We are indebted to Dr Rita H. Cornforth of the National Institute for Medical Research for the synthesis of [2-¹⁴C]-mevalonic acid; to Professor A. Wormal, F.R.S., for a gift of squalene and to Horlicks Ltd. for a supply of β -mercaptoethylamine (Becaptan). The spectroscopic examination of the microsomes were done by Dr F. A. Holton. It is a pleasure to acknowledge the skilled technical assistance of Mr K. Clifford and Mr T. Flynn. A grant from the Eli Lilly Foundation to the Medical Research Council for the purchase of our Spinco Model L centrifuge is also gratefully acknowledged.

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The Nature of the Thyroid Auto-antibodies Present in Patients with Hashimoto's Thyroiditis (Lymphadenoid Goitre)

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(Received 7 October 1957)

The chronic disease of the thyroid known as Hashimoto's thyroiditis is characterized by invasion of the gland with lymphoid tissue and plasma cells which gradually proliferate and replace the normal follicular structure, giving rise to thyroid deficiency in the patient. The serum of these patients contains abnormally raised concentrations of γ -globulins and these have been shown to reflect the presence of precipitating auto-antibodies against thyroglobulin, the specific protein (or proteins) in which the thyroid hormones are stored in the colloid of the thyroid follicles (Roitt, Doniach, Campbell & Hudson, 1956); (Doniach & Roitt, 1957).

It may be postulated that the confinement of thyroglobulin in closed follicles prevents the establishment of immunological tolerance in early life so that any subsequent release of thyroglobulin might set up an auto-immunization process. This could be responsible for the gradual destruction of the gland by a self-perpetuating chain reaction in which further release of colloid from follicles damaged by interaction with antibody leads to enhancement of the immunity. The finding by Witebsky, Rose, Terplan, Paine & Egan (1957) that the formation of auto-antibodies against thyroid extracts could be induced in rabbits, dogs and guinea pigs and that this led to thyroiditis

lends support to the hypothesis that a similar sequence of events occurs in the human in Hashimoto's disease.

The level of antibody found in the sera of patients with Hashimoto's thyroiditis affords an opportunity to study the characteristics not only of an auto-antibody but also of a human precipitating antibody.

A preliminary account of some aspects of this work has already appeared (Roitt, Doniach & Campbell, 1956).

MATERIALS AND METHODS

Radioactive compound. Algal protein hydrolysate containing a mixture of [¹⁴C]amino acids (100 μ c $\equiv$ 2.5 mg.) was obtained from The Radiochemical Centre, Amersham, Bucks.

Sera containing the auto-antibody. These were obtained from patients with lymphadenoid goitre, untreated or treated with thyroid hormones, but not subjected to surgery, since the antibody content of thyroidectomized patients is low.

Rabbit antisera. Rabbits were immunized against human γ -globulin (kindly given by Dr R. A. Kekwick) and against pooled Hashimoto sera by means of a Freund-type adjuvant (Freund & McDermott, 1942). The animals were injected subcutaneously at several sites with an emulsion of antigen-stock adjuvant mixture (1:3, v/v). The stock mixture was prepared by homogenizing 1 vol. of Arlacel A (Atlas

Powder Co.) with 3 vol. of Bayol F (Esso Standard Oil Co.) containing 2 mg. of heat-killed human tubercule bacilli/ml. Antisera against human thyroglobulin were obtained by intravenous injection of 50 mg. of the alum-precipitated protein (Proom, 1943) over a period of 3 weeks.

Human thyroglobulin. This was purified by precipitation with $(\text{NH}_4)_2\text{SO}_4$ from saline extracts of fresh thyroids removed at operation and of post-mortem material, by the method of Derrien, Michel & Roche (1948). The purified thyroglobulin was freeze-dried and stored over silica gel at -20° . The protein was dissolved in 0.9% NaCl before use.

