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LEUCOCYTE MIGRATION INHIBITION WITH MITOCHONDRIA IN HUMAN AUTOIMMUNE THYROID DISORDERS

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SUMMARY

Leucocyte migration inhibition tests (LMT) with rat and human liver mitochondria are reported in forty-nine thyroid patients and forty-seven healthy controls. Whereas normal subjects and colloid goitre cases were inactive in this test, patients with autoimmune thyroiditis and thyrotoxicosis gave positive mitochondrial reactions which paralleled the organ-specific thyroid microsomal LMT obtained in the same patients and were not species dependent. The active antigen may be in the inner membranes of the particles.

As with thyroid microsomes, intense inhibition with mitochondria was seen in the hypercellular variant of Hashimoto goitre characterized serologically by low or absent thyroglobulin antibodies, and the lowest LMT values occurred in the rare cases showing poor response to thyroxine therapy. An inverse correlation was found between mitochondrial LMT and thyroglobulin antibody titres. Surprisingly, weak LMT was also found in four thyroid patients who happened to have mitochondrial antibodies in the serum in addition to the usual thyroid-specific reactivities. The mitochondrial LMT appears to be of widespread occurrence in autoimmune diseases and also develops following tissue injury. Its possible significance in relation to cellular immunity, cell destruction and the inflammatory response is discussed.

INTRODUCTION

Circulating autoantibodies to mitochondria have been extensively studied in primary biliary cirrhosis where they occur in over 90% of cases and have proved of diagnostic significance (Walker *et al.*, 1965; Doniach, 1972). They are also found in 5–8% of patients with collagen disorders some of whom have a subclinical form of 'autoimmune' hepatitis (Walker *et al.*, 1970) and in chronic false-positive reactors for syphilis (Catterall, 1972; Fulford *et al.*, 1972). Their incidence in healthy subjects is about 0·7% and in patients with

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thyroiditis and other autoimmune disorders they occur in about 2% of cases. Mitochondrial antibodies which are found in the three main immunoglobulin classes, are detectable by the typical immunofluorescence pattern produced on human and animal tissues, and are complement fixing. The autoantigen is a lipoprotein located in the mitochondrial inner membranes, apparently unassociated with the respiratory enzymes (Berg *et al.*, 1969).

In experimental animals antibodies against mitochondrial components occur spontaneously from an early age (Weir *et al.*, 1966) and a rise in titre has been demonstrated after chemical liver injuries in rats (Weir & Elson, 1969) and after myocardial infarction in dogs (Pinckard *et al.*, 1971). These natural antibodies differ in many respects from those found in human disease: although they fix complement, they do not react in the immunofluorescence test, they have poor keeping qualities and are often confined to the IgM class. In rats, spontaneous mitochondrial antibodies are often directed against the outer membrane while chemically induced reactions are either against outer or inner mitochondrial membranes (Deheer *et al.*, 1972).

Leucocyte migration inhibition studies with mitochondria were undertaken in the first instance (Brostoff, 1970) to see whether patients with primary biliary cirrhosis had any evidence of cellular immunity in addition to the high titres of circulating mitochondrial antibodies, and to compare this disease with secondary biliary cirrhosis due to mechanical strictures, and with patients having mitochondrial antibodies without evidence of liver disease. When Hashimoto patients were included as 'negative controls' it became apparent that the great majority showed a pronounced inhibition in Bendixen & Søborg's (1969) leucocyte migration test (LMT) when purified liver mitochondria were added to peripheral leucocyte cultures. The present studies were undertaken to analyse this unexpected phenomenon, and to look for a possible relation to the disease process, especially in relation to cellular immune mechanisms of organ-specific nature involving thyroid antigens (Wartenberg *et al.*, 1973).

MATERIALS AND METHODS

Patients

Forty-nine cases of thyroid disease were studied and clinical details are given in a previous communication (Wartenberg *et al.*, 1973). Of thirty-two Hashimoto thyroiditis cases, twelve were untreated at the time of LMT and the others had received thyroxine in full replacement doses for many years in most instances. All had thyroid antibodies in the serum, and in addition three patients had circulating mitochondrial antibodies. Five patients had a persistent goitre showing poor response to thyroxine. Of twelve thyrotoxic patients tested, two were untreated, four had active disease controlled with carbimazole and six were in permanent remission. Nine had thyroid antibodies and one mitochondrial antibodies. Two cases of primary myxoedema and three with proven non-toxic colloid goitres were also included.

Controls

Forty-seven healthy medical students and laboratory staff, twenty-seven males and twenty females, were tested. None of these had goitres though two had thyroid antibodies. One female control had antinuclear antibodies to a titre of 1:10.

Leucocyte migration test (LMT)

This was carried out according to Bendixen & Søborg (1969) as described in Wartenberg *et al.* (1973). With each group of patients one or two normal controls were included. The results for each dose of antigen was calculated from quadruplicate cultures but when repeat tests were done, up to twelve culture chambers were included for calculation of the mean percentage migration for a given dose of antigen.

Antigens

Rat liver mitochondria

These were prepared by differential centrifugation in 0·25 M sucrose as described by Parsons *et al.* (1966). After removal of nuclei at 125 ***g***, the liver homogenates were spun at 9000 ***g*** to sediment the mitochondria. After discarding the supernatant which contained microsomes, lysosomes and soluble proteins, the mitochondrial pellet was washed three times and then lyophilized.

Human liver mitochondria and thyroid microsomes

The mitochondria were prepared from a normal liver taken 6 hr post-mortem by the same method as for the rat. Thyroid microsomes were obtained by differential centrifugation from a fresh operative specimen of thyrotoxic thyroid as described in Roitt *et al.* (1964).

For the LMT, an appropriate weight of the lyophilized antigens was resuspended in Eagle's minimum essential medium (MEM). It was not found necessary to rehomogenize or sonicate the mitochondrial preparations. Tests were performed with concentrations of 5, 50, 250 and 500 μg lyophilate/ml culture medium.

Mitochondrial outer and inner membranes

Two preparations were used. In the first batch of experiments the membranes were separated by hypotonic swelling followed by contraction of the inner membranes with ATP (Sottocasa *et al.*, 1967) and separation was done by differential centrifugation only. The outer membrane fraction in these experiments was probably contaminated with other fragments. In the second preparation ATP was not used (Parsons *et al.*, 1966) but the outer membranes were further purified on a sucrose gradient.

Serological methods

The sera of patients and controls were tested for mitochondrial and other autoantibodies by the methods described in the W.H.O. manual of autoimmune serology (Roitt & Doniach, 1969).

RESULTS

The overall results of leucocyte migration inhibition with rat liver mitochondria are shown in Fig. 1. With all antigen dosages, patients with Hashimoto goitre, primary myxoedema and thyrotoxicosis showed a significant inhibition of migration. Maximal inhibition