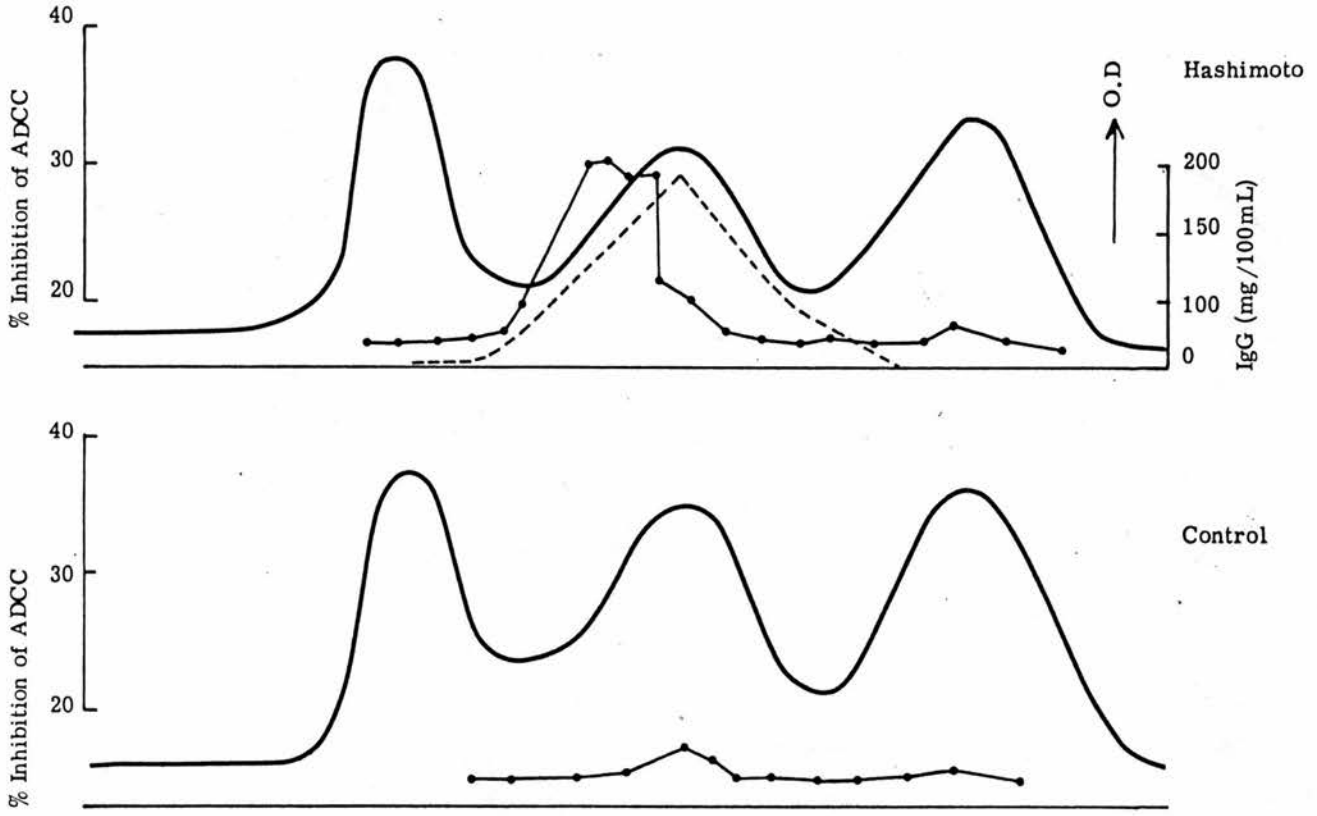


Fig. 13 Chromatography of Hashimoto and control sera on Sephadex G-200 showing the IgG content and the percentage inhibition of ADCC associated with each fraction.

------- IgG; •—• percentage inhibition.



observed in the second elution peak of the control serum. Similar results were obtained from the 2 of other Hashimoto sera and their controls.

Fractions obtained from heat-inactivated Hashimoto sera showed additional inhibition in the first elution peak as shown in Figure 14. Similarly, heat-inactivation of control serum resulted in the appearance of inhibitory activity in the first peak. These observations suggest the presence of aggregates formed as a result of heat inactivation. Other 4 Hashimoto sera gave inconclusive results.

3.1.8.2. Hypothyroid serum

The inhibition of ADCC by fractions of hypothyroid serum is shown in Figure 15. Inhibitory activity was detected in the ascending limb of the second elution peak. The degree of inhibition by these fractions was less than that of the comparable fractions of Hashimoto sera.

3.1.9. Correlation of ADCC inhibition by sera from patients with thyroid disorders with their clinical data

The results of serological investigations of each serum for the presence of anti-thyroid antibodies using the TCH and CFT techniques and for the presence of anti-microsomal antibody (IC), and anti-parietal cell antibody (PC) were compared with the capacity of the serum to inhibit ADCC.

As shown in Tables 3, 4, 5 and 6, no correlation was found between the percentage inhibition (which was regarded as positive if the percentage inhibition in the test serum was more than that of the mean of the controls or of its particular control, whichever was the greater), and the presence or levels of the antibodies investigated. Similarly, the degree of inhibition could not be correlated with the duration of illness (date of diagnosis to date of study).

Fig. 14 Chromatography of heat-inactivated Hashimoto and control sera on Sephadex G-200 showing the percentage inhibition of ADCC associated with each fraction.

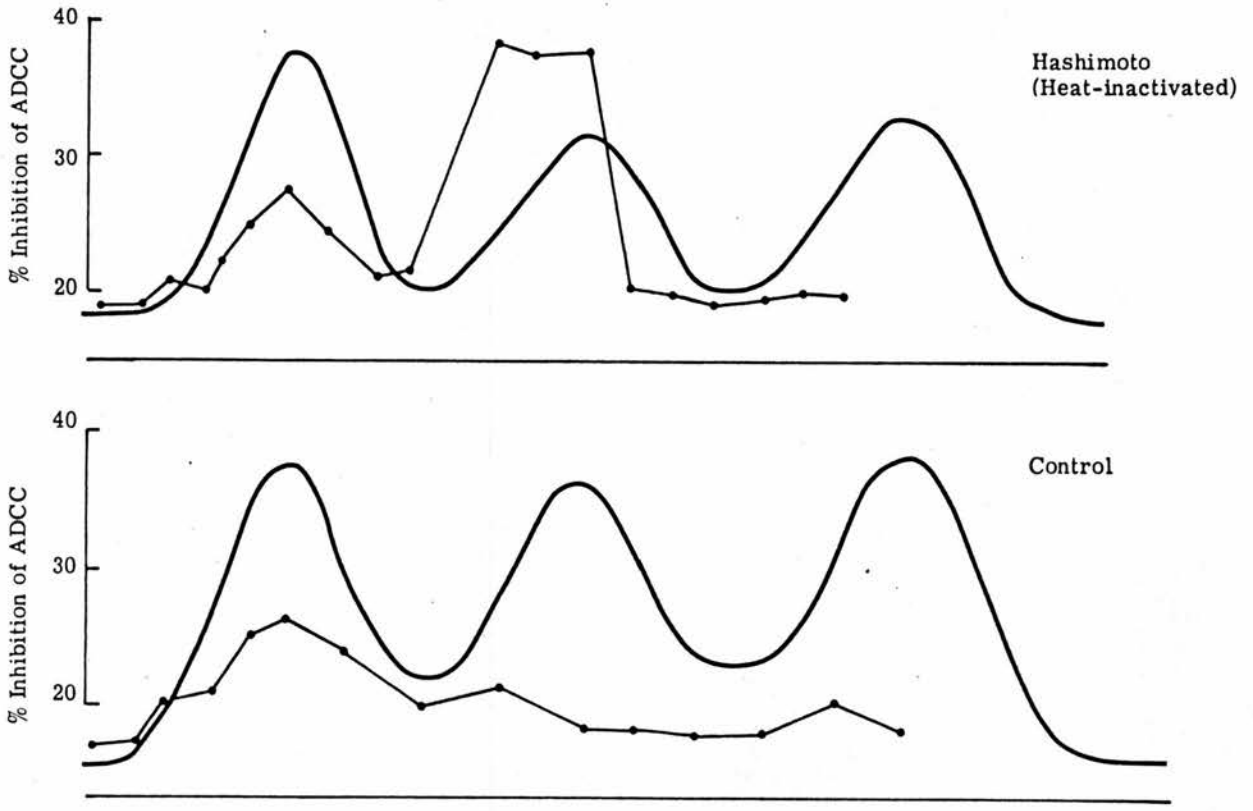


Fig. 15 Chromatography of sera from a patient with primary hypothyroidism and its control on Sephadex G-200 showing IgG content and the percentage inhibition of ADCC associated with each fraction.
------ IgG; •—• percentage inhibition.

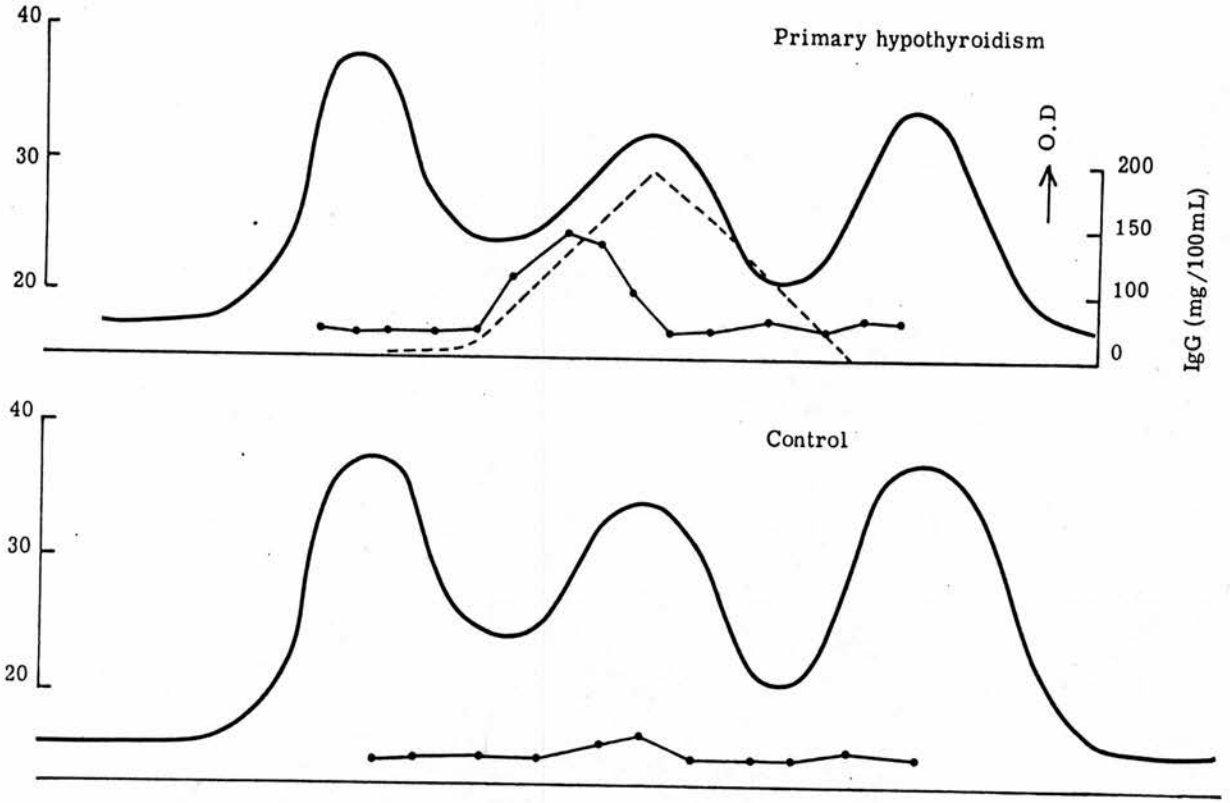


Table 3 Correlation between percentage inhibition of ADCC by sera from patients with Hashimoto thyroiditis and clinical data.

* : date of diagnosis to date of study (months)

** : Eu = Euthyroid; Hypo = Hypothyroid

*** : difference in percentage inhibition of ADCC between test serum and control

\+ = 10 difference

++ = 20 〃

+++ = 30 〃

patient	age	duration*	thyroid status**	treated with	TCH	CFT	IC	PC	inhibition of ADCC***
M.T.	45	12	Eu	T4	-	-	++	-	+
J.R.	47	28	Eu	T4	2500	32	++	-	-
F.G.	51	14	Eu	T4	-	-	-	-	+
D.S.	49	23	Eu	T4	250	32	+	+	-
E.B.	63	18	Eu	T4	2500	-	++	-	++
D.P.	47	45	Eu	T4	-	64	++	-	-
W.H.	54	58	Eu	T4	250	-	-	++	+
J.D.	60	1	Eu	T4	25000	32	++	-	-
E.B.	43	5	Eu	T4	-	-	++	-	++
I.M.	63	1	Hypo	T4	25000	64	-	+	-
M.N.	68	60	Eu	T4	25000	-	-	-	-
I.C.	62	113	Eu	T4	-	-	+	-	++
K.M.	49	2	Eu	-	-	32	++	-	+++
M.W.	57	42	Eu	T4	-	-	++	-	+
H.H.	66	13	Eu	T4	25	-	-	-	+
J.F.	53	23	Hypo	T4	-	64	++	-	+++
J.T.	71	42	Eu	T4	25	-	-	-	+
E.S.	41	-	Eu	-	25	64	++	-	-
L.M.	46	34	Eu	T4	2500	-	++	-	++
M.L.	72	71	Eu	T4	25000	-	++	-	++
H.B.	48	1	Eu	T4	25	32	+	-	++
M.H.	65	31	Eu	-	-	-	++	++	++
J.W.	71	49	Eu	T4	250	-	++	-	+
M.Mc.	60	30	Eu	T4	2500	-	++	-	-
E.A.	59	49	Eu	T4	-	-	-	-	+
J.S.	49	45	Eu	T4	-	32	++	-	++

Table 4 Correlation between percentage inhibition of ADCC by sera from patients with primary hypothyroidism and clinical data. Details as in Table 3.

patient	sex	age	duration	thyroid status	treated with	TCH	CFT	IC	PC	inhibition of ADCC
J.Q.	M	60	-	Hypo	-	-	-	+	-	++
A.L.	F	61	2	Eu	T4	2500	-	+	-	+
A.W.	F	83	-	Hypo	-	2500	32	++	-	+++
L.W.	F	46	-	Hypo	-	-	-	-	-	-
G.H.	M	63	4	Eu	T4	25000	-	++	-	-
M.A.	F	62	-	Hypo	-	25000	-	++	-	-
J.B.	F	77	-	Hypo	-	25000	-	+	-	+
J.B.	F	65	6	Hypo	T4	25000	-	++	-	++
M.B.	F	52	-	Hypo	-	-	-	++	-	-
M.R.	F	73	7	Eu	T4	25	-	+	-	++
W.G.	M	72	-	Hypo	-	-	-	++	-	-
M.B.	F	33	13	Eu	T4	25	-	++	-	++
S.A.	F	37	-	Hypo	-	-	-	++	-	-
J.S.	F	54	-	Hypo	-	-	-	++	-	+
W.F.	M	70	22	Eu	T4	25	-	+	+	++
A.S.	M	62	46	Eu	T4	-	-	+	-	-
A.S.	F	59	1	Eu	T4	-	-	++	-	+
M.G.	F	70	12	Eu	T4	25	-	+	-	+
A.H.	F	71	15	Eu	T4	25	-	++	-	++
M.E.	F	60	33	Eu	T4	-	-	+	-	-
R.W.	M	15	17	Eu	T4	-	-	-	-	-
J.S.	F	57	99	Eu	T4	-	-	+	+	++
G.C.	F	63	54	Eu	T4	-	-	+	-	-
S.N.	F	57	6	Eu	T4	-	-	+	-	-
A.L.	F	75	58	Eu	T4	25	-	++	-	++
M.K.	F	73	47	Eu	T4	25	-	++	-	++
M.C.	F	59	3	Eu	T4	-	-	++	-	-
J.D.	F	69	20	Eu	T4	2500	-	++	-	-
J.C.	M	78	-	Hypo	-	2500	-	++	+	-

Table 5 Correlation between percentage inhibition of ADCC by sera from patients with untreated thyrotoxicosis and clinical data.