Preparation of ^{14}C -labelled human thyroglobulin. Three batches of approximately 2.5 g. of thinly sliced fresh human thyrotoxic gland were incubated in 10 ml. of a bicarbonate medium (Peters & Anfinsen, 1950) containing $10\text{ }\mu\text{C}$ of the ^{14}C -labelled algal protein hydrolysate at 37° for 4 hr. in an atmosphere of $\text{CO}_2 + \text{O}_2$ (5:95, v/v) with gentle shaking. The slices were removed by centrifuging and the thyroglobulin was precipitated from the supernatant with $(\text{NH}_4)_2\text{SO}_4$ as in the method of Derrien *et al.* (1948). The final precipitate was washed with 41% saturated $(\text{NH}_4)_2\text{SO}_4$, dialysed against running water overnight and against changes of distilled water for 3 days. After freeze-drying, 17.2 mg. of ^{14}C -thyroglobulin was obtained.

Zone electrophoresis. Electrophoresis of human sera was carried out in phosphate-borate buffer (pH 8.4), I 0.05, on treated cellulose columns according to the methods described by Flodin & Kupke (1956) and Porath (1956). The serum (1.0 ml.) was run for 40 hr. with a current of 10 ma on a column of cellulose (45 cm. $\times$ 1.5 cm.). Paper electrophoresis with veronal buffer (pH 8.6), I 0.1 (Flynn & de Mayo, 1951), was used to identify the eluted fractions which were run side by side with whole serum.

Immuno-electrophoresis. The method described by Grabar & Williams (1955) was used. The agar was stained when this was required by immersion for 12 hr. in a 0.1% solution of azocarmin B in acetic acid-ethanol-water (10:50:40, by vol.); the agar was then washed with aq. 10% acetic acid until the background was colourless.

Precipitation in agar gel. This was carried out by the method of Ouchterlony and by a modified Oudin technique at 2° as previously described (Doniach & Roitt, 1957). Sodium azide (1%) was incorporated into the medium as a bactericide. The wells were not recharged. Except in Fig. 4 the plates were photographed by the method described by Lawson (1957).

Quantitative precipitation. The method of Heidelberger & Kendall (1935) was used: increasing amounts of thyroglobulin solution (2.0 mg./ml.) were added to a series of tubes containing 0.10 ml. of serum and the volumes made up to 1.0 ml. with 0.9% NaCl. The mixtures were incubated at 37° for 30 min. and kept at 2° for 5 days. The specific precipitates were then centrifuged, washed twice with 2 ml. of ice-cold saline and finally dissolved in 2.0 ml. of 0.1 M- Na_2CO_3 . The protein content was estimated by the absorption at $280\text{ m}\mu$.

Complement fixation. The micro-method described by Belyavin (1953) was used. The antigen was a saline extract of fresh thyrotoxic thyroid.

Determination of radioactivity. The protein was dissolved in aq. 5N- NH_3 , transferred to a 2 cm.² polythene disk and dried under an infrared lamp. The sample was counted at infinite thinness in a Geiger-Müller counter with a mica end-window. Self-absorption was negligible up to 1 mg. of protein (cf. Campbell & Stone, 1957).

RESULTS

Zone electrophoresis

Figs. 1a and 1b show typical examples of the fractionation of serum proteins on cellulose columns in a normal subject (Fig. 1a) and a Hashimoto patient before treatment (Fig. 1b). Only the β - and γ -globulin peaks are shown in Fig. 1, since under the conditions used for electrophoresis the albumin and α -globulins migrated off the column.

Fig. 1. Zone electrophoresis of human serum proteins. Serum (1.0 ml.) was added to the column (45 cm. $\times$ 1.5 cm.) packed with treated cellulose. A current of 10 ma (450 v) was applied for 42 hr. Borate phosphate buffer (see Materials and Methods) was used. The proteins of fraction A on paper electrophoresis moved with the mobility of β -globulin, and those of fraction B with the mobility of γ -globulin. (a) Fractionation of a normal serum containing 67 mg. of protein/ml.; (b) fractionation of a Hashimoto serum containing 80 mg. of protein/ml.

The β -globulins show a composite peak which is of comparable size and configuration in the normal and Hashimoto subjects. The γ -globulin fraction, which is also partially resolved on these columns, is not qualitatively different in the two sera, but the Hashimoto patient has a greatly increased amount of γ -globulin. There was general agreement between the relative amounts of γ -globulin found for different sera as determined by the area under the curve and the results previously obtained by paper electrophoresis (Doniach & Hudson, 1957).

Characterization of the antibody

The fractions eluted from the column were tested for the presence of precipitating thyroglobulin antibodies and these were demonstrable only in the γ -globulins of the pathological serum. Characterization of the antibody as a γ -globulin was further confirmed by immuno-electrophoresis of the serum in agar. On termination of the run, and after a longitudinal portion of the strip had been removed for staining, a solution of thyroglobulin was placed in an adjacent parallel channel. The precipitation arc which appeared was confined to the region opposite the γ -globulin fraction (Fig. 2) both for sera of the 'rabbit precipitin' and 'flocculating' types (see under *Precipitation curves*).

After incubation with rabbit antibody to human γ -globulin, a Hashimoto serum failed to give precipitation with thyroglobulin in the Ouchterlony test, further confirming the identity of the auto-antibody protein as a γ -globulin.

Fig. 2. Immuno-electrophoresis of Hashimoto serum (R.S.) in agar. Serum (0.2 ml.) was incorporated with agar into the slit S in the buffered agar plate [veronal buffer (pH 8.2), I 0.05, in 1.35% (w/v) agar] and run for 5 hr. with a current of 37 mA (205 V). The position of the albumin, α_2 , β -, and γ -globulin fractions was revealed by staining a longitudinal strip of the agar. The plate was immersed in 0.067 M-phosphate buffer (pH 7.2) for 20 min. then human thyroglobulin (5 mg./ml. of 0.6% agar) was run into the channel Ch. and the plate left at 2°. The precipitation arc appeared at the side of the γ -globulin fraction. Tracing of photograph taken after 10 days. The density of shading represents the degree of staining.

Immunological comparison of normal and Hashimoto sera

Although electrophoresis failed to reveal any qualitative differences between normal and Hashimoto sera, it was of interest to apply the more sensitive techniques of immunology to this question. Antibodies produced by injecting a rabbit with Hashimoto serum were completely absorbed with normal human serum; the absorbed rabbit antiserum showed no residual lines when made to react with a Hashimoto serum in an Ouchterlony plate, showing that the antigens present in Hashimoto serum are constituents of normal serum. Conversely, rabbit antiserum to normal purified human γ -globulin gave identical precipitation patterns against the sera of Hashimoto patients and normal subjects. Owen & McConahey (1956) have reported the presence of an abnormal iodinated protein in the serum of Hashimoto patients, but the concentration of this protein would appear to be too low for it to be detected by the methods described above.

Nature of the antigen

It has previously been shown (Doniach & Roitt, 1957) that some Hashimoto sera, when tested against either purified thyroglobulin or crude saline extracts from pooled glands, give two distinct precipitation bands. It has now been found that as many as three lines may be visible in some instances (Pl. 1a), although multiple lines appearing after 2 weeks have been treated with reserve. Identical results were given by individual extracts made from eleven different thyroids, some of which were fresh thyrotoxic specimens and some normal post-mortem glands, suggesting that the number of lines depends on the immune response of the patient rather than on the composition of the antigen.

A Hashimoto serum giving a well-defined double line was set up in serial dilution in Oudin tubes against constant amounts of thyroglobulin. The double line was visible in the tubes containing excess of antibody; with progressive dilution the system reached equivalent proportions, the precipitation lines became more compact and the two lines merged together, only to become distinct again with further dilution of the serum. The two lines vary from one serum to another in their relative densities and in the characteristics of their upper and lower edges.

It has been possible to demonstrate that these two lines represent two distinct antibody-antigen systems. Fig. 3 shows an Ouchterlony plate in which the antigen, thyroglobulin, was placed in the centre well, while the sera of four different Hashimoto patients were put in the outer wells: two of them gave double lines, and these lines crossed

each other, suggesting non-identity of the antigens involved.