$\not$: Carb. = Carbimazole; Prop. = Propranalol

Other details (except thyroid status) as in Table 3.

patients	sex	age	duration	thyroid status	treated with	TCH	CFT	IC	PC	inhibition of ADCC
A.H.	M	30	-	toxic	-	-	64	++	-	-
I.R.	F	44	-	toxic	-	-	-	-	-	+
T.M.	M	14	-	toxic	-	2500	-	+	-	-
M.H.	F	51	-	toxic	-	-	32	±	-	-
J.C.	M	25	2	toxic	Carb.	-	-	++	-	-
M.R.	F	27	14	toxic	Prop. Carb.	-	32	++	-	+
E.S.	F	27	-	toxic	Prop.	-	-	-	-	+
B.L.	F	23	-	toxic	-	-	64	++	-	+
M.W.	F	23	-	toxic	-	-	-	-	-	+
H.S.	F	25	-	toxic	-	25	-	-	-	-
H.G.	F	54	-	toxic	-	-	32	++	-	-
R.D.	F	38	-	toxic	-	-	-	++	-	-
M.S.	F	27	-	toxic	Prop.	250	64	++	-	-
M.S.	F	53	-	toxic	Prop.	-	32	++	-	-
M.D.	F	32	-	toxic	-	-	-	-	-	-
S.W.	F	28	-	toxic	-	-	-	-	-	-
J.B.	F	62	-	toxic	-	-	-	-	-	-
I.C.	F	59	-	toxic	-	25	-	-	-	-
E.W.	F	44	1	toxic	-	25	-	+	-	-
E.R.	F	41	-	toxic	-	-	-	±	-	-
G.M.	F	50	-	toxic	-	-	-	++	-	-
M.C.	F	46	-	toxic	-	2500	64	++	-	+
A.G.	F	21	-	toxic	Prop.	-	-	+	-	+
J.I.	F	33	-	toxic	-	25000	-	++	-	+
W.L.	F	24	-	toxic	-	-	-	++	-	-
E.B.	F	66	-	toxic	-	-	-	++	-	-

Table 6 Correlation between percentage inhibition of ADCC by sera from patients with treated thyrotoxicosis and clinical data. Details as in Table 3.

patient	sex	age	duration	thyroid status	treated with	TCH	CFT	IC	PC	inhibition of ADCC
P.C.	M	45	6	Eu	^{131}I	-	32	+	++	-
R.L.	F	47	15	Eu	^{131}I	-	-	+	-	+
W.D.	F	53	57	Hypo	^{131}I	250	-	++	++	+
W.T.	M	53	51	Eu	^{131}I	-	32	++	-	-
J.H.	F	51	50	Eu	^{131}I	-	-	+	++	+
M.H.	F	48	39	Eu	^{131}I	-	32	+	+	-
E.H.	M	57	20	Eu	^{131}I	250	-	++	+	-
J.A.	F	57	32	Eu	^{131}I	-	-	+	-	-
J.L.	M	81	54	Eu	^{131}I	-	-	+	+	++
E.H.	F	56	40	Eu	^{131}I	-	32	++	-	-
A.F.	F	56	34	Eu	^{131}I	-	-	-	-	++
D.D.	F	55	35	Eu	^{131}I	-	32	++	-	-
E.C.	F	58	58	Hypo	^{131}I	-	-	-	-	+
A.G.	F	63	19	Eu	^{131}I	-	-	-	-	+++
J.S.	F	49	15	Eu	^{131}I	2500	32	++	+	+
D.H.	M	64	28	Eu	^{131}I	-	-	-	-	+
A.P.	F	59	50	Eu	^{131}I	-	-	-	-	+
I.W.	F	53	25	Eu	^{131}I	-	-	++	-	+
M.M.	F	44	40	Hypo	^{131}I	-	-	+	-	-
J.V.	M	45	25	Eu	^{131}I	-	-	-	-	-
M.H.	F	53	51	Eu	^{131}I	-	-	-	-	-
M.G.	F	54	19	Eu	^{131}I	-	32	++	-	-
J.M.	F	53	53	Eu	^{131}I	-	-	-	-	+
S.C.	F	43	14	Eu	^{131}I	-	-	-	-	-
W.L.	M	45	57	Eu	^{131}I	25	-	++	-	-
M.E.	F	45	24	Eu	^{131}I	-	-	++	-	-

3.2. C1q DEVIATION TEST

3.2.1. Sensitivity of the C1q deviation test to aggregated IgG

The deviation of ^{125}I C1q binding to sensitized sheep erythrocytes by C1q-reactive material in the form of aggregated IgG added to pooled normal human serum was measured by comparing the amount of radio-labelled C1q bound to the indicator cells with that detected in the supernatant. ^{125}I C1q was found to deviate from binding to sheep erythrocytes in the presence of aggregated IgG (Figure 16) and also in the presence of heat-inactivated serum.

The results indicate that this assay is relatively insensitive, since the lower limit of detection of added aggregates was approximately 100µg/ml. Moreover, this limit of detection varied according to the human serum used (Figure 17).

3.3. RAJI CELL RADIOIMMUNOASSAY

3.3.1. Antibody activity of fractionated anti-human IgG antiserum

Anti-human IgG antiserum was fractionated on DEAE cellulose as described in 2.2.3., and the fractions (Figure 18) tested for antibody activity.

Antibody activity was found in all fractions of the first and second elution peaks. However, this activity was lower than that shown by the solubilized precipitate formed by treatment of the antiserum with ammonium sulphate, even after bulking and concentration of the fractions. Accordingly, iodination of anti-human IgG antibodies was carried out using the proteins isolated by ammonium sulphate precipitation.

Fig. 16 Deviation of radiolabelled C1q from binding to sensitized sheep erythrocytes by various amounts of aggregated IgG added to normal human serum. Each point represents the mean and standard error of four experiments.

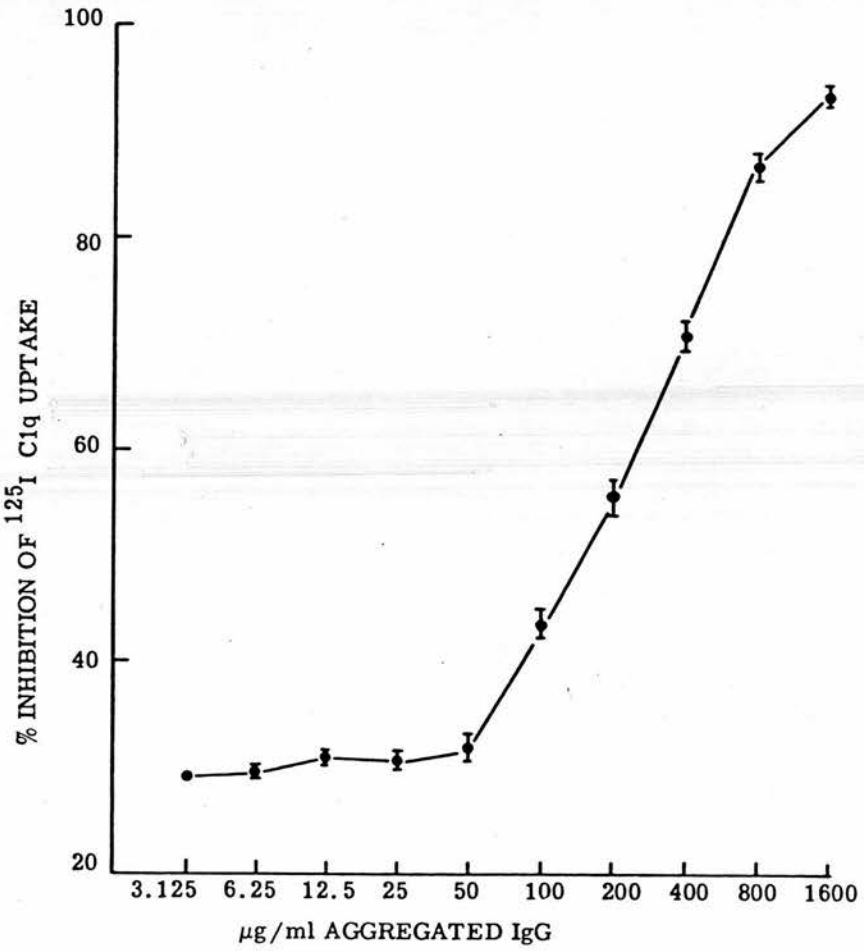
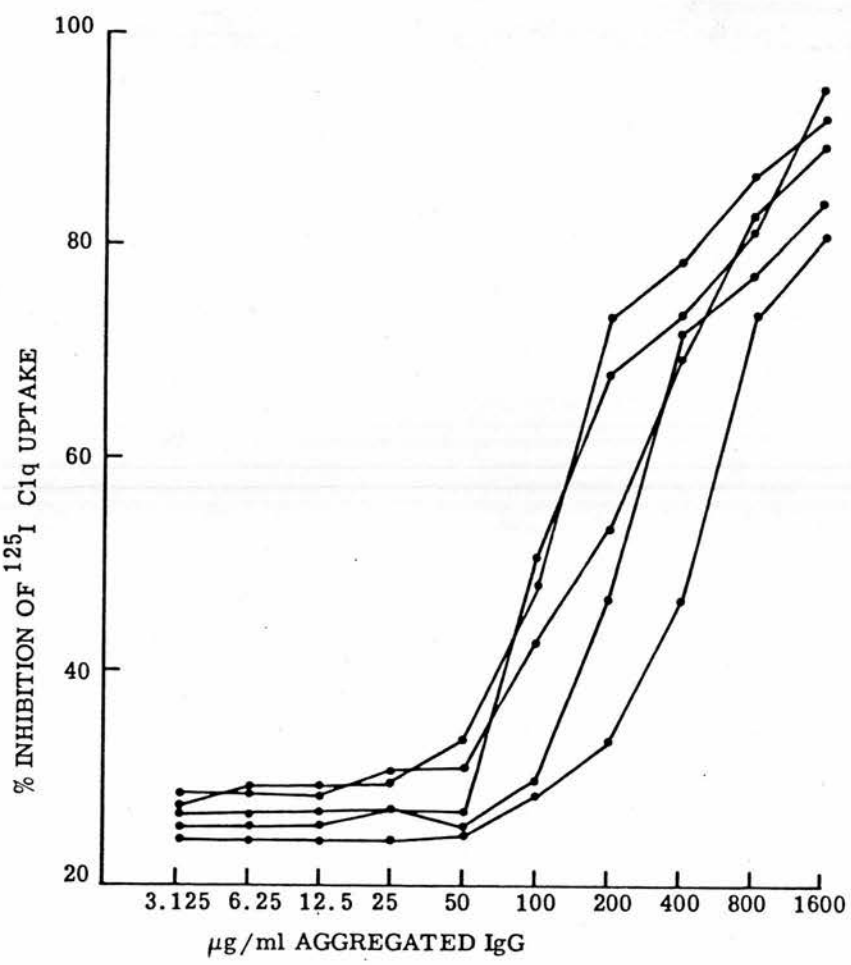


Fig. 17 Deviation of radiolabelled C1q from binding to sensitized sheep erythrocytes by various amounts of aggregated IgG added to each of five normal human sera.



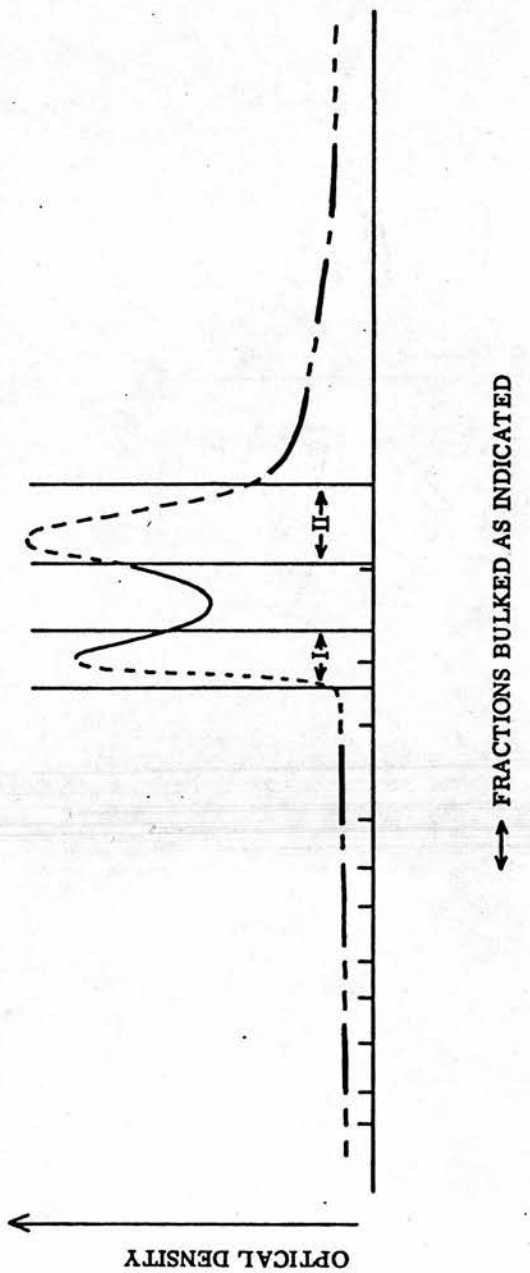


Fig. 18 The DEAE cellulose separation profile of goat anti-human IgG antiserum.

3.3.2. Optimal conditions for the assay

Increasing amounts of radiolabelled anti-human IgG were added to a constant amount of aggregated IgG bound to $2x10^6$ Raji cells. As shown in Figure 19, 30µl of 1% BSA in phosphate buffer containing approximately 15µg radiolabelled anti-human IgG was found to give the highest level of specific uptake (uptake by cells incubated with NHS and added aggregated IgG minus uptake by cells incubated with NHS alone).

3.3.3. Sensitivity of the assay to aggregated IgG

The sensitivity of the Raji cell radioimmunoassay in detecting immune complexes in serum was estimated by adding different concentrations of aggregated IgG to a 1:4 dilution of pooled normal human serum, and measuring the uptake of label after incubation with 30µl of 1% BSA in phosphate buffer containing 15µg radiolabelled goat anti-human IgG. At a concentration of 6µg/ml in 1:4 dilution of serum the uptake of radiolabelled antibody by the Raji cells was considerably greater than that using serum alone. The amount of radiolabelled antibody taken up by the cells was directly related to the amount of aggregated IgG present in the serum as shown in Figure 20. The sensitivity of the assay was therefore 24 µg/ml in neat serum.

3.3.4. Detection and quantitation of immune complexes in sera of patients with thyroid disorders

Raji cells were incubated with twenty-three Hashimoto, twenty-eight hypothyroid and twenty thyrotoxic sera and then reacted with an optimal amount of radiolabelled anti-human IgG. Subsequently the uptake was determined and referred to a standard curve of radioactive antibody uptake by cells previously incubated with various amounts of aggregated IgG in a 1:4 dilution of serum (Figure 20). The radiolabelled

Fig. 19 Titration of radiolabelled anti-human IgG for the demonstration of aggregated IgG in serum: Raji cell radioimmunoassay.

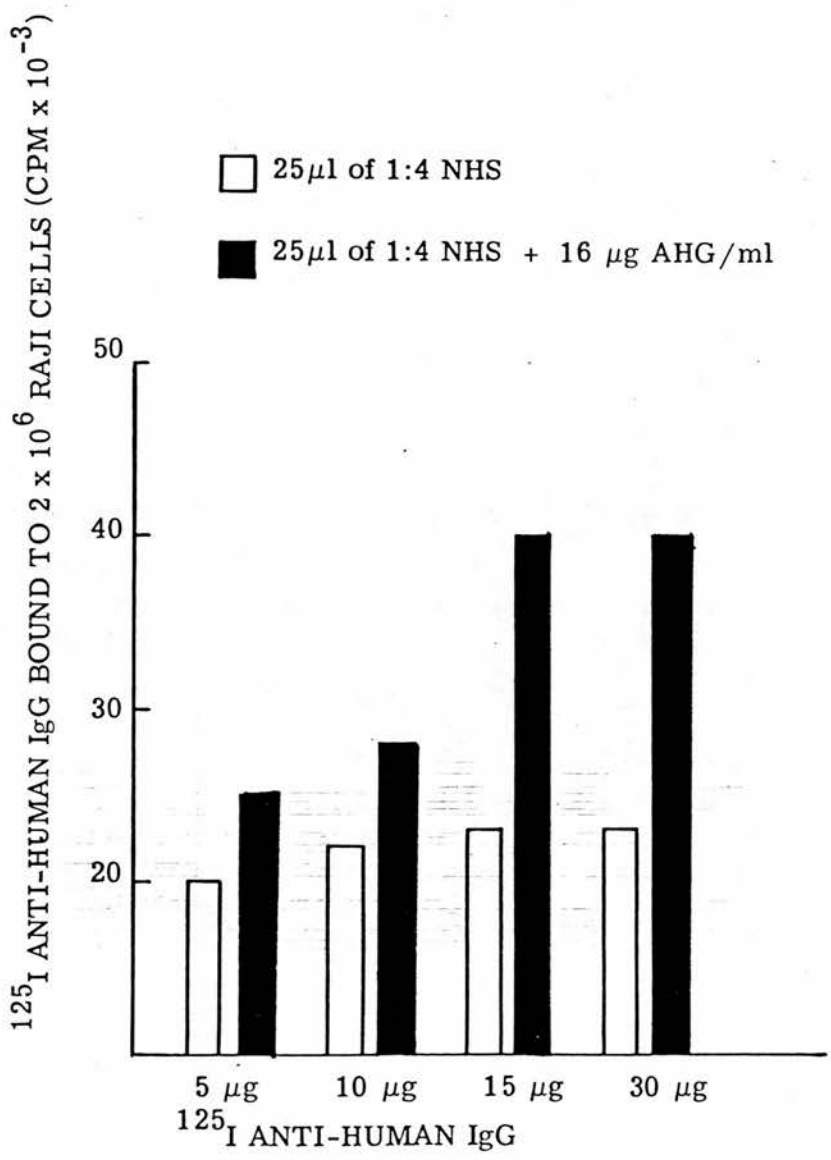
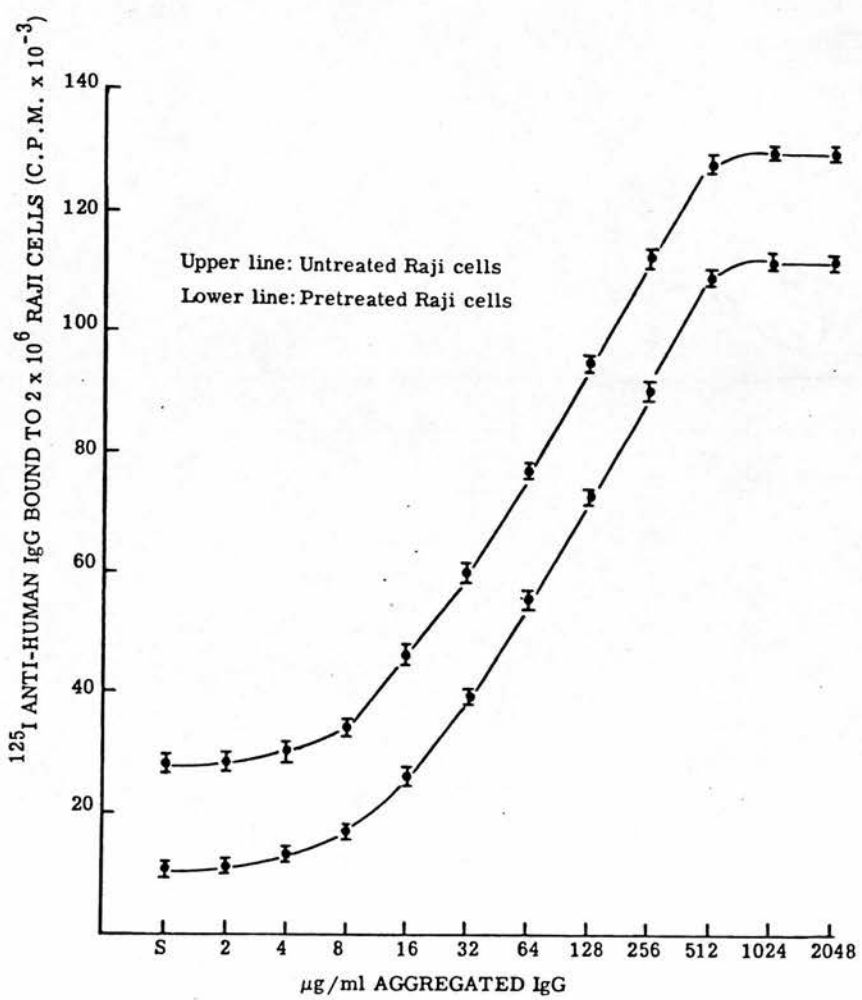


Fig. 20 Standard curve of radiolabelled anti human IgG bound to untreated or NRS-treated Raji cells incubated with various amounts of aggregated IgG. Each point represents the mean and standard error of four experiments.



antibody uptake by cells incubated with a 1:4 dilution of pooled NHS was considered as a baseline in the reference curve. The estimated amount of complexes in each serum tested was expressed as ug aggregated IgG equivalents per ml serum (µg eq.AggIgG/ml). More than 6µg eq.AggIgG/ml, a level exceeding the upper limit of controls, was found in 1:4 dilutions of 78% Hashimoto, 36% hypothyroid and 20% thyrotoxic sera (Table 7). The results indicate that the greatest amount of complexes was detected in sera from patients with Hashimoto thyroiditis.