The characterization of the antigen as thyroglobulin is based upon the following observations. As judged by the Ouchterlony test, the antigens present in crude thyroid extracts are also present in the 'thyroglobulin' preparation obtained from it by ammonium sulphate fractionation. This preparation migrates as one band when subjected to paper electrophoresis with a mobility lying between those of human α_1 - and α_2 -globulins (cf. Robbins, 1954). Immuno-electrophoresis in agar shows that the antigens in the crude thyroid extract and in the thyroglobulin preparation have identical mobilities (Fig. 4). When a rabbit antiserum prepared against the thyroglobulin preparation and a Hashimoto serum known to give at least three lines when tested against thyroglobulin were set up in an Ouchterlony plate with thyroglobulin in the centre well, the precipitation lines formed merged completely. This suggests that the same antigens were responsible for stimulating production of antibody in both the rabbit and man.

The possibility that one of the antigens in the thyroglobulin preparation might have an appreciably different molecular weight from the others was investigated. In view of the demonstration by Korngold & Van Leeuwen (1957) that the curvature of a precipitation line in an Ouchterlony plate is concave towards the reactant of higher molecular

weight, thyroglobulin and a Hashimoto serum were allowed to react together under conditions which permitted a study of the curvature of lines (Pl. 1b). The curvature of the lines towards the antigen well accords with the known high molecular weight of thyroglobulin (650 000) relative to that of human γ -globulin (170 000) and suggests further that at least two of the antigens present have comparable molecular weights. In a similar experiment with a serum known to give three and possibly four lines in the Ouchterlony test (serum L.M.; cf. Pl. 1a), all the precipitation lines formed were concave towards the thyroglobulin well.

These experiments suggest that the term 'thyroglobulin' covers a system of three or more closely related proteins; it may be recalled that similar conclusions were reached by Roche, Michel, Deltour & Michel (1952) from 'salting-out' studies.

Precipitation curves

Quantitative precipitation curves were established for Hashimoto sera against purified thyroglobulin. These fell into two distinct groups: some sera gave curves, exemplified by Fig. 5, which correspond to the type obtained with rabbit-precipitin systems in which the antigen is completely precipitated in the region of antibody

Fig. 3. Ouchterlony plate in which human thyroglobulin (2 mg./ml. of 0.9% NaCl) in the centre well has reacted with sera from different patients with Hashimoto's thyroiditis. The lines obtained with the sera H.S. and J.R. crossed, indicating the presence of two distinct antigen-antibody systems. Tracing of photograph taken after 22 days.

Fig. 4. Immuno-electrophoresis of human thyroid extract (S_1) and of purified human thyroglobulin (S_2) in agar. Conditions as described in Fig. 2, Hashimoto serum (W.A.) being placed in the channel Ch. The precipitation arcs show that the antigens present in the extract have the same mobility as those present in the thyroglobulin preparation and that these antigens move with the same mobility as the bulk of the protein constituting the preparation (shown to move between the α_1 - and α_2 -globulins on paper electrophoresis). Tracing of photograph taken after 10 days. The density of shading represents the degree of staining.

excess, and other sera gave curves which resembled those usually associated with the horse flocculating systems, in that the antigen-antibody complex was soluble in the presence of excess of antibody. A typical example of the latter type is shown in Fig. 6.

Fig. 5. Amount of precipitate formed on addition of increasing weights of human thyroglobulin to 0.10 ml. of Hashimoto serum (R.S.). The washed precipitates were dissolved in 2.0 ml. of 0.1M- Na_2CO_3 and the protein content estimated by measurement of the extinction coefficient at 280 $\text{m}\mu$.

Fig. 6. Amount of precipitate formed on addition of increasing weights of human thyroglobulin to 0.10 ml. of Hashimoto serum (W.A.). The precipitates were treated as described in Fig. 5.

Since these sera fixed complement very weakly or not at all when the purified thyroglobulin preparation was used as antigen, no particular precautions were taken to allow for any effect of complement on the weight of the specific precipitates in these experiments. Ring tests on the supernatants obtained after spinning down the precipitates showed that all the antigen was precipitated at the maximum point of the curve in Fig. 5. The curve obtained with a serum which gave well-marked double lines in the gel tests showed a broad zone of maximum precipitation and this probably reflects the presence of the two precipitating systems mentioned above; tests on the supernatants showed that precipitation of antigen was incomplete in this zone.