3.3.5. Detection and quantitation of immune complexes in Hashimoto sera after blocking the Fc receptors on Raji cells

The binding of immune complexes to Raji cells is mediated via complement receptors and Fc receptors. The latter receptor also binds monomeric IgG, and sera of patients with Hashimoto thyroiditis contain higher levels of IgG than control sera. Therefore, to avoid false positive results which might arise due to the binding of monomeric IgG to Fc receptors on the Raji cells, the cells were pre-incubated with NRS for 1 hour at 37°C in order to block Fc receptors by monomeric rabbit IgG. The cells were then washed and incubated with a 1:4 dilution of test and control sera for 45 minutes at 37°C. After washing the cells 3 times with Eagle's medium, 30µl of ^{125}I goat anti-human IgG were added. Unbound antibody was removed by washing the cells 3 times with 1% BSA in PBS. The amount of complexes bound to the cells was estimated by referring to a standard curve of radioactive antibody uptake by cells pre-incubated with NRS, and subsequently incubated with various amounts of aggregated IgG in a 1:4 dilution of pooled normal human serum (Figure 20). It will be observed that the blocking of the Fc receptors on Raji cells led to

Table 7 Detection and quantitation of immune complexes in sera of patients with thyroid disorders using the Raji cell radioimmunoassay

patients	no.	no. positive	% positive	µg eq.AggIgG/ml range (mean)
Hashimoto thyroiditis	23	18 $P<0.002$	78	32 - 512 (200)
controls	23	2	9	
Primary hypothyroid	28	10 $P<0.01$	36	32 - 64 (42)
controls	28	1	4	
Thyrotoxic	20	4 $P<0.05$	20	32 - 64 (40)
controls	20	0	0	

a reduction in the amount of radioactivity bound to these cells. This suggests that the binding of monomeric human IgG to Raji cells was prevented by pre-incubating these cells with NRS.

The amount of complexes detected in each serum sample using Raji cells pre-treated with NRS was compared with that detected using untreated Raji cells. As shown in Table 8, the blocking of Fc receptors did not significantly affect the degree of binding of complexes to the cells.

3.3.6. Characterization of immune complexes

Sera from eighteen patients with Hashimoto thyroiditis, ten patients with primary hypothyroidism and four patients with thyrotoxicosis found positive for the presence of soluble immune complexes were incubated with Raji cells. The cells were then incubated with ^{125}I-rabbit anti-human thyroglobulin antiserum in order to test for the presence of thyroglobulin in the bound complexes. As shown in Figure 21, the degree of binding of radiolabelled anti-human thyroglobulin antibodies to Raji cells incubated with 8 out of 18 Hashimoto sera and 3 out of 10 hypothyroid sera was higher than that detected using Raji cells incubated with control sera.

3.3.7. Correlation of clinical data of patients with thyroid disorders with the presence of immune complexes in their sera

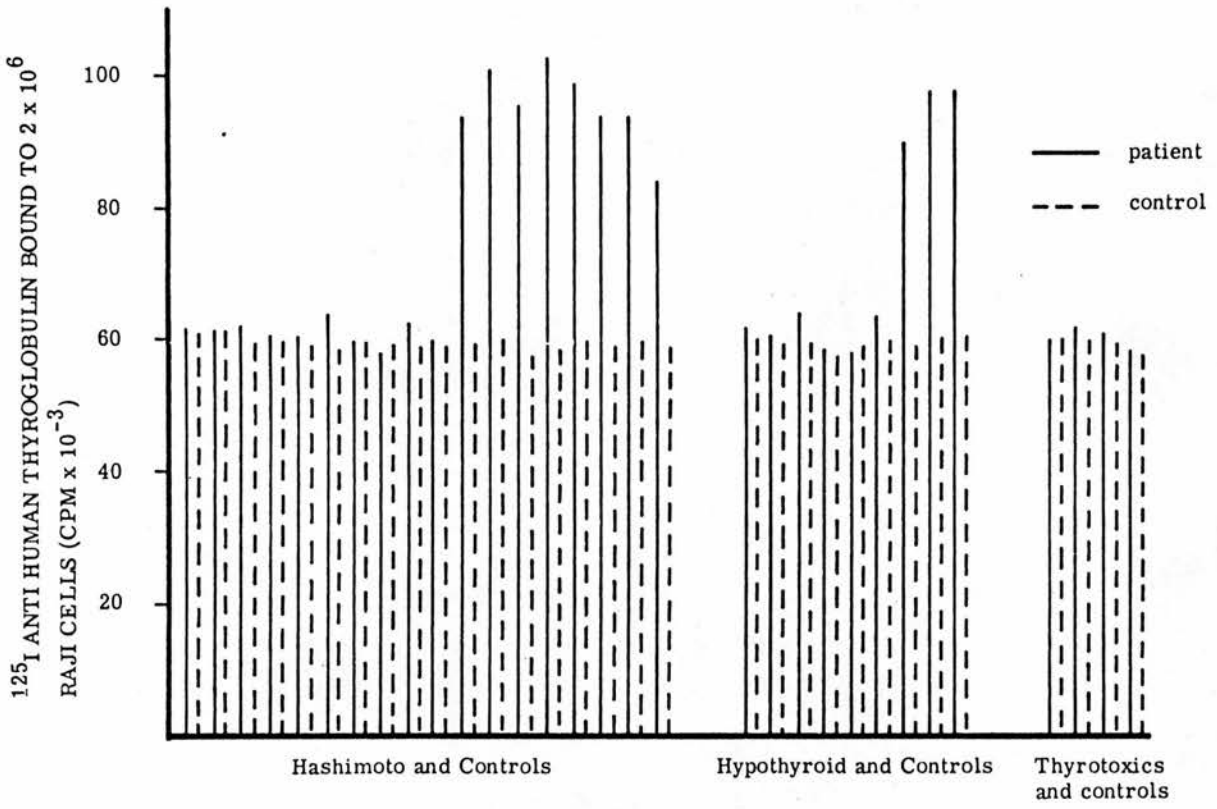
The results of serological investigations of each serum for the presence of anti-thyroid antibodies using the TCH and CFT techniques and for the presence of anti-microsomal antibody (IC) and anti-parietal cell antibodies (PC), together with the duration of the illness (date of diagnosis to date of study) and the treatment, if any, were compared with the presence and the amounts of immune complexes

Table 8 The binding of complexes present in the sera of patients with Hashimoto thyroiditis to untreated Raji cells and to Raji cells pretreated with normal rabbit serum.

patient	complexes µg eq.AggIgG/ml (untreated cells)	complexes µg eq.AggIgG/ml (pretreated cells)
C.B.	135	90
H.L.	32	32
J.R.	38	45
J.Mc.	256	185
A.B.	75	70
M.M.	512	450
D.P.	500	500
J.D.	60	32
E.H.	450	175
H.G.	275	250
S.W.	220	240
M.M.	55	40
M.G.	45	45
A.E.	32	32
J.R.	400	250
M.M.	160	200
E.W.	320	300
O.M.	40	32
Mean ± SE	200 ± 40.54	165 ± 34.41

$P > 0.25$

Fig. 21 The binding of radiolabelled anti-human thyroglobulin antibodies to Raji cells pre-incubated with sera from patients with thyroid disorders.



detected by the Raji cell radioimmunoassay. The results indicate that no correlation exists between serological findings, duration and treatment and the presence or absence of immune complexes in the sera of patients with Hashimoto thyroiditis (Table 9), with primary hypothyroidism (Table 10) and with thyrotoxicosis (Table 11).

3.3.8. Detection and quantitation of immune complexes in diabetic sera

Raji cells were incubated with sera from thirteen newly diagnosed insulin-dependent diabetics, thirty-two insulin-treated diabetics, thirty-one diabetics treated with oral hypoglycaemic agents (OHA) and twenty-one diabetics requiring restriction of diet alone. Sera from 53.8% of newly diagnosed, 53% insulin-treated, 9.6% OHA-treated and 9.5% diet-treated diabetics were found to have immune complex-like activity exceeding the limit of controls (Table 12).

3.3.9. Detection of anti-viral antibodies in sera of newly diagnosed diabetics

The sera of the thirteen newly diagnosed insulin-dependent diabetics and their age- and sex-matched controls were screened and titrated for antibodies to common viral infections, as shown in Table 13. The prevalence and titres of antibodies to all viruses investigated other than Coxsackie B4 were similar in diabetics and in their controls. Coxsackie B4 antibodies were found in 6 out of 13 test sera compared with only 2 out of 13 controls, but this difference between the two groups was not significant.

3.3.10. Correlation of the presence of anti-islet cell antibodies, anti-Coxsackie B4 antibodies and immune complexes

All diabetic sera were investigated for the presence of anti-islet cell antibodies.

Table 9 Correlation between the presence of complexes detected by the Raji cell radioimmunoassay in the sera of patients with Hashimoto thyroiditis and clinical data.

* : date of diagnosis to date of study (months)

** : Eu = Euthyroid; Hypo = Hypothyroid

patient	age	duration*	thyroid status**	treatment	TCH	CFT	IC	PC	complexes	µg eq. AggIgG/ml
C.B.	76	1	Eu	T4	2500	-	+	-	+	135
H.L.	28	1	Eu	T4	-	4	++	-	+	32
J.R.	48	46	Eu	T4	25000	8	+	-	+	38
J.Mc.	57	26	Eu	T4	-	2	++	-	+	256
M.H.	43	77	Eu	T4	-	-	++	-	-	-
E.H.	65	180	Eu	T4	25	-	-	-	-	-
A.B.	59	69	Hypo	-	-	64	++	±	+	75
M.M.	75	138	Eu	T4	25000	-	++	±	+	512
D.P.	48	61	Eu	T4	-	-	+	-	+	500
J.D.	61	18	Eu	T4	2500	-	-	-	+	60
E.H.	72	120	Eu	T4	250	-	-	-	+	450
H.G.	73	108	Eu	T4	-	2	++	+	+	275
S.W.	18	9	Eu	T4	-	8	++	++	+	220
C.B.	66	21	Eu	T4	250	-	++	±	-	-
M.M.	54	3	Eu	T4	250000	-	++	++	+	55
M.G.	72	8	Eu	-	25	128	++	+	+	45
J.S.	50	59	Eu	T4	25	2	+	-	-	-
A.E.	96	0	Hypo	-	-	128	++	-	+	32
J.R.	67	47	Eu	T4	-	4	++	-	+	400
M.M.	48	15	Eu	T4	25000	-	+	++	+	160
E.W.	20	7	Eu	T4	-	16	++	neg	+	320
E.L.	44	25	Eu	T4	-	-	+	-	-	-
O.M.	55	0	Hypo	-	250	32	+	+	+	40

Table 10 Correlation between the presence of complexes detected by the Raji cell radioimmunoassay in the sera of patients with primary hypothyroidism and clinical data. Details as in Table 9.

patient	sex	age	duration	thyroid status	treatment	TCH	CFT	IC	PC	complexes	µg eq. AggIgG/ml
A.H.	F	63	20	Eu	T4	25	-	-	-	-	-
H.Mc.	F	63	5	Eu	T4	-	-	+	-	-	-
M.R.	F	74	26	Eu	T4	2500	8	±	-	-	-
C.R.	F	76	86	Eu	T4	25	-	-	-	+	64
M.H.	F	57	12	Eu	T4	2500	-	-	-	-	-
M.G.	F	67	27	Eu	T4	2500	-	++	+	+	32
J.F.	F	64	17	Eu	T4	-	-	+	+	-	-
M.M.	F	73	116	Eu	T4	-	-	-	-	+	32
C.C.	F	60	85	Eu	T4	2500	-	-	-	-	-
A.L.	F	62	22	Eu	T4	2500	-	±	-	-	-
C.T.	M	52	3	Hypo	T4	-	-	++	-	+	32
J.Mc.	M	41	8	Eu	T4	-	-	+	-	-	-
S.G.	F	45	150	Eu	T4	-	-	-	-	-	-
E.T.	F	62	3	Eu	T4	-	-	-	-	-	-
D.W.	F	36	16	Eu	T4	-	-	++	-	+	32
J.W.	F	67	120	Eu	T4	-	-	-	-	-	-
J.B.	F	72	25	Eu	T4	-	-	-	-	-	--
K.F.	F	74	69	Eu	T4	-	-	++	-	+	32
J.D.	F	70	62	Eu	T4	-	-	-	-	-	-
A.M.	F	50	8	Eu	T4	2500	4	++	-	+	55
J.P.	F	64	37	Eu	T4	-	-	-	++	-	-
M.C.	F	66	13	Eu	T4	-	32	++	-	-	-
J.M.	F	68	0	Hypo	-	-	-	++	+	+	45
J.B.	F	35	0	Hypo	-	2500	-	+	-	-	-
I.L.	F	65	0	Hypo	-	-	32	++	±	+	32
A.G.	F	64	0	Hypo	-	-	256	++	++	-	-
B.G.	F	61	0	Hypo	-	-	-	+	±	+	70
L.H.	F	78	0	Hypo	-	-	-	++	++	-	-

Table 11 Correlation between the presence of complexes detected by the Raji cell radioimmunoassay in the sera of patients with thyrotoxicosis and clinical data.

$\not$: Prop. = Propranalol

Other details (except thyroid status) as in Table 9.

patient	sex	age	duration	thyroid status	treated with/	TCH	CFT	IC	PC	complexes	µg eq. AggIgG/ml
J.T.	F	41	1	toxic	-	-	-	+	-	-	-
D.S.	M	45	-	toxic	Prop.	-	-	++	-	-	-
M.R.	F	55	-	toxic	Prop.	25	32	++	-	-	-
R.S.	M	57	1	toxic	-	-	2	++	-	+	32
P.Mc.	M	28	1	toxic	Prop.	-	-	+	-	-	-
A.H.	F	61	-	toxic	-	25	-	+	±	+	50
J.M.	F	41	-	toxic	-	-	-	-	-	-	-
S.T.	F	29	-	toxic	Prop.	25	-	+	-	-	-
J.L.	F	43	-	toxic	Prop.	250	-	++	±	-	-
M.W.	F	33	-	toxic	-	-	-	++	-	-	-
E.K.	F	33	-	toxic	Prop.	2500	-	-	-	-	-
T.G.	M	62	-	toxic	Prop.	-	-	-	-	+	32
E.Mc.	F	42	-	toxic	-	-	-	+	-	-	-
R.R.	F	38	-	toxic	Prop.	-	-	++	-	-	-
G.H.	F	35	-	toxic	-	-	-	++	±	-	-
L.R.	F	21	-	toxic	Prop.	-	-	++	-	-	-
L.S.	F	22	-	toxic	Prop.	-	-	+	+	-	-
S.S.	F	32	-	toxic	Prop.	-	-	++	-	-	-
L.B.	F	35	-	toxic	-	250	-	+	-	+	32
M.S.	F	68	-	toxic	-	-	-	-	-	-	-

Table 12 Detection and quantitation of immune complexes in diabetic sera using the Raji cell radioimmunoassay

patients	No.	No. positive		% positive	µg eq.AggIgG/ml range (mean)
newly diagnosed insulin-dependent	13	7	$P<0.05$	53	32 - 64 (46)
controls	13	0		-	
insulin-treated	32	17	$P<0.001$	53	32 - 128 (60)
controls	32	2		6	(32)
oral hypoglycaemic agents	31	3	N.S.	10	32 - 64 (42)
controls	31	2		6	(32)
restriction of diet	21	2	N.S.	10	(32)
controls	21	1		5	(32)

N.S. = not significant

Table 13 Anti-viral antibodies in the sera of newly diagnosed insulin-dependent diabetics

/ : P. = patient; C. = control

a) Influenza A
b) Influenza B
c) Influenza C
d) Parainfluenza
e) Adenovirus
f) Chlymydia group B
g) Coxiella burneti phase 2
h) Mumps S
i) Mumps V
j) Respiratory Syncytial virus
k) Mycoplasma pneumoniae
l) Measles
m) Cytomegalovirus
n) Varicella zoster
o) Herpes simplex
p) Epstein Barr virus (IgG)
q) Epstein Barr virus (IgM)
r) Coxsackie B1 virus
s) Coxsackie B2 virus
t) Coxsackie B3 virus
u) Coxsackie B4 virus
v) Coxsackie B5 virus

P.#	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	u	v
1	<16	*16	*16	<16	<16	<16	<16	<16	<16	*16	*16	<16	*32	<16	<16	Pos	Neg	<32	<32	<32	<32	<32
2	<16	<16	*32	<16	<16	<16	<16	<16	<16	<16	*16	<16	<16	<16	<16	Pos	Neg	<32	<32	<32	<32	<32
3	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	Neg	Neg	<32	<32	<32	<32	<32
4	*16	<16	*16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	*64	Pos	Neg	<32	<32	<32	<32	<32
5	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	Pos	Neg	<32	<32	<32	*256	<32
6	<16	<16	<16	*16	<16	<16	<16	<16	<16	*16	<16	<16	<16	<16	<16	Neg	Neg	<32	<32	<32	<32	<32
7	<16	<16	*16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	Neg	Neg	<32	<32	<32	<32	<32
8	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	*32	Neg	Neg	<32	<32	<32	*32	<32
9	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	*32	<16	*32	Pos	Neg	<32	<32	<32	*32	<32
10	*32	<16	*32	<16	*16	<16	<16	<16	<16	<16	<16	<16	<16	<16	*64	Pos	Neg	<32	<32	<32	*32	<32
11	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	*16	Pos	Neg	<32	*32	<32	*32	<32
12	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	*128	<16	<16	Neg	Neg	<32	*128	<32	*256	<32
13	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	*32	Pos	Neg	<32	<32	<32	<32	<32
C.																						
14	*32	<16	*64	<16	<16	<16	<16	*16	<16	<16	<16	<16	<16	<16	<16	Pos	Neg	*32	<32	<32	*64	<32
15	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	*32	<16	<16	<16	<16	Pos	Neg	<32	*32	<32	<32	<32
16	*64	<16	*64	*16	<16	<16	<16	*16	*32	*32	<16	<16	<16	<16	*64	Pos	Neg	<32	<32	<32	*32	<32
17	<16	<16	*32	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	*128	<16	*32	Pos	Neg	<32	<32	<32	<32
18	*32	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	*32	Pos	Neg	<32	<32	<32	<32
19	<16	*64	*64	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	*16	<16	*32	Pos	Neg	<32	<32	<32	<32
20	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	*32	<16	*32	Neg	Neg	<32	<32	<32	<32
21	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	*64	Pos	Neg	<32	<32	<32	<32
22	<16	<16	*32	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	*64	Pos	Neg	<32	<32	<32	<32
23	<16	<16	*32	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	*32	<16	*32	Pos	Neg	<32	<32	<32	<32
24	<16	*16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	*32	<16	<16	Pos	Neg	<32	<32	<32	<32	<32
25	*32	<16	*32	<16	<16	<16	<16	<16	<16	*32	<16	<16	<16	<16	*64	Pos	Neg	<32	<32	<32	<32	<32
26	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	*32	Pos	Neg	<32	<32	<32	<32	<32

The occurrence of soluble immune complexes in the sera of seven out of thirteen newly diagnosed insulin-dependent diabetics exhibited a high degree of correlation with the presence of anti-islet cell antibodies ($p<0.01$) while no correlation was found with the prevalence and titre of anti-Coxsackie B4 antibodies.

3.3.11. Detection of anti-insulin antibodies

All diabetic sera were tested for the presence of anti-insulin antibodies. No antibodies were detected in the newly diagnosed insulin-dependent diabetics.

The sera of all the thirty-two diabetics treated with bovine or porcine insulin had increased insulin-binding activity indicating the presence of antibody to insulin. The titre varied from 26-286μU/ml irrespective of the duration of insulin therapy (between 2-42 years), the dosage of insulin used or the sex of the patient (Figure 22). The sera of all insulin-independent diabetics were negative for anti-insulin antibodies.

3.3.12. Correlation of the titre of anti-insulin antibodies and the presence of complexes and duration of treatment

All patients with high titres of anti-insulin antibodies (>300uU/ml) had soluble immune complexes in their serum (Figure 23). When the insulin-treated diabetics were subdivided according to whether they developed diabetes before or after the age of 30 years, immune complexes were found more frequently in the presence of moderate titres of anti-insulin antibodies in the early onset, compared to the late onset, diabetics ($p<0.05$) (Figure 24). Immune complexes occurred more commonly in patients treated with insulin for 10 years or more (16 out of 23) than in those receiving insulin for less than 10 years (1 out of 9) ($p<0.001$).

Fig. 22 The correlation between the occurrence of immune complexes and insulin antibodies in the thirty-two insulin-treated diabetics and the duration of insulin treatment.

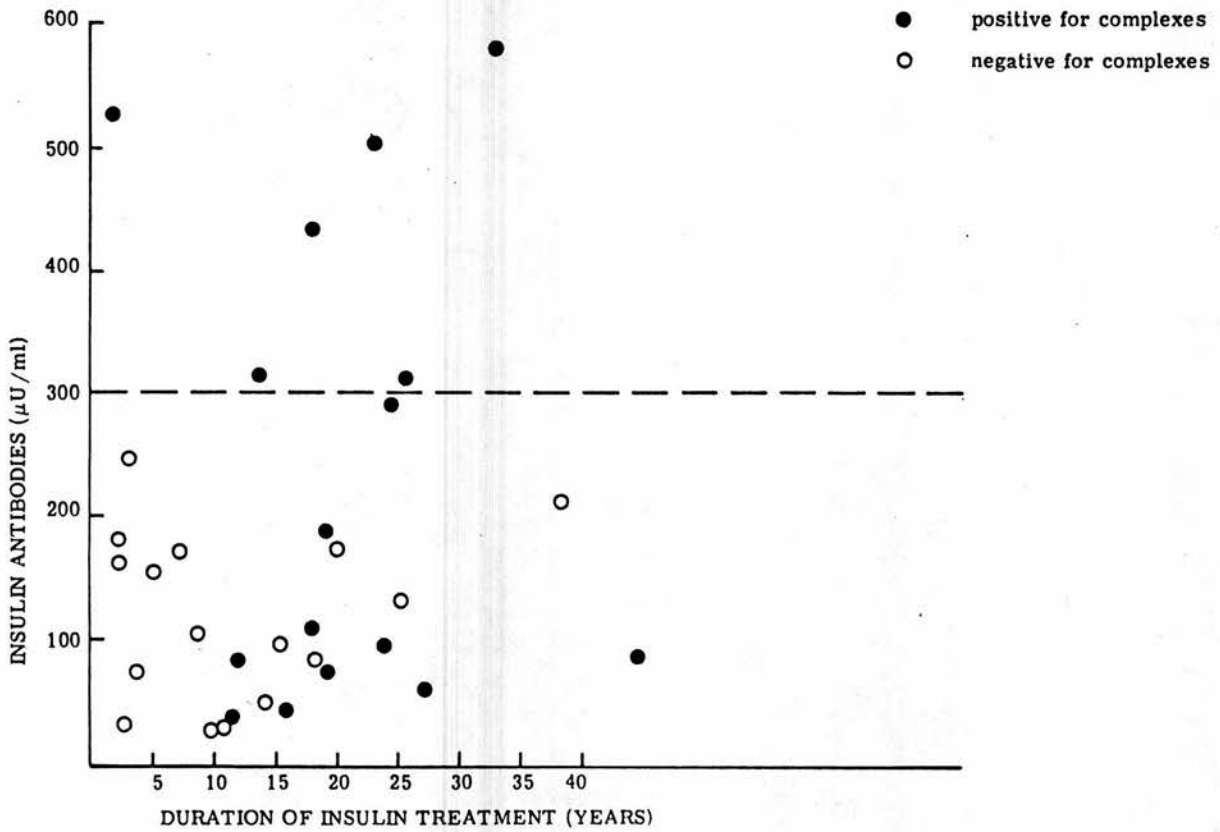


Fig. 23 The correlation between the titre of insulin antibodies and the amount of immune complexes in the sera from thirty-two insulin-treated diabetics.

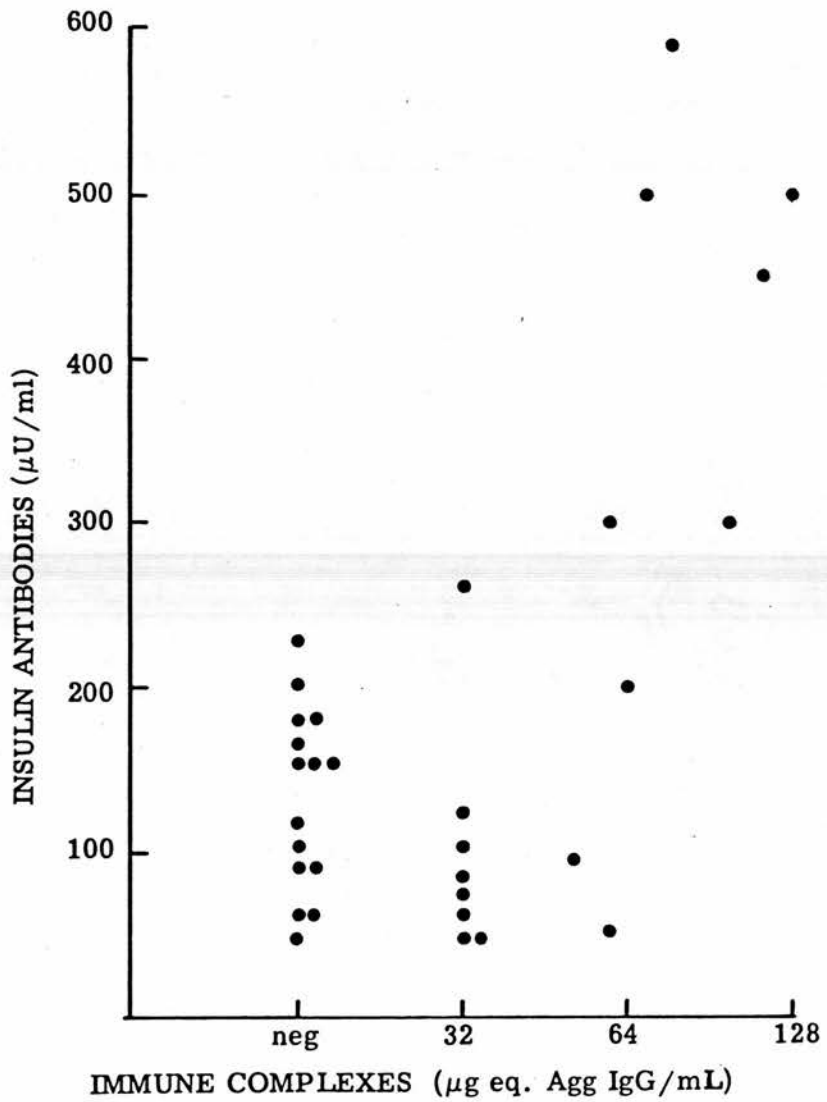
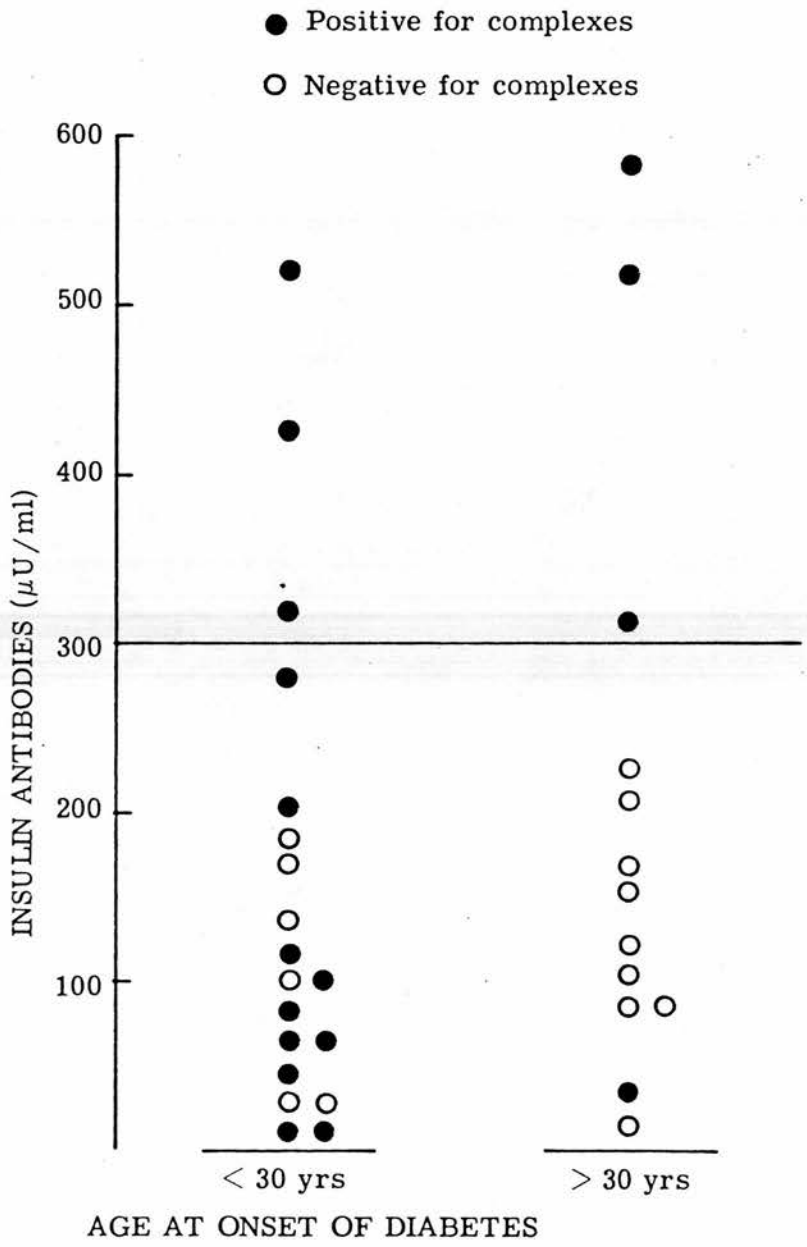


Fig. 24 The prevelance of immune complexes and titres of insulin antibodies in insulin-treated diabetics according to the age at onset of the diabetes.



3.3.13. Diabetic complications and the presence of complexes

Thirty-two insulin-dependent diabetics were examined for clinically manifested complications including diabetic retinopathy (defined by the presence of more than 3 microaneurisms, retinal haemorrhages, exudate or new blood vessel formation), or diabetic nephropathy (defined by a permanent proteinurea with or without abnormal kidney function).

As shown in Figure 25, sixteen patients had complications, and twelve of these had soluble immune complexes compared with only five of the remaining sixteen patients. It was found that immune complexes in the serum was associated with late diabetic complications only in patients who had suffered from diabetes for more than 13 years.

High titres of anti-insulin antibodies ($>300\mu U/ml$) were associated with the presence of diabetic complications. Four of the six patients with insulin antibodies in the serum at a titre of $>300\mu U/ml$ had late diabetic complications and has suffered from diabetes for 15 years or more. In the other two, the duration of diabetes was 2 and 13 years respectively.

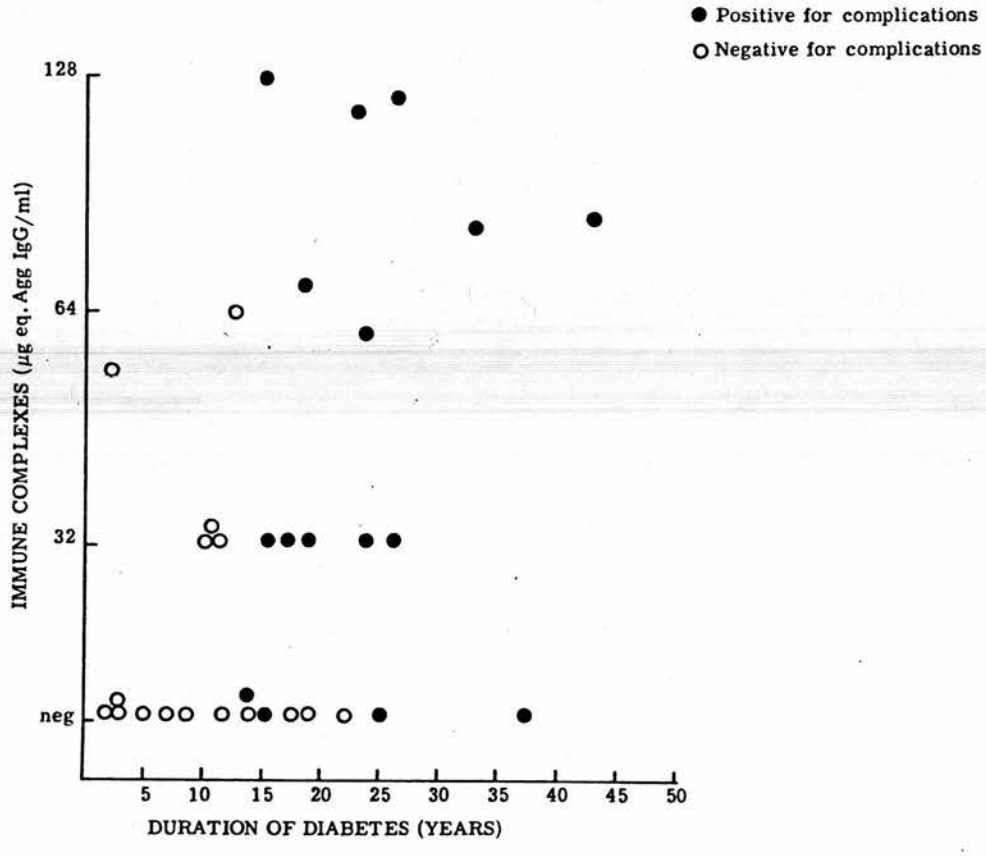
3.4. RAT SPLEEN LEUCOCYTE RADIOIMMUNOASSAY

3.4.1. Optimal conditions for the assay

3.4.1.1. Concentration of RSL

100ul volumes of different concentrations of RSL were incubated with various concentrations of aggregated IgG in 50µl NHS inactivated by 20mM EDTA and absorbed by rat erythrocytes or medium alone. The cells were incubated for 1 hour at $37^{o}C$, washed 3 times with Eagle's medium and then incubated at $4^{o}C$ for 30 min with 30µl ^{125}I anti-human

Fig. 25 The correlation between the amount of immune complexes and the presence of late diabetic complications according to the duration of diabetes in the thirty-two insulin-treated patients.