Sera of the 'flocculation' type gave precipitation over a narrow range of concentrations of antigen and when tested by the Oudin technique these sera tended to form compact precipitation bands. Ring tests on the supernatants failed to detect incomplete precipitation of antigen at the point of maximum precipitation but the validity of this test for the demonstration of minute amounts of antigen using an antiserum of this type is questionable. It was therefore decided to investigate the problem using radioactive antigen which would also permit calculation of the antibody-antigen ratios in complexes obtained in the regions of excess of both antigen and antibody.

Radioactive thyroglobulin

Although thyroglobulin labelled with ^{131}I can readily be obtained from the thyroid gland of patients treated with ^{131}I before thyroidectomy, it was felt that labelling with ^{14}C would be more convenient in view of its much greater half-life. Since Peters & Anfinsen (1950) and Campbell & Stone (1957) have demonstrated the synthesis of labelled serum albumin on incubation of liver slices with radioactive amino acids, it was decided to apply similar methods to the synthesis of labelled thyroglobulin (see Methods section). Owing to the high radioactivity of the amino acids in the medium from which the thyroglobulin was isolated it was necessary to ensure that the radioactivity of the protein was due to the presence of amino acids in peptide linkage rather than to adsorption phenomena. Characterization of the protein as radioactive thyroglobulin was accomplished by the following procedures: (i) The protein moved on paper electrophoresis substantially as one band with the mobility of human thyroglobulin (i.e. between α_1 - and α_2 -globulin). A radioautograph of the stained electrophoretic strip showed most of the radioactivity to be coincident with this band. A small amount of radioactivity remained at the origin associated with denatured material, and a

faint radioactive band was seen having a mobility intermediate between those of serum β - and γ -globulins. (ii) When the radioactive protein was mixed with Hashimoto serum under conditions such that the antibody and antigen were present in equivalent proportions, approximately 90% of the radioactivity was recovered in the precipitate. (iii) Inactive thyroglobulin was precipitated by ammonium sulphate from a solution containing the same amount of radioactive algal-protein hydrolysate as was used in the slice experiments. After washing and exhaustive dialysis, the resulting protein had a specific activity of 2% of that of the biosynthetic preparation of radioactive thyroglobulin. (iv) When the radioactive protein was mixed with inactive thyroglobulin, reprecipitation with ammonium sulphate under conditions in which only part of the total protein was precipitated produced a reduction in counts proportional to the dilution factor.

*Quantitative precipitation reaction
with [14 C]thyroglobulin*

A quantitative curve was constructed based on the precipitation of radioactive thyroglobulin by a Hashimoto serum. The results are presented in

Fig. 7. Precipitates formed on addition of increasing weights of human [14 C]thyroglobulin to 0.06 ml. of Hashimoto serum (M.M.). ●, Wt. of protein precipitated (μ g.); ○, wt. of antibody protein precipitated (μ g.); ▲, molar ratio of antibody:antigen in the precipitate.

Fig. 7. From the radioactivity of the specific precipitates, the antigen content could be calculated throughout the range studied, assuming that the specific activity of the precipitated antigen was the same as that of the thyroglobulin preparation used. By allowing for the different absorptions at 280 $m\mu$ of thyroglobulin and γ -globulin, the antibody content of each precipitate could be calculated. At the point of maximum precipitation it was possible to account for 89% of the added radioactivity in the specific precipitate; the addition of Hashimoto serum to the supernatant, followed 24 hr. later by treatment with rabbit antiserum to human γ -globulin to carry down any small amount of remaining thyroglobulin, failed to precipitate more than a small fraction of the residual radioactivity, which suggests that substantially all the thyroglobulin had been precipitated at this point. The specific activity of the precipitate obtained by treatment of another portion of the supernatant with [14 C]thyroglobulin and rabbit anti-human γ -globulin showed the anti-thyroglobulin content of the supernatant to be negligible. Thus, at the point of maximal precipitation, no thyroglobulin or anti-thyroglobulin were demonstrable in the supernatant.

The molar ratio of antibody to antigen in the specific precipitates was found to vary from 4:1 in the region of antibody excess to 2:1 at the point of maximum precipitation and throughout the range of antigen excess studied; similar ratios were obtained from the precipitation curve for the 'rabbit-precipitin' type of serum shown in Fig. 6. These low ratios contrast with the much higher values obtained with heterologous thyroid-immune systems; Heidelberger (1938) found ratios of up to 40:1 for sera of rabbits hyperimmunized against hog thyroglobulin. A low ratio indicates a small number of combining sites on the antigen, and it may be that only restricted parts of the molecule provide antigenic stimuli during auto-immunization.