IgG. Unbound radiolabelled antibody was removed by washing the cells in 1% BSA in PBS. As shown in Figure 26, $4x10^6$ RSL was found to be the optimal concentration of cells required for the assay. Further experiments using other volumes of NHS (25µl, 100µl) did not detect any increase in the sensitivity of the assay (Figure 27).

3.4.1.2. Concentration of ^{125}I anti-human IgG

Increasing amounts of radiolabelled anti-human IgG were added to a constant amount of aggregated IgG bound to $4x10^6$ RSL. As shown in Figure 28, 30µl of 1% BSA in phosphate buffer containing approximately 15µg radiolabelled anti-human IgG was found to give the highest level of specific uptake (uptake by cells incubated with NHS and added aggregated IgG minus uptake by cells incubated with NHS alone).

3.4.1.3. Incubation time

$4x10^6$ RSL in 100µl Eagle's medium were incubated with various concentrations of aggregated IgG in 50µl inactivated and absorbed serum for 30 min, 1 hour, 2 hours or 4 hours. The cells were washed and radiolabelled anti-human IgG was added. Figure 29 shows the radiolabel uptake after each period of incubation. The degree of binding was maximal at 1 hour.

3.4.1.4. EDTA concentration

The concentration of EDTA needed to inactivate the serum was estimated. $4x10^6$ RSL in 100µl Eagle's medium were incubated with a constant amount of aggregated IgG added to 50µl inactivated and absorbed serum. As shown in Figure 30, serum was totally inactivated by 8mM EDTA. In all subsequent experiments, a final concentration of 10mM EDTA in serum was used.

Fig. 26 The uptake of radiolabelled anti-human IgG by various concentrations of RSL incubated with different amounts of aggregated IgG. S = normal human serum.

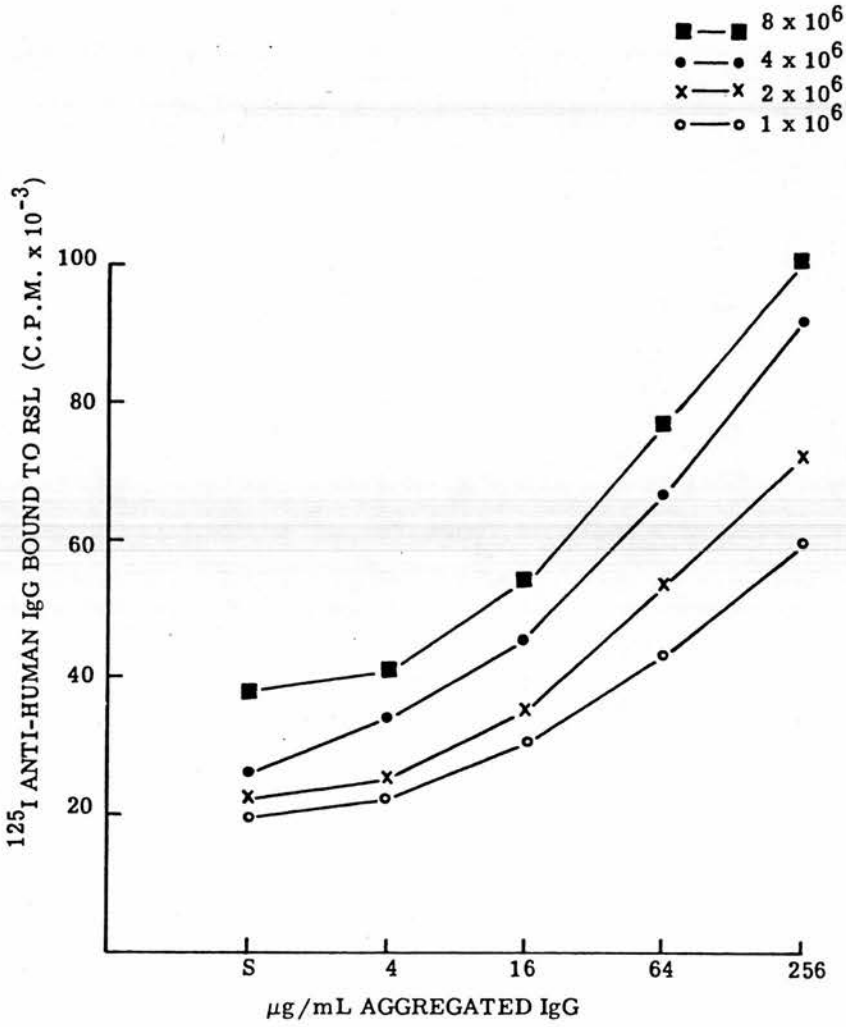


Fig. 27 The uptake of radiolabelled anti-human IgG by RSL incubated with different volumes of normal human serum containing various amounts of aggregated IgG. S = normal human serum.

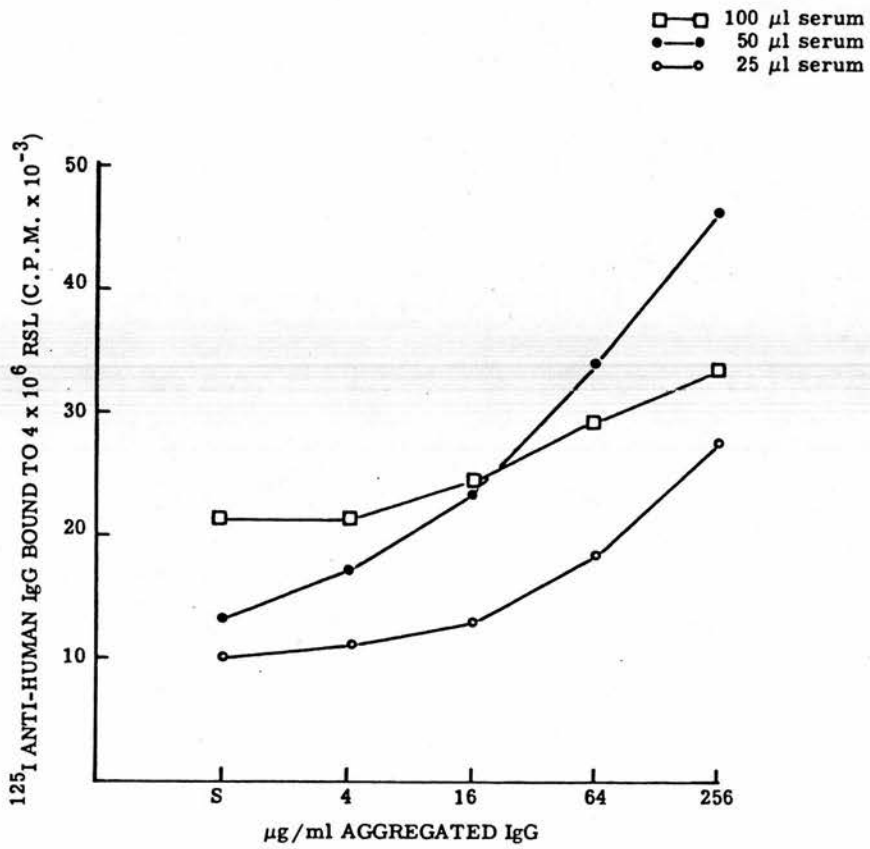


Fig. 28 Titration of radiolabelled anti-human IgG for the demonstration of aggregated IgG in serum: RSL radioimmunoassay.

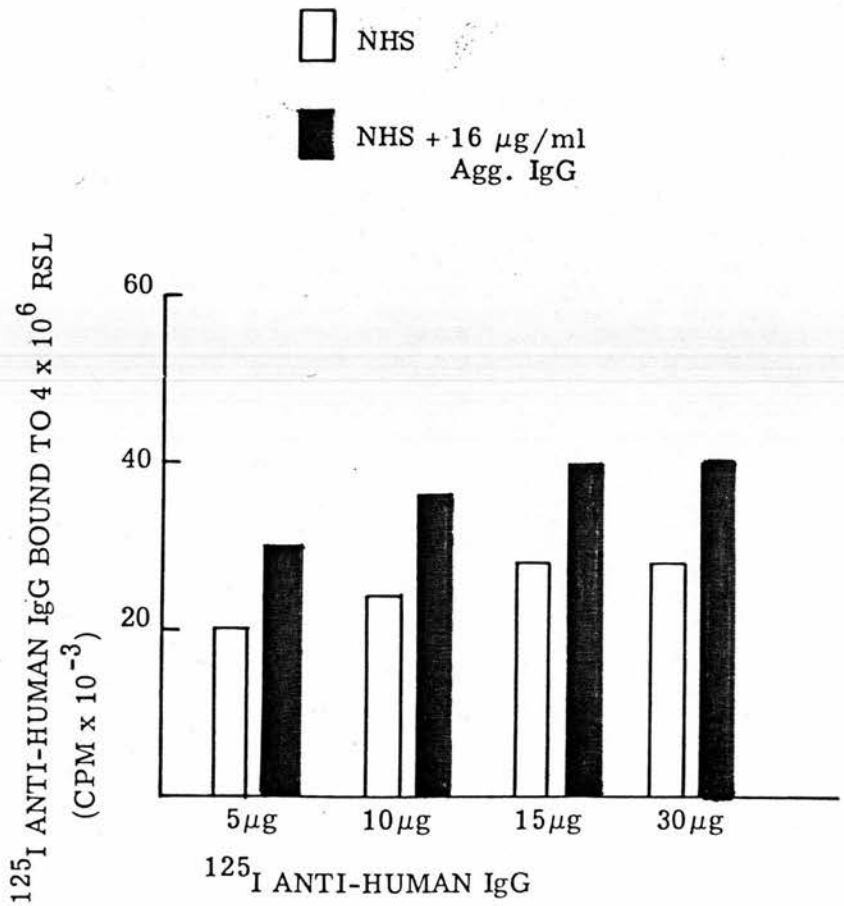


Fig. 29 The uptake of radiolabelled anti-human IgG by RSL incubated with various amounts of aggregated IgG: the effect of different incubation times. S = normal human serum.

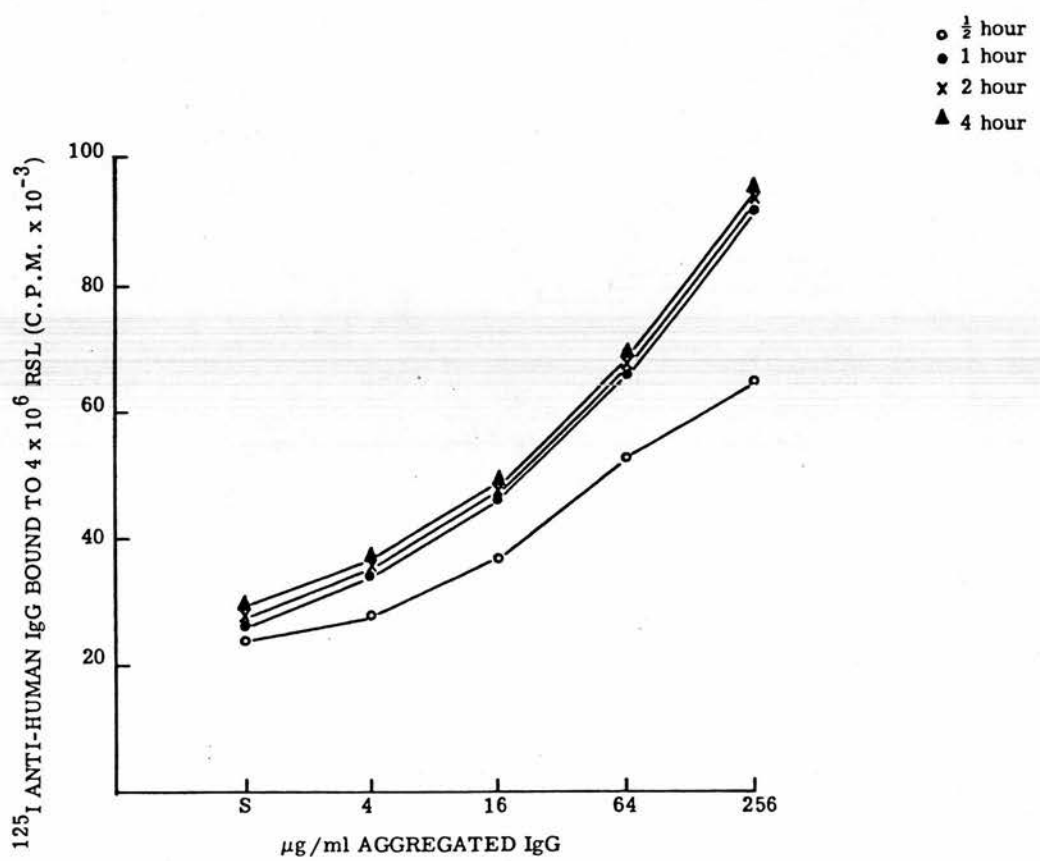
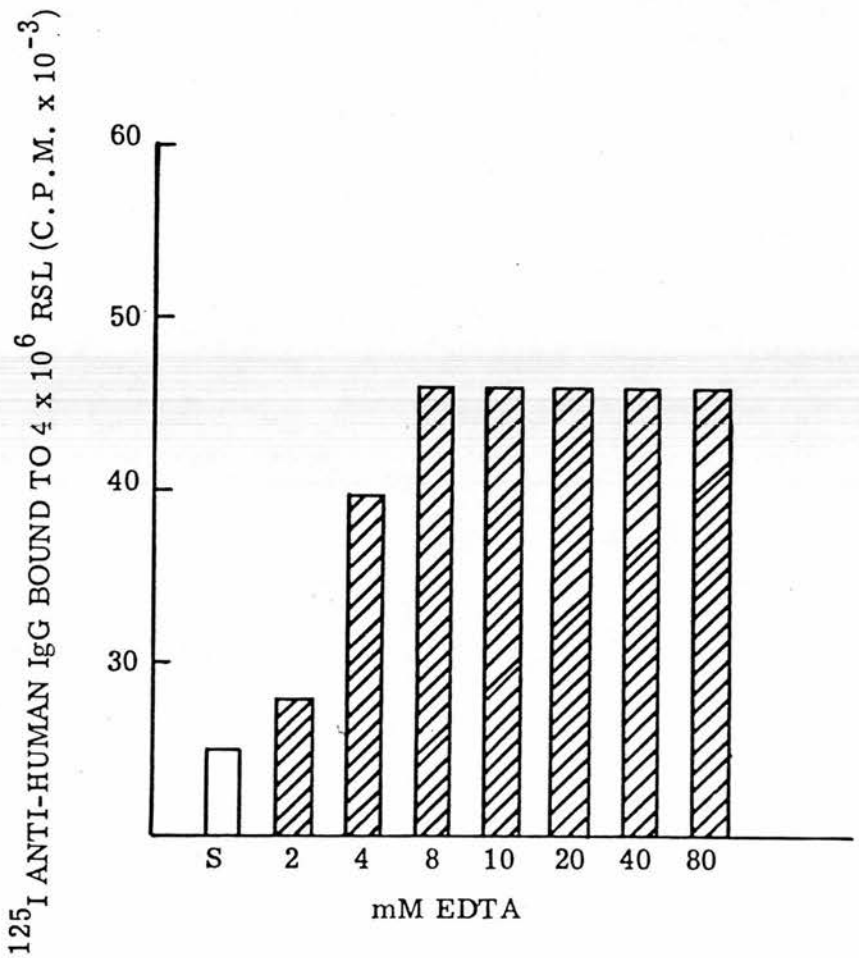


Fig. 30 Inactivation of normal human serum with various concentrations of EDTA as shown by the effect on binding of a fixed amount of aggregated IgG (16μg/ml) to RSL. S = normal human serum.



3.4.2. Standard curve and sensitivity of the assay

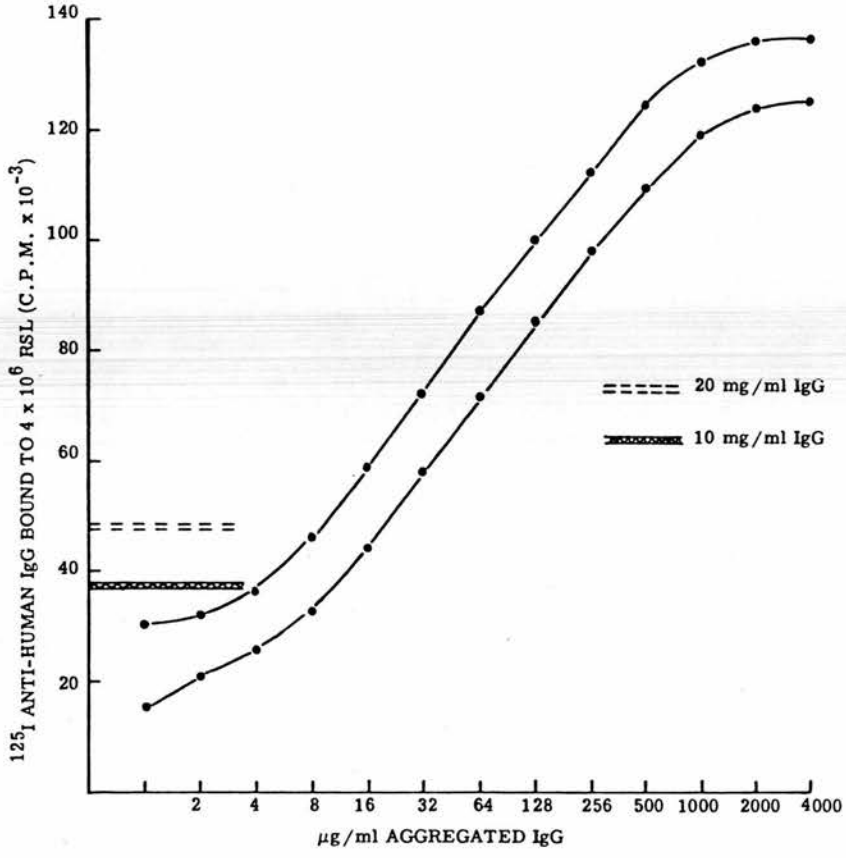
$4x10^6$ RSL in 100µl Eagle's medium were incubated with 50µl pooled NHS (inactivated with 10mM EDTA and absorbed with rat erythrocytes) containing varying concentrations of aggregated IgG (2µg - 4mg/ml). The amount of radiolabelled anti-human IgG bound to RSL was estimated as described in 3.4.1.1. As shown in Figure 31, the radioactivity bound to $4x10^6$ RSL in medium containing 2µg/ml aggregated IgG was higher than that bound to the same number of cells in medium alone. Similarly the radioactivity bound to $4x10^6$ RSL in serum containing 4µg/ml aggregated IgG was higher than that bound to the same number of cells in serum alone. The amount of radiolabelled antibody uptake by the cells was directly related to the amount of aggregated IgG present in the serum. In further experiments on the detection of immune complexes or of aggregates present in human serum, the results were referred to this curve and expressed as µg aggregated IgG equivalent per ml (µg eq.AggIgG/ml). The sensitivity of the assay was therefore 4ug/ml in neat serum.

3.4.3. Binding of monomeric IgG to RSL

$4x10^6$ RSL in 100µl Eagle's medium were incubated with 50µl of EDTA-inactivated and rat erythrocyte-absorbed NHS with or without 10mg/ml or 20mg/ml added IgG. Human sera with low, medium, and high IgG levels were incubated in 50µl volumes with RSL after inactivation and absorption. The binding of IgG to RSL was estimated by referring to the standard curve of radiolabelled antibody uptake by RSL previously incubated with various amounts of aggregated IgG in serum.

The results indicate that the difference in the uptake of label between RSL incubated with serum of low and high IgG levels was equivalent to approximately 4µg eq.AggIgG/ml. The difference in

Fig. 31 Standard curve of radiolabelled anti-human IgG bound to RSL incubated with various amounts of aggregated IgG added to serum or medium. Horizontal bars indicate the degree of binding of label to RSL incubated with two different concentrations of monomeric IgG. Upper line: serum; lower line: medium.



the uptake of label by cells incubated with NHS and those incubated with NHS plus added IgG was equal to 4µg eq.AggIgG/ml in the case of 10mg/ml added IgG, and 8µg eq.AggIgG/ml in the case of 20mg/ml (Figure 31).

3.4.4. Effects of complement on aggregates binding to RSL

Experiments were carried out to determine whether complement activity present in NHS inhibits the binding of aggregated IgG to RSL and/or induces the release of bound aggregates.

3.4.4.1. Inhibition of binding

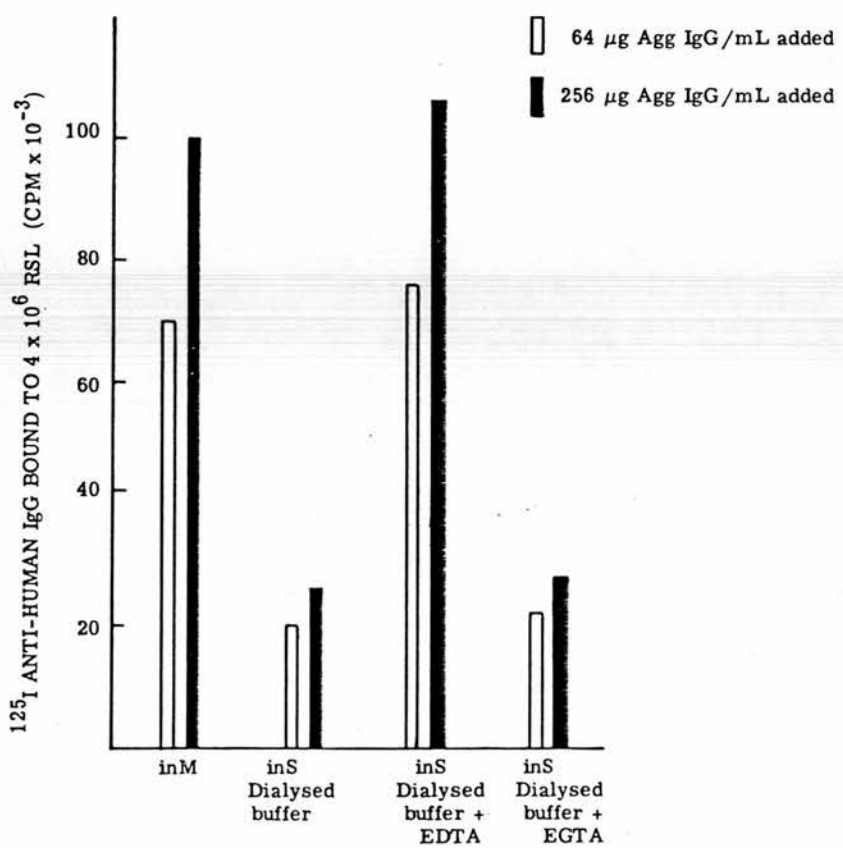
Serum samples were dialysed against CFD or CFD containing a final concentration of 20mM EDTA or EGTA. Aggregated IgG at 256 or 64µg/ml were added to the dialysed sera. The sera then were incubated for 1 hour at 37°C in 50µl volumes with 100µl medium containing $4x10^6$ RSL. The binding of aggregated IgG to RSL was determined after incubation with 30µl radiolabelled anti-human IgG.

The inactivation of serum with EGTA was found to have no effect on the binding of aggregated IgG to RSL. In contrast, inactivation with EDTA resulted in a significant increase in the degree of binding compared to that found using serum dialysed against CFD alone (Figure 32).

3.4.4.2. Release of bound aggregates

Aggregated IgG in medium (256 and 64µg/ml) was incubated for 1 hour at 37°C with $4x10^6$ RSL in 100µl medium. After washing the cells with Eagle's medium and reconstituting the volume to 100µl, 50µl EDTA-treated or heat-inactivated NHS were added to the cell suspensions. The cells and added serum were incubated for 1 hour at 37°C, and

Fig. 32 The uptake of radiolabelled anti-human IgG by RSL incubated with medium or with serum containing aggregated IgG: the effect of inactivation by EDTA or EGTA. M = medium; S = serum.



then the cells were washed 3 times with Eagle's medium. The degree of binding of aggregated IgG to the cells was assessed as described above.

As shown in Figure 33, the radiolabel uptake by RSL incubated with aggregated IgG and subsequently with NHS was negligible and comparable with that shown by RSL incubated with medium alone. In contrast, the uptake by cells incubated with aggregated IgG and then with inactivated sera was comparable with that of cells incubated with aggregated IgG and not treated with serum. These results indicate that NHS induces the release of bound aggregates from RSL.

3.4.5. Formation of aggregates by heat treatment of serum

Serum samples obtained from 20 normal donors were inactivated with 10mM EDTA and absorbed with rat erythrocytes. Each serum sample was divided into 2 aliquots, one of which was heated at 56°C for 30 min. 50µl of each aliquot was incubated for 1 hour at 37°C with $4x10^6$ RSL in 100µl Eagle's medium. The cells were then washed 3 times with Eagle's medium and incubated at 4°C for 30 min with 30µl ^{125}I anti-human IgG.

By referring to a standard curve of radiolabelled antibody uptake by RSL previously incubated with various concentrations of aggregated IgG, the amount of aggregates formed in each serum tested was determined. As shown in Figure 34, 0-128 (mean 37) µg eq.AggIgG/ml were detected in the heat-treated normal sera.

3.4.6. Detection of anti-rat erythrocyte antibodies

Serum samples obtained from 40 human donors were tested by haemagglutination assay for the presence of anti-rat erythrocyte antibodies. 20µl neat or serially diluted (1:2 to 1:256) serum were

Fig. 33 The effect of fresh, heat-inactivated and EDTA treated serum on the aggregates bound to RSL.

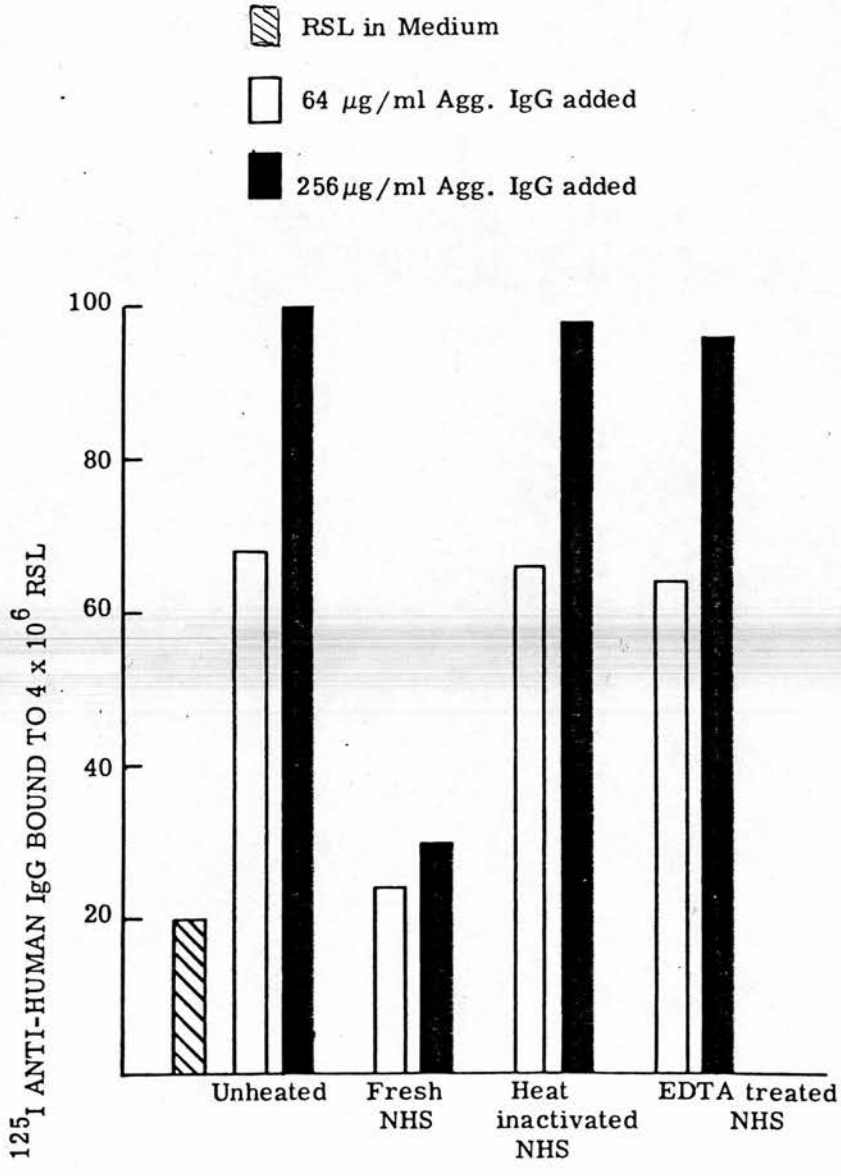
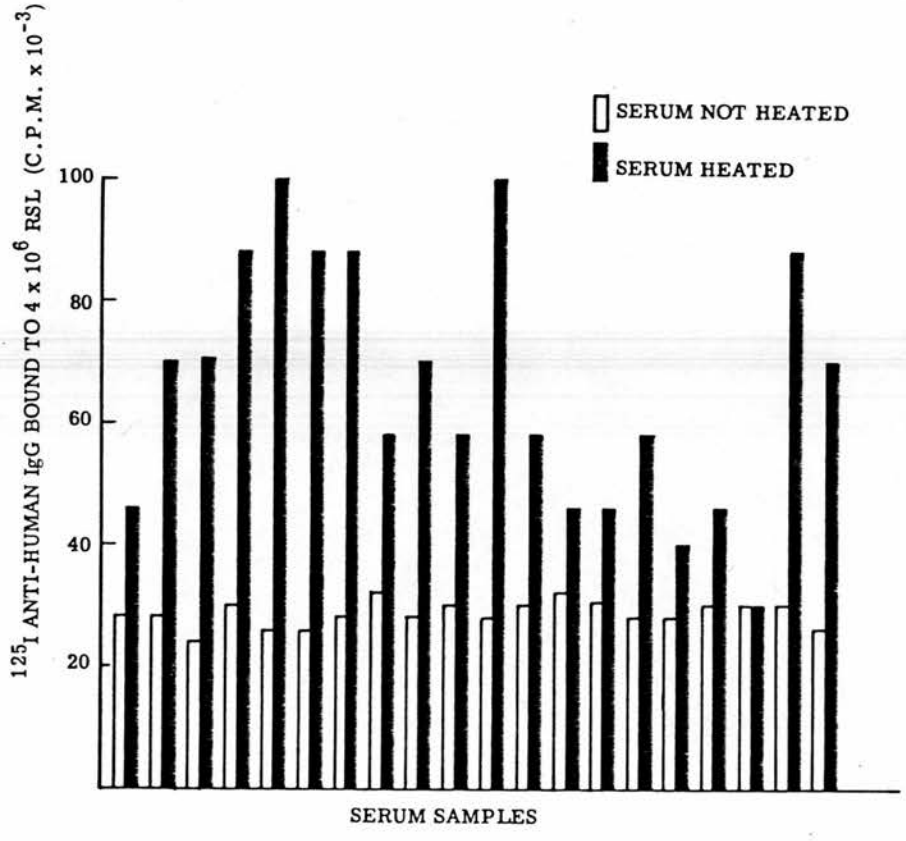


Fig. 34 The uptake of radiolabelled anti-human IgG by RSL incubated with normal human sera: the effect of heat-inactivation of the sera.



incubated at room temperature overnight with 20µl 2% rat erythrocytes in PBS. The results indicate that all sera tested had anti-rat erythrocyte antibodies with titres up to 1:128 (Figure 35). 10 serum samples from patients with thyroid disorders or diabetics were compared with their controls for haemagglutination titre. No significant differences were detected. The activity was completely removed by a single absorption with rat erythrocytes.

3.4.7. Absence of residual anti-rat leucocyte antibodies in sera absorbed with rat erythrocytes

Although one absorption with rat erythrocytes was effective in removing all anti-rat erythrocyte activity from serum samples, the possibility existed that residual antibodies directed toward other antigenic determinants, for example those present on rat leucocytes, could still be interferring with the RSL assay. Further experiments were therefore carried out in which sera were absorbed once or twice with rat erythrocytes or once with rat erythrocytes and once with rat thymocytes and then tested for binding to RSL in the assay. These extra absorption stages had no significant effect on the amount of binding to RSL by any of the absorbed sera (74,230±6,940 cpm after one absorption with rat erythrocytes; 72,700±6,270 cpm after two absorptions; 70,576±4,320 cpm after one absorption with rat erythrocytes and one with rat thymocytes). Further evidence that the elevated levels of binding found in positive sera were due to the presence of immune complexes and not to the presence of anti-leucocyte antibodies was provided by an experiment in which binding of label was completely inhibited by prior incubation of the RSL with aggregated autologous rat IgG (Table 14).

Fig. 35 Anti-rat erythrocyte antibody titres of forty normal human sera.

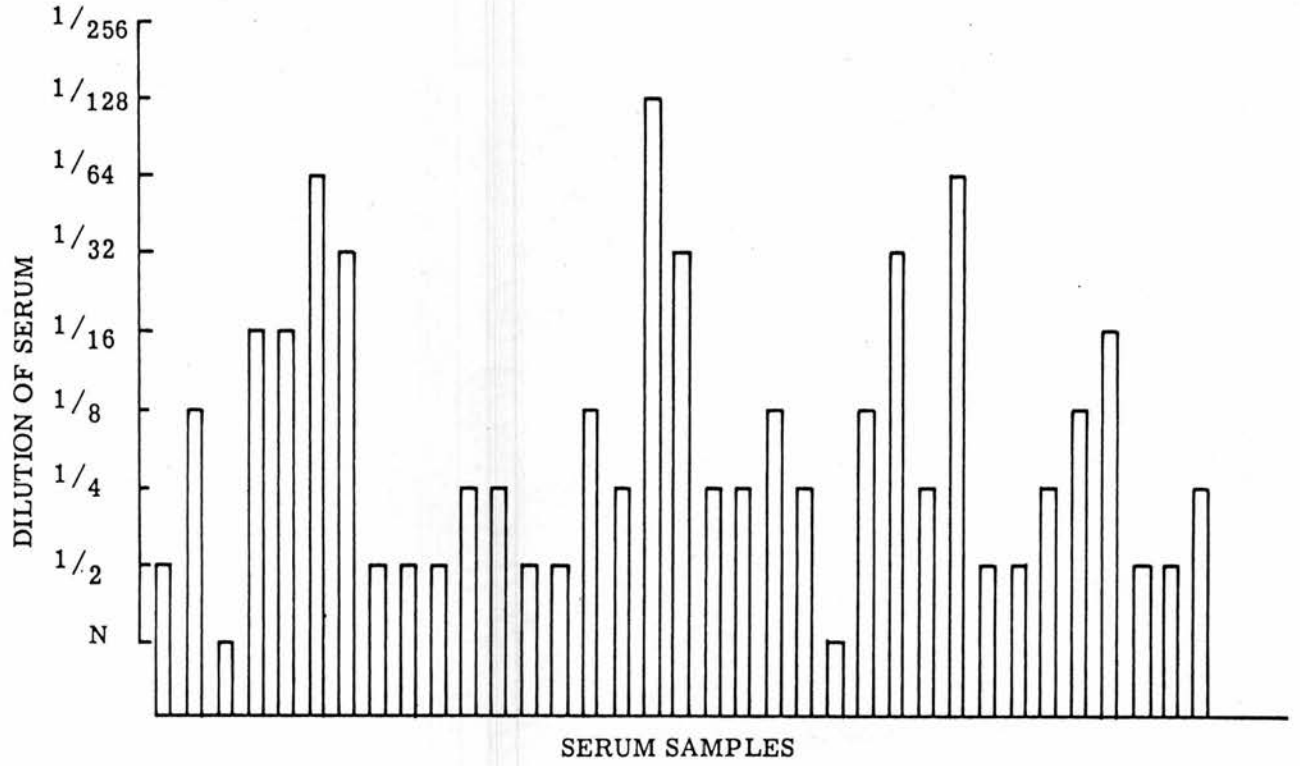


Table 14 Inhibition of binding of aggregated human IgG by aggregated autologous rat IgG.