Antibody content of Hashimoto sera

From the data shown in Fig. 7, the serum M.M. was found to contain 5.2 mg. of antibody protein/ml. At the point of maximum precipitation obtained with serum W.A. (Fig. 6), ring tests failed to demonstrate the presence of thyroglobulin in the supernatant, and in view of the results described above with [14 C]thyroglobulin it seems reasonable to assume complete precipitation of antigen; on this basis an antibody content of 4.8 mg. of protein/ml. may be calculated for this serum. The serum R.S. (Fig. 5) had an antibody content of 4.7 mg./ml. These sera were the most potent of those tested.

*Cross-reactions of the human auto-antibody
with other mammalian thyroid extracts*

By means of the Oudin technique, the Hashimoto antibody was found to cross-react with saline extracts of rhesus-monkey and chimpanzee thyroids, though it failed to give precipitation when tested against similar extracts of thyroids from the rat, rabbit, sheep, beef, hog and horse. In contrast, Hektoen, Fox & Schulhof (1927) have shown that rabbit anti-human thyroid serum cross-reacted with a wide variety of mammalian thyroid extracts. There appeared to be considerable variation in the degree to which different Hashimoto sera cross-reacted with the monkey antigen. Of seven patients tested, five cross-reacted strongly and two gave only weak bands. 'Double-line' sera still showed two bands with monkey thyroid extract.

Complement-fixation reactions

Hashimoto sera containing precipitating antibody either failed to fix complement or fixed complement very weakly when extracts of post-mortem normal thyroid gland or thyroglobulin purified from these extracts were used as the antigen. The findings of Trotter, Belyavin & Wadhams (1957) showing that complement fixation can be readily demonstrated when fresh thyrotoxic gland is used as the antigen have been confirmed. Complement fixation could then also be demonstrated in Hashimoto sera which failed to react by the precipitation tests.

Failure to demonstrate visible precipitation does not preclude the presence of thyroglobulin antibodies, since these could be either 'non-precipitating' or present in amounts detectable only by more sensitive techniques. Preliminary experiments with [^{14}C]thyroglobulin in conjunction with rabbit anti-human γ -globulin serum have shown that the latter possibility may occur.

DISCUSSION

The abnormally high concentrations of serum γ -globulin present in patients with Hashimoto's thyroiditis and the presence of precipitating auto-antibodies to human thyroglobulin in these sera pose a number of problems concerned with the nature of the γ -globulins, their relation to the auto-antibody content of the serum, the type of precipitation curve obtained with the auto-antibody, the nature of the antigen against which the immunity is directed and the mechanism of its possible cytotoxic action. Investigation of these problems is greatly facilitated by the potency of the antisera in many individuals, and provides at the same time a convenient opportunity to study some of the characteristics of a human precipitating antibody.

The zone-electrophoresis studies support the previous demonstration of raised concentrations of γ -globulins in Hashimoto's disease. A direct link between the elevated concentrations of serum globulins and the thyroid auto-immunity is provided by the characterization of the auto-antibodies as proteins of the serum γ -globulin fraction. In order to evaluate the extent to which the auto-antibodies contribute towards the increase in the concentrations of these globulins above normal, classical precipitation curves were established with purified thyroglobulin from which it was possible to calculate the serum-antibody content. A striking feature of the fully developed thyroid auto-immunity seen in lymphadenoid goitre is the extremely high level of circulating antibody. Only rarely have precipitin levels greater than 2 mg. of antibody protein/ml. of serum been described in the human after immunization with foreign antigens, whereas Hashimoto patients sometimes have as much as 5 mg./ml. of serum. This reflects a state of hyperimmunization and is probably a consequence of the continuous synthesis and release of the antigen from the damaged thyroid gland. The high concentrations of circulating antibody could account for the greater part of the rise in γ -globulins in these sera, but appreciably elevated values were sometimes found in sera in which the precipitins were either weak or not demonstrable. The finding that the precipitation curves obtained with a number of Hashimoto sera fell into two distinct groups—'rabbit precipitin' and 'horse flocculating' types—is of interest in that the latter have not been described previously in human immune systems. The failure to observe precipitation in the presence of excess of flocculating antibody which is a characteristic of this type of curve has not been satisfactorily explained, although inhibition by 'non-precipitating' antibody has been suggested as the cause of this phenomenon. The simultaneous presence of 'incomplete' and precipitating antibodies directed against the same antigen is well recognized in the immunization of animals. Some animals produce only 'incomplete' antibodies at first and give precipitins after more prolonged immunization, whereas a few immunized animals never show precipitins (Pappenheimer, 1940). In the human patient neither the absence of precipitating antibody nor the type of precipitation curve could be correlated with the duration of the disease, the degree of thyroid destruction as evidenced by myxoedema or the size of the goitre. The majority (75%) of subjects produced precipitating antibodies and, of those, patients with large goitres tended to have the highest titres, which decreased as the goitre regressed under thyroid treatment. Several patients with large goitres and abnormal concentrations of γ -globulins