Percentage binding of test samples relative to controls	Untreated RSL	Treated RSL
	171, 143, 157	107, 92, 111
Mean	153	103

3.4.8. Detection and quantitation of immune complexes in sera from patients

RSL were incubated with sera from twenty-one Hashimoto, twenty-eight hypothyroid and twenty thyrotoxic patients, and with thirteen newly diagnosed insulin-dependent diabetics, thirty-two diabetics treated with insulin, thirty-one diabetics treated with OHA, twenty-one diabetics treated by restriction of diet and one hundred and sixty normal controls. The sera were inactivated with 10mM EDTA and absorbed with rat erythrocytes before being used.

Radiolabelled anti-IgG antibody was added to the washed cells. The amounts of radioactivity bound to the cells were referred to a standard curve of radiolabelled antibody uptake by cells previously incubated with varying amounts of aggregated IgG. More than 4µg eq.AggIgG/ml were detected in 52.1% of sera from patients with thyroid disorders and in 39.5% of diabetic sera (Table 15).

3.4.9. Comparison of results obtained using the RSL and Raji cell radioimmunoassays

The results obtained using the RSL assay were compared with those obtained using the Raji cell assay. As shown in Table 15, a higher percentage of sera tested were positive using RSL than using Raji cells. In addition, the amounts of complexes detected by the Raji cell radioimmunoassay were higher in all positive sera. All sera found positive using Raji cells were also positive by the RSL assay.

Table 15 Detection and quantitation of soluble immune complexes in sera from patients with thyroid disorders and from diabetics, using the RSL and Raji cell radioimmunoassays.

		Raji cell radioimmunoassay				RSL radioimmunoassay		
	Diagnosis	No	No. positive	%	µg eq.AggIgG/ml	No. positive	%	µg eq.AggIgG/ml
Thyroid disorders	Hashimoto thyroiditis	21	17	80.9	32-500, mean 182	19	90.5	16-256, mean 116
	Primary hypothyroidism	28	10	35.7	32-64, mean 42	13	46.4	6-64, mean 24
	Thyrotoxicosis	20	4	20	32-64, mean 40	6	30	6-32, mean 22
	Controls	69	2	2.8	32	4	5.7	6-16, mean 10
Diabetics	Newly diagnosed insulin-dependent	13	7	53.8	32-64, mean 46	8	61.5	6-64, mean 22
	Insulin-dependent	32	17	53	32-128, mean 60	21	65.6	8-128, mean 32
	OHA	31	3	9.6	32-64, mean 42	5	16.1	6-32, mean 19
	Diet	21	2	9.5	32	3	14.3	6-16, mean 10
	Controls	91	5	5.4	32	8	8.7	6-32, mean 12

4. DISCUSSION

Speculation as to the role of immune complexes in the pathogenesis of disease has prompted many workers to attempt to develop methods for the detection of these complexes. At the present time there are a large number of methods available for the detection of immune complexes in serum, although most if not all of these methods are of limited value, for while they suggest that immune complexes may be present, they can give no indication of the nature of the antigenic component involved or even if antigen is present.

In the present study efforts were made both to use established methods with or without modification and also to develop a new and sensitive method for the detection and quantitation of soluble immune complexes in the sera of patients with thyroid disorders or with diabetes mellitus.

At the initiation of this study few of the available methods were thought to be sufficiently sensitive. The method of inhibition of antibody-dependent cell-mediated cytotoxicity (ADCC) which was described by Jewell and MacLennan (1973) to detect immune complexes in the sera of patients with inflammatory bowel disease appeared to be the most sensitive and reproducible. A modification of this method was under trial in this laboratory in which chicken red blood cells were substituted for the Chang liver cells used as target cells in the Jewell and MacLennan assay. The variable results obtained using different samples of human lymphocytes as effector cells together with the difficulties encountered in obtaining a regular supply of human blood prompted the development of a further modification using other lymphocytes as effector cells. Rat spleen lymphocytes were found to be effective in inducing antibody-dependent cell-mediated cytotoxicity and were used in this study in an inhibition assay for the detection of immune complexes.

Using this assay it was possible to demonstrate a marked inhibition of ADCC by the sera of patients with Hashimoto thyroiditis and primary hypothyroidism and a less marked inhibition in the case of thyrotoxic patients as compared with their age- and sex-matched controls.

It is possible that this inhibition may be due not to the presence of antigen-antibody complexes but to the presence of aggregated IgG formed as a result of heat inactivation of the sera. Heat inactivation was found to be necessary to prevent lysis of the target cells by human serum in the absence of added antibody. This lysis could be mediated either by natural antibody or by complement. The finding that chicken serum lysed autologous erythrocytes would appear to exclude the presence of natural antibody. The participation of target cell-bound complement in cell-mediated cytotoxicity as reported by many workers (Perlmann et al., 1969; O'Neill et al., 1973; Lustig and Bianco, 1976) and suggested by the results of Eden and co-workers (1973) and Scornik and Drewinko (1975), does not appear to be involved, since the effect was also observed in the absence of lymphoid cells or in the presence of cytochalasin B, an agent which effectively blocks cell-mediated cytotoxicity (Gelfand et al., 1975). Consequently, it is likely that the lysis was mediated directly by complement activated by the chicken erythrocytes in a manner similar to that reported for mouse spleen cells (Pepys, 1974), tumour cells (Okada and Baba, 1974), rabbit cells (Platts-Mills and Ishizaka, 1974) and human and rabbit platelets (Zimmerman and Kolb, 1976). The role of complement in target cell lysis is clearly indicated by the observation that complement activity and inhibitory activity could be separated by Sephadex G-200 fractionation and that the inhibitory activity was demonstrable in the absence of heat inactivation.

The observed inhibition of ADCC by serum samples from patients with thyroid disorders is thought more likely to be due to the presence of immune complexes than to that of aggregated IgG. This was initially suggested by the observation that the degree of inhibition did not correlate with the levels of serum IgG. At a given amount of IgG in both test sera and their controls there was still a significant difference in the percentage of inhibition. Further evidence was provided by the results of experiments in which serum samples were fractionated on Sephadex G-200. These demonstrated that inhibitory activity was localized predominantly in front of the ascending limb of the IgG profile, and not in those fractions in which aggregated IgG should appear. Heat inactivation of serum was in fact found to induce the formation of aggregated IgG; sera which were heat-inactivated and then fractionated by Sephadex G-200 showed the formation of inhibitory material in the 19S peak. Thus it appears that the inhibition of ADCC by heat-inactivated test sera is due to the presence of both antigen-antibody complexes and aggregated IgG. The formation of aggregates and their quantitation in heat-inactivated normal serum is discussed below with respect to the RSL assay.

The sensitivity of the ADCC inhibition assay was found to be around 250µg/ml in neat serum and it was felt that this degree of sensitivity was insufficient to detect small amounts of immune complexes in the test sera. Efforts to increase the sensitivity of the ADCC inhibition assay by using a low effector to target cell ratio were unsuccessful due to problems associated with higher levels of inhibition by control sera. Further work was carried out to increase the sensitivity of the assay and to circumvent the need for heat inactivation (Barkas, Al-khateeb and Irvine, submitted for publication).

In order to confirm and extend the data obtained in the ADCC inhibition assay, experiments were carried out using the C1q deviation test which was reported by Sobel and co-workers (1975) to be a more sensitive and reproducible method for the detection of immune complexes. These workers described a new method based on the deviation of radiolabelled C1q from binding to sensitized sheep red blood cells. According to these authors, the sensitivity of the assay was very high in the sense that it could detect very minute amounts of complexes. In the present study this assay was found to be moderately sensitive in detecting aggregated IgG in medium but not in the detection of aggregates added to serum samples at levels less than approximately 100µg/ml. It is likely that this lack of sensitivity was due to the fact that aggregates were already present in the sera as a consequence of heat inactivation. The sensitivity of the assay with respect to added aggregates was found to vary markedly from one sample of normal human serum to another.

Many of the difficulties encountered with these two techniques may be attributed to the need to heat-inactivate sera before testing for the presence of immune complexes. During the progress of this work, a new method for the detection of complexes was reported in which heat inactivation was not required. Theofilopolous and co-workers (1974a) screened different lymphoblastoid cell lines for the presence of membrane-bound Ig and receptors for IgG Fc and complement in order to find a suitable model for measuring the binding of immune complexes. They found that Raji cells were the most suitable as these cells lacked membrane bound IgG and possessed receptors for IgG Fc, C3b and C3d. Using these cells they developed a radioimmunoassay (Theofilopolous et al., 1976) which is based on the binding of immune

complexes via complement receptors and the subsequent detection of these complexes using radiolabelled anti-human IgG. Using this technique they reported the presence of immune complexes in a number of pathological sera. Quantitation of the complexes was carried out by referring to a standard curve of radiolabelled antibody uptake by cells previously incubated with varying amounts of aggregated IgG in normal human serum. This was based on the observation that aggregated IgG possesses many of the properties of immune complexes in vitro (Christian, 1960a; Ishizaka et al., 1965).

In the present study, this assay was used to demonstrate and quantitate immune complexes in the sera of patients with thyroid disorders and diabetes. Among those patients with thyroid disorders, the highest levels were detected in sera from patients with Hashimoto thyroiditis while lesser amounts were found in the hypothyroid and thyrotoxic groups. In patients with diabetes, immune complexes were detected in the sera from juvenile insulin-dependent diabetics and from insulin-treated diabetics but not in the sera from patients treated with oral hypoglycaemic agents or by restriction of diet.

The sensitivity of the Raji cell radioimmunoassay was found to be approximately 6µg/ml of added aggregated IgG, corresponding to 24µg/ml in neat serum since all sera were used at a dilution of 1:4. This level of sensitivity is lower than that reported by Theofilopolous and co-workers, who were able to detect 1.6µg/ml (equivalent to 6µg/ml in neat serum. This apparent difference in sensitivity could be due to differences in the method of establishing the standard curve of radioactivity, since Theofilopolous and co-workers used lower amounts of IgG to be aggregated and subsequently added to 1:4 serum. However, experiments using similar amounts of IgG to form aggregates did not result in an increase in the sensitivity of the assay in the present

study. Alternatively, the difference in sensitivity might be attributed simply to the inherent variability associated with the culture of cell lines in different laboratories.

The failure to detect complexes in certain sera from the thyroid disorders and diabetes groups might be due either to the absence of complexes in these sera or to the fact that the complexes exist in a form unsuitable for detection in the Raji cell assay. Raji cells are thought to bind large complexes formed in 2-6X antigen excess (Theofilopolous et al., 1974b), and smaller complexes or those formed in antibody excess may not be detected.

Raji cells have been shown to bind monomeric IgG as well as aggregated IgG via their Fc receptors (Theofilopolous et al., 1974b). On this basis, the apparent detection of complexes in sera from the Hashimoto group could be attributed to the presence of higher levels of monomeric IgG in these sera as compared to their controls. In order to eliminate this possibility, experiments were carried out in which Fc receptors on Raji cells were blocked by incubation of these cells with normal rabbit serum. This treatment was found to prevent the binding of monomeric human IgG, contrary to the finding of Theofilopolous and co-workers (1974b) who reported that rabbit IgG did not bind to the Fc receptors on Raji cells. The observed difference in binding between Hashimoto sera and their controls was not affected by the use of cells pretreated with normal rabbit serum in the Raji cell radioimmunoassay, and this would appear to rule out the possibility that the higher levels of binding to Raji cells by Hashimoto sera were due to high levels of monomeric IgG in these sera.

The Raji cell radioimmunoassay was used in an attempt to characterize the antigenic component of the complexes detected in the sera from patients with thyroid disorders. Using radiolabelled anti-

human thyroglobulin antibody instead of radiolabelled anti-IgG antibody, it was possible to demonstrate significantly higher levels of binding of label to cells incubated with one third of the sera scored positive for the presence of complexes. This result indicates that in at least a proportion of these sera, the complexes present contain thyroglobulin or other antigens which react with antibody to thyroglobulin. The significance of this finding is discussed below.

A new and sensitive method for the detection of soluble complexes was developed during this study. This method is a radioimmunoassay using rat spleen leucocytes, and is based on the ability of these cells to bind complexes via Fc receptors and the subsequent detection of the IgG in the complexes by radiolabelled anti-human IgG. By referring to a standard curve of radiolabelled antibody uptake by cells previously incubated with varying amounts of aggregated IgG in EDTA-treated normal human serum, it is possible to estimate the amount of complexes in test sera. The radiolabelled antibody uptake was found to be proportional to the amount of aggregates added to the serum or medium. The amount of soluble complexes present in test sera was equated to an amount of aggregated IgG on the standard curve and expressed as µg aggregated IgG equivalents/ml.

The binding of aggregated IgG to RSL in medium was comparable to that in EDTA-inactivated serum. This indicates that the binding was mediated by Fc receptors and not complement receptors, possibly due to the presence of more Fc receptors than complement receptors or to the higher binding affinity of Fc receptors. The presence of complement activity was found to inhibit the binding of aggregates and also to induce the release of aggregates bound to RSL. The inactivation of serum samples by treatment with EDTA but not with

EGTA abolished this inhibitory activity, suggesting that complement was acting via the alternative pathway. Similar findings have been reported by Miller and co-workers (1973, 1974) for mouse lymphocytes and platelets. These authors also showed that complement serves as a sensitive regulator of the interaction between immune complexes and cell membranes.

The binding of label to RSL incubated with aggregated IgG in serum was slightly greater than that in medium, possibly due to the non-specific binding of small amounts of monomeric IgG to the cells. It was found that the uptake of label after the addition of 10mg/ml monomeric IgG to RSL was equivalent to that observed after the addition of 4µg/ml of aggregated IgG. Although this indicates that small amounts of monomeric IgG can bind to RSL, it is also possible that this finding may be attributed to the presence of small amounts of aggregates in the preparation of IgG used. The addition of 10mg/ml monomeric IgG to sera containing complexes did not significantly increase the binding of label to the cells. If it is assumed that differences in IgG levels among different human sera are unlikely to be much greater than 10mg/ml, this would appear to rule out the possibility that the higher levels of binding detected by this assay are due to the presence of monomeric IgG. In addition, only small differences in the uptake of label were detected using a wide range of normal human sera.

RSL have previously been used to detect immune complexes by the inhibition of ADCC (Barkas et al., 1976). The sensitivity for the detection of aggregates has subsequently been increased to 0.6µg/ml in medium or 6µg/ml in serum (Barkas, Al-khateeb and Irvine, submitted for publication). The RSL radioimmunoassay has considerable advantages over this technique, being extremely simple and rapid to

perform and also somewhat more sensitive to the presence of aggregates in serum. The assay is also considerably simpler than the rather similar assay using Raji cells. A further advantage over the Raji cell radioimmunoassay is that the maintenance of a cell-line in culture is not required.

The RSL radioimmunoassay was used to detect and quantitate complexes in those serum samples used for the Raji cell assay. Complexes were found in a higher percentage of sera from patients with thyroid disorders or diabetes using the RSL assay than using the Raji cell assay. This may be due to the higher sensitivity of the RSL assay which allows the detection of 4µg/ml aggregated IgG in neat serum. In the Raji cell radioimmunoassay it was necessary to dilute the test serum to 1:4 in order to minimise the inhibitory effect of complement on the binding of aggregates to these cells (Theofilopolous et al., 1976), and the limit of detection was 6µg/ml aggregated IgG (equivalent to 24µg/ml aggregates in neat serum). In general, the amount of complexes detected in a given serum was higher by the Raji cell method, possibly reflecting the different modes of binding in the two methods. Binding to the Raji cells is mediated by complement receptors and to a lesser extent by Fc receptors whereas binding to the RSL is via Fc receptors alone, as indicated by the similar levels of binding of aggregates in medium or EDTA-treated serum.

One possible factor which could give rise to spurious results in the RSL assay is the presence, in the sera tested, of IgG antibodies to rat erythrocytes or leucocytes. Anti-rat erythrocyte antibodies in human sera have been described by McDonald (1973) and by Salaman and co-workers (1975), who also mentioned the presence of anti-leucocyte antibodies. These authors suggested that these antibodies arise probably as a consequence of previous infection by gram-negative

bacteria, since they have been found to cross-react with these bacteria. In the RSL experiments, the presence of anti-rat erythrocyte antibodies was demonstrated by haemagglutination. Antibody activity was, however, completely abolished by one absorption with rat erythrocytes. It appears that any IgG antibodies to rat leucocytes are also removed by this single absorption, since further absorption using rat thymocytes did not significantly decrease the amount of binding to RSL in the assay system. In addition, no differences in binding of radiolabelled anti-human IgG antibody to rat thymocytes were detected among sera of patients or controls after one absorption with rat erythrocytes. A final argument against the possible influence of anti-rat antibodies in the RSL assay is the demonstration of complete inhibition of the binding by prior treatment of the RSL with aggregated autologous rat IgG, demonstrating that binding does indeed occur via the Fc receptors.

It has been repeatedly suggested that heating serum at 56°C for 30 min in order to inactivate the complement could lead to the formation of aggregates (Zubler et al., 1976a; WHO Scientific Group, 1977), and that this could influence assays for immune complexes, although little evidence for this has been presented. In the present study, it has been shown that heating serum induces the formation of aggregates detectable by the ADCC inhibition assay. Furthermore, using the RSL assay it was possible to quantitate these aggregates. Up to 128µg equivalents of aggregated IgG were detected in heated sera.