gave no precipitins and in these instances the globulins may represent 'non-precipitating' antibodies, or a concomitant production of non-specific globulins by the stimulated reticulo-endothelial system, or perhaps antibodies to a different antigen.

The antigens responsible for the precipitating antibody appear to be fractions of normal human thyroglobulin and there is no evidence that any alteration in structure is necessary to stimulate auto-immunity. This view is supported by our results and by the fact that antibodies cytotoxic to the thyroid could be produced in animals with carefully prepared homologous thyroid extracts as well as with purified thyroglobulin (Witebsky *et al.* 1957). However, an additional antigen may be implicated in human auto-immune processes since all Hashimoto sera fix complement in the presence of thyrotoxic thyroid extracts, but do so only weakly or not at all with purified thyroglobulin. Further, the lack of parallelism between precipitin and complement fixation titres in the same individual suggests that the antigens concerned in these two reactions are distinct from each other.

The mechanism by which the auto-immune process destroys the thyroid gland is not completely understood. Presumably damage results from interaction of either circulating or 'cell-bound' antibodies with thyroid antigens. A number of clear-cut examples of cell damage caused by the injection of serum antibodies are known: the passive transfer of heterologous antibodies against placenta (Seegal & Loeb, 1940), kidney (Smadel, 1936), erythrocytes (Damashek & Schwartz, 1940), leucocytes (Chew, Stephens & Lawrence, 1936) and blood platelets (Ledingham, 1914) causes disruption of the elements against which they are directed, while the intraperitoneal injection of rabbit anti-Ehrlich ascites-tumour serum plus complement increases the survival time of mice bearing the tumour and results in progressive cellular degeneration of the ascites cells (Flax, 1956). The lysis of red cells, Ehrlich ascites-tumour cells (Flax, 1956; Easty & Ambrose, 1957), Bragg rat-lymphosarcoma cells (Schreck & Preston, 1956) *in vitro* and human skin and placenta in tissue culture (Bassett, Campbell, Evan & Earle, 1957) by their respective antisera have been demonstrated, but the presence of complement was obligatory. In these examples of cell damage produced both *in vivo* and *in vitro* by circulating antibodies, it is probable that the antibodies were directed against 'cell-surface' antigens. With thyroglobulin conditions are not quite comparable in that it is predominantly intrafollicular and therefore not so easily accessible to its antibody. There is little experimental evidence of cytotoxic action produced by circulating antibodies directed against intracellular antigens. Lilien (1954) describes the de-

struction or disorganization of thyroid follicles after repeated injections into rats of a rabbit anti-serum specific to rat thyroid, although it was not established whether a cell-surface antigen was involved. The characterization of the antigen responsible for complement fixation with thyrotoxic glands in Hashimoto patients may have considerable bearing on this problem if it can be shown to be a cell-surface antigen.