The RSL assay has a number of important advantages over other methods used for the detection of immune complexes. In contrast to the ADCC inhibition assay and those methods using C1q, heat inactivation of sera is not required. The sensitivity of the RSL

radioimmunoassay is significantly greater than that of other new methods proposed for the detection of soluble immune complexes. For example, the selective precipitation by polyethylene glycol of radiolabelled C1q bound to complexes (Zubler et al., 1976a) detects aggregated IgG at a minimum concentration of approximately 100µg/ml. In the present study, the Raji cell assay was found to detect 24µg/ml added aggregates in normal human serum. In addition, the use of an assay in which the binding of complexes is mediated through Fc receptors is to be preferred to one which involves binding through complement receptors since in the latter case the binding is influenced by the amount of complement fixed to the complexes, which in turn depends on the size of the complexes (Christian, 1960; Ishizaka et al., 1965; Agnello et al., 1970). Moreover, not all the subclasses of IgG fix complement with the same affinity. IgG_1 and IgG_3 are more effective in this respect than IgG_2 and IgG_4 (Augener et al., 1971).

Using the ADCC inhibition assay, the Raji cell radioimmunoassay and the RSL radioimmunoassay, it was possible to demonstrate the presence of immune complexes in the sera of patients with thyroid disorders. The greatest frequency was observed in sera from patients with Hashimoto thyroiditis. In addition, using the Raji cell assay it was possible to demonstrate the presence of thyroglobulin in 44% of the Hashimoto sera scored positive for the presence of immune complexes and in 30% of the positive hypothyroid sera. The earlier study of Calder and co-workers (1974) using the anti-complementary assay also detected immune complexes in sera of patients with thyroid diseases, but no attempt was made to identify the antigenic component involved. The apparent absence of thyroglobulin in the remaining positive sera suggests either that other antigens may be involved or

that the thyroglobulin antigenic determinants are inaccessible. Alternatively, the assay used in this study may be insufficiently sensitive. Recently, Takeda and Kriss (1977) demonstrated a radiometric two-site assay for soluble thyroglobulin-anti-thyroglobulin complexes applicable to human sera. Their preliminary data indicated that thyroglobulin-antibody complexes were detected in the sera of 7 out of 29 patients with Graves' disease but not in the sera of 10 normal subjects.

It should be noted that the increased binding of labelled anti-human thyroglobulin antibody to cells incubated with positive sera demonstrated in this study may reflect the presence in the complexes not of thyroglobulin but of thyroxine. All the Hashimoto and hypothyroid patients in which the detected complexes were found to contain thyroglobulin were on thyroxine, and it has been reported that anti-thyroglobulin antibodies in mice, guinea pigs and rabbits are able to bind thyroxine (McKenzie and Haibach, 1967; Margherita and Premachandra, 1969).

The role of detected complexes in the pathogenesis of autoimmune thyroid disease is uncertain. In the present study, thyroglobulin was shown to be a constituent of the detected complexes in a proportion of sera. Circulating thyroglobulin has been detected in the sera of normal individuals (Hjort and Pedersen, 1962; Assem, 1964; Roitt and Torrigiani, 1967; Torrigiani et al., 1969; Van Herle et al., 1972). It is possible that the deposition in the thyroid tissue of complexes formed from thyroglobulin and specific antibody induces an inflammatory response and the subsequent destruction of thyroid tissue. These complexes may form in the circulation and then subsequently be deposited in organs other than the thyroid gland. A significant

correlation between the presence of thyroid disease and the involvement of other organs has been reported. A high incidence of pernicious anaemia, idiopathic hypoparathyroidism, diabetes mellitus and idiopathic Addisons disease has been found in patients with thyroid disorders (Irvine, 1967; Rose, 1974). Bloch and co-workers (1960) reported that Sjogren's syndrome was associated with high titres of antibody to thyroglobulin. One might speculate that the presence of circulating immune complexes may be a common cause for multiple lesions in some patients with autoimmune disease, but there is no evidence for such a presumption.

The deposition of complexes on the surface of thyroid epithelial cells may render the cells susceptible to antibody-dependent cell-mediated cytotoxicity. It has been demonstrated by Calder and co-workers (1973c) that lymphoid cells from normal donors are rendered cytotoxic to target cells coated with thyroglobulin and preincubated with serum samples from patients with thyroid disorders. These authors also reported a significant correlation between this cytotoxicity and the serum antibody titre, although Wasserman and co-workers (1974) were unable to demonstrate such a correlation. It was found in the present study that there was no correlation between the presence or titre of anti-thyroid antibodies and the presence or quantity of immune complexes.

The presence of immune complexes in the circulation was not associated with immune complex diseases such as arteritis or glomerulonephritis and the presence of these complexes may be merely a physiological phenomenon with no pathological significance. An alternative explanation for the lack of pathogenic effect is that these complexes may not be of a size suitable for deposition in vessel walls or tissues. Cochrane (1971) reported that only large complexes

(greater than 19S) were found in the vessel walls. The observation in the present study that complexes in the sera of patients with thyroid disorders are detected in front of the ascending limb of the IgG profile on Sephadex G-200 fractionation may indicate that these complexes are not of a suitable size for deposition. This finding does not mean necessarily that larger complexes are not deposited in the thyroid gland or in other tissue, since the presence of small complexes in serum might be reflecting the presence of injurious complexes that are deposited elsewhere (WHO Scientific Group, 1977). It is worthy of note that even in rheumatoid arthritis, a disease in which immune complexes play an important role, there is a striking absence of nephritis in most patients. Accordingly, the absence of an association between thyroid diseases and nephritis does not rule out the possibility that immune complexes are involved in the pathogenesis of thyroid disease. If complexes have no role in the production of thyroid lesions, then the demonstration of complement components C1q and C3 and of IgG, IgM and IgE in thyrotoxic glands (Werner et al., 1972) together with the presence of electron dense depositis in the follicular basement membranes of Hashimoto thyroid glands and the high cytotoxic activity of lymphoid cells obtained from Hashimoto patients (Calder et al., 1973b) are phenomena which require some explanation.

Further studies on immune complexes in thyroid disorders are required in order to further characterize the antigenic components in the detected complexes. More sensitive methods for the detection of thyroglobulin, such as the two-site radiometric assay (Takeda and Kriss, 1977), could be applied to this problem and these techniques could also be exploited to detect other antigenic components. Separation of complexes from pathological sera could be achieved using the Raji cell

or RSL assays to bind these complexes followed by elution of the complexes from the cells. These complexes could then be injected into experimental animals in order to define the clinical and histological changes, if any, induced by such complexes. The presence of complexes in the sera of patients with Hashimoto thyroiditis or primary hypothyroidism could be studied both before and after treatment with thyroxine in order to define any differences in the quantity or quality of these complexes.

Using the Raji cell radioimmunoassay, it was possible to demonstrate the presence of soluble immune complexes in 24 out of 45 sera from patients with newly diagnosed insulin-dependent diabetes or insulin-treated diabetes, compared with only 2 out of 45 age- and sex-matched controls. The percentage of positive sera from diabetic patients treated with oral hypoglycaemic agents or diet restriction was similar to that of their age- and sex-matched controls. These results were confirmed using the more sensitive RSL radioimmunoassay.

In newly diagnosed insulin-dependent diabetics, there was a close correlation between the presence of soluble complexes and islet cell antibodies, while no such correlation was apparent between the presence of complexes and anti-viral antibodies. There was some indication that anti-Coxsackie B4 virus antibodies tended to occur in a high percentage of diabetic sera as compared to their age- and sex-matched controls, but the prevalence and titres of antibodies to other viruses were comparable in both diabetic and control groups. Similar findings were reported by Gamble and co-workers, who found that insulin-dependent diabetics within 3 months of onset had higher antibody titres to Coxsackie viruses, particularly B4, than either normal subjects or patients with diabetes of longer duration (Gamble

et al., 1969, 1973). The possible role of Coxsackie B4 virus in inducing diabetes is not clear, although it has been reported that the virus can induce necrotic lesions in the pancreas (Kibrick and Benirschke, 1958) and that it may be isolated from the pancreas of patients with pancreatitis (Fechner et al., 1963; Murphy and Simmul, 1964).

The correlation between the presence of complexes and islet cell antibodies in sera from newly diagnosed diabetics suggests that the antigens involved in these immune complexes may be derived from islet cells. These antigens may be present in the circulation either through a recent release of islet cell antigens or because they are being continually released after the onset of clinical diabetes. The fact that the symptoms of diabetes were present before diagnosis for as long as 4 months in patients found to have immune complexes at the time of diagnosis supports the latter alternative. The continuous release of antigen from islet cells might be due to a slow inflammatory process in the pancreatic B cells. These cells have been found to persist after the clinical onset of this type of diabetes as evidenced by their ability to produce C peptide (Heding and Rasmussen, 1975). Islet cell antibody has been shown to react with all islet cells although only B cells are markedly affected (Doniach, 1974; Egeberg et al., 1976). Similarly, complexes deposited in the islets may affect only B cells, although they may also affect the A and D cells which possibly have greater powers of regeneration. Islet cell antibodies were also found in the sera from two insulin-treated diabetics and one patient treated with oral hypoglycaemic agents. The fact that these three sera were found to contain complexes supports the concept of a correlation between the presence of islet cell antibodies and immune complexes. Detected complexes comprising islet

cell antigen and antibody could be important in relation to antibody-dependent cell-mediated cytotoxicity, with cytotoxic cells being specifically armed by such complexes. On the other hand the presence of complexes in newly diagnosed juvenile diabetics may have no pathological significance and may be merely a consequence of islet cell damage.

Soluble immune complexes were also detected and quantitated in insulin-treated diabetics. These complexes most probably result from treatment with heterologous insulin. A correlation was observed between high titres of insulin antibodies and the presence of immune complexes. The failure to detect complexes in some sera with low or moderate levels of insulin antibodies may be due to technical reasons or to the varying affinities that insulin antibodies may have in different patients (Dixon et al., 1975). Alternatively, immune complexes present in these sera might be removed from the circulation more rapidly than in other sera.

It was found that anti-insulin antibody titres appeared to be a characteristic of the patient rather than of the dosage or duration of insulin therapy. In this connection, it is worth noting that patients on insulin treatment who are HLA.BW15 tend to have higher titres of insulin antibodies than patients of other HLA types (Bertrams et al., 1976). This might suggest that certain patients with insulin-dependent diabetes might have a greater tendency than others to form complexes, and this could be an appropriate subject for further study.

The possibility exists that late complications of diabetes may be related, at least in part, to the production of immune complexes. It is therefore noteworthy that juvenile-onset insulin-dependent diabetics tended to have more immune complexes in relation to moderate or low titres of insulin antibodies than did subjects who developed insulin-

dependent diabetes after the age of 30 years, even though the duration of diabetes and of insulin treatment was comparable in the two groups. Insulin-dependent diabetes developing in young people is considered to be associated with a greater risk of severe late diabetic complications (Bradley and Ramos, 1971). It is possible that this is due to differences in the size of complexes formed in these two groups. Complexes of intermediate size appear to be the most injurious. Small complexes do not bind complement, while larger complexes are rapidly removed from the circulation (Cochrane and Koffler, 1973). Less severe late diabetic complications do occur in mainly elderly patients with maturity-onset diabetes who have received no insulin (Ortved Andersen, 1976). Taken together with the findings in this study that few patients on oral hypoglycaemic agents or diet restriction have complexes, this would suggest that complications can develop in the absence of complexes.

The presence of circulating soluble complexes in sera of insulin-dependent diabetics may reflect the presence of complexes deposited in the tissues. Both insulin and anti-insulin antibodies have been detected in the renal glomeruli of diabetics with nephropathy (Berns et al., 1962; Farrant and Shedden, 1965). Insulin-binding capacity has also been demonstrated in the vessels of the diabetic eye (Coleman et al., 1962). Bloodworth (1968) reported the presence of fine granular deposits in the renal tissue of diabetics resembling those found in certain types of glomerulonephritis. It was found in the present study that after prolonged insulin therapy there is a tendency for immune complexes to occur. Since insulin treatment has to be continued for at least 10 years before clinical complications manifest themselves, it is likely that the formation of complexes is a relatively slow process and that the progressive accumulation of these

complexes in different tissues over a long period of time leads to the appearance of complications. Westberg and Michael (1972) demonstrated that the basement membrane in diabetic glomerulosclerosis contains not only insulin, IgG, IgM and components of complement but also "non-immunological" serum proteins deposited in linear fashion. Such a finding suggests that there may be non-specific lesions involving the trapping of serum proteins.

Further work on the extent to which soluble immune complexes are implicated in the pathogenesis of juvenile insulin-dependent diabetes is required. It was found in the present study that six newly-diagnosed juvenile diabetics had titres of Coxsackie B4 antibodies greater than or equal to 1:32, but only three of these had evidence of immune complexes in their serum. Studies involving larger numbers of newly-diagnosed juvenile diabetics may reveal a significant correlation between the presence of complexes and the presence of viral antibodies. It is of great importance not only to detect complexes but also to determine the antigenic component(s) involved. This could be acheived by a modification of the Raji cell or RSL radioimmunoassay using radiolabelled anti-viral antibodies and radiolabelled anti-islet cell antibodies. Further studies should be undertaken in order to define the pathological role of the complexes formed when diabetic patients are treated with insulin. The use of highly purified insulin in the treatment of diabetics has been reported not to induce a significant increase in serum antibodies (Ortved Andersen, 1975). A comparative study of patients treated with highly purified insulin or with crude insulin might reveal differences in the levels of complexes in the sera of these two groups of patients and subsequent differences in the development of complications.

The development of sensitive and reproducible methods for the detection of circulating immune complexes which avoid the use of living cells or unstable molecules, do not require heat inactivation of sera and which are capable of processing large numbers of sera, is likely to be of paramount importance in future work on the presence and role of antigen-antibody complexes in disease.

A number of such techniques have recently been reported or are currently under trial. These include assays involving the use of bovine conglutinin (Eisenberg et al., 1977; Casali et al., 1977) and latex particles with low affinity IgM antibodies (Levinsky and Soothill, 1977) and a modified solid phase C1q radioimmunoassay (Hay Nineham and Roitt, personal communication). The application of one or more of these methods to the testing of serum samples from patients with thyroid disorders or diabetes may prove to be of great value.

APPENDICES

APPENDIX I

CULTURE MEDIA, CHEMICALS, ETC.

Eagle's medium (1x strength, without glutamine)	--- Gibco: Bio-Cult Ltd
Foetal calf serum (FCS)	--- Gibco: Bio-Cult Ltd
L-glutamine (200mM)	--- Gibco: Bio-Cult Ltd
Sephadex G-200 and G-50	--- Pharmacia
DE52 cellulose	--- Whatman
Chloramine T	--- Fluorochem Ltd
Tryptose phosphate broth	--- Oxoid Ltd
Ethylenediamine-tetraacetic acid: disodium salt (EDTA)	--- Hopkins and Williams Ltd
Ethyleneglycol bis-(aminoethyl)-tetraacetic acid (EGTA)	--- Koch-Light Laboratories Ltd
Bovine serum albumin	--- Koch-Light Laboratories Ltd
Goat anti-human IgG antiserum ..	--- Searle Diagnostic
Radial immunodiffusion plates ..	--- Oxford Laboratories
^{51}Cr-sodium chromate	--- Radiochemical Centre, Amersham
^{125}I-sodium iodide (IM30)	--- Radiochemical Centre, Amersham

EQUIPMENT

Automatic Well-type counter ..	--- Wallac Ltd
Fractionation equipment:	
1. Ultrarac fraction collector	--- LKB
2. Uvicord MKII Spectrophotometer . ..	--- LKB
3. Strip chart recorder ..	--- LKB

LP3 round-bottomed plastic tubes .. .. --- Luckhams Ltd

10ml tissue-culture tubes .. .. --- Sterilin Ltd

APPENDIX II

PREPARATION OF BUFFERS

1. 0.1M phosphate buffered saline pH7.2

KH_2PO_4	0.34g
K_2HPO_4	1.21g
NaCl	8.5g

Made up to 1 litre with distilled water

2. 0.1M phosphate buffer pH7

KH_2PO_4	5.3g
K_2HPO_4	10.6g

Made up to 1 litre with distilled water

3. 0.5M phosphate buffer pH8

945ml 0.5M K_2HPO_4 (87g/litre)

55ml 0.5M KH_2PO_4 (68g/litre)

4. Complement fixation diluent (CFD) pH7.2

Barbitone	0.575g
Barbitone sodium	0.185g
NaCl	8.5g
$MgCl_2$	0.168g
$CaCl_2$	0.028g

Made up to 1 litre with distilled water

PUBLICATIONS

1. Al-khateeb, S.F., and Barkas, T. Rat spleen leucocyte (RSL) radioimmunoassay for the detection and quantitation of soluble immune complexes. In preparation.

2. Al-khateeb, S.F., and Irvine, W.J. Detection, quantitation and characterization of soluble immune complexes in sera of patients with thyroid disorders. Submitted for publication.

3. Barkas, T., Al-khateeb, S.F., and Irvine, W.J. Serum factors influencing antibody-directed cell-mediated cytotoxicity (ADCC) and their effect on the detection of immune complexes by inhibition of ADCC. Submitted for publication.

4. Barkas, T., Al-khateeb, S.F., Irvine, W.J., Davidson, McD.N., and Roscoe, P. (1976) Inhibition of antibody-dependent cell-mediated cytotoxicity (ADCC) as a means of detection of immune complexes in the sera of patients with thyroid disorders and bronchogenic carcinoma. Clin. Exp. Immunol. 25, 270.

5. Irvine, W.J., Al-khateeb, S.F., Di Mario, U., Feek, C.M., Gray, R.S., Edmond, B., and Duncan, L.J.P. (1977) Soluble immune complexes in the sera of newly diagnosed insulin-dependent diabetics and in treated diabetics. Clin. Exp. Immunol. 30, 16.

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