It is possible that the circulating antibodies, both precipitating and complement-fixing, are not the effective agents in the destruction of the gland but are only indicators of the immune process. Although Rose & Witebsky (1956), in their work on the auto-immunization of rabbits with thyroid extracts, found an approximate parallelism between the level of circulating antibody and the extent of the lesions in the thyroid gland, histological damage was also found in some animals in which only low concentrations of antibody were demonstrable. In similar experiments with dogs and guinea pigs (Witebsky *et al.* 1957), dense cellular infiltration and striking follicular changes were evident in association with very low titres of circulating antibody. It may be that the lymphocytes which infiltrate the thyroid so extensively in auto-immune goitres are implicated in the cellular destruction, since these cells play a dominant role in tissue immunity where no circulating antibodies can be demonstrated. Billingham, Brent & Medawar (1956) have shown that grafting of lymph-node cells from a normal animal results in the rejection of a skin graft previously accepted by a tolerant animal. Weaver, Algire & Prehn (1955) have shown that the lymphoid cells, and not the serum of animals which have rejected a homograft, are responsible for the destruction of any further graft from the same donor. Similarly, allergic encephalomyelitis could be passively transferred by cells but not by the serum of rats immunized with isologous brain extracts (Lipton & Freund, 1953). The mechanism by which cellular destruction is effected in auto-immune thyroiditis may be more readily elucidated when the conditions required for its passive transfer can be established and the key antigens involved have been characterized more fully.

SUMMARY

1. Fractionation of Hashimoto sera by zone electrophoresis on cellulose columns has confirmed the high γ -globulin content but failed to demonstrate qualitative differences from the normal.
2. The thyroglobulin precipitins were localized entirely in the γ -globulin fraction.
3. Some patients were immunized against two, or sometimes even three, distinct antigens present in purified thyroglobulin.

4. Two types of precipitation curves were obtained in different patients: one resembled the familiar rabbit-precipitin system and the other was similar to that seen with horse flocculating antibodies.

5. High levels of circulating auto-antibodies were found in Hashimoto patients, ranging up to 5.2 mg. of antibody protein/ml. of serum.

6. The preparation of [^{14}C]thyroglobulin is described. The labelled protein was used to calculate the molar ratio of antibody:antigen in the specific precipitates. This was found to vary from 4:1 to 2:1 in the range studied.

7. The human auto-antibody has a narrow species specificity, reacting only with monkey and chimpanzee thyroid extracts.

8. The antigens involved in thyroid auto-immunity and the possible mechanism of cell destruction by their antibodies are discussed.

We are grateful to Professor F. Dickens, F.R.S. and to Mr R. Vaughan Hudson, F.R.C.S., for their constant co-operation and interest. We thank Mr H. E. H. Jones for immunizing the rabbits, Dr W. Wood of Glaxo Laboratories for monkey thyroids, Dr O. Hill of the Zoological Society of London for chimpanzee thyroid, and Dr J. G. Feinberg of Beecham Research Laboratories Ltd. for helpful advice on the Ouchterlony technique. We particularly wish to thank members of the photographic department of the Middlesex Hospital for preparation of the plate. This work was made possible by a grant to the Medical School by the British Empire Cancer Campaign. Finally, we should like to thank Miss P. Wilson for technical assistance and Miss B. Kernot for the preparation of the diagrams.

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EXPLANATION OF PLATE 1

- Pl. 1a. Ouchterlony plate in which human thyroglobulin (5 mg./ml. of 0.9% NaCl) in the centre well has reacted with dilutions of Hashimoto serum (L.M.). With the serum diluted 16-fold, three distinct lines are visible. Photographed after 21 days.
- Pl. 1b. Ouchterlony plate in which human thyroglobulin (2 mg./ml. of 0.9% NaCl) has reacted with a 'double-line' Hashimoto sera. Curvature of the precipitation lines towards the thyroglobulin indicates that the molecular weights of the antigens present in this preparation are greater than that of human γ -globulin. The denseness of the precipitate leads to the darkness in the precipitation lines visible in the photograph which was taken after 26 days. The wells at the top and bottom of the plate were not filled.

(a)

(b)

I. M. ROITT, P. N. CAMPBELL, AND DEBORAH DONIACH—THE NATURE OF THE THYROID AUTO-ANTIBODIES PRESENT IN PATIENTS WITH HASHIMOTO'S THYROIDITIS (LYMPHADENOID GOITRE)

(Facing p. 256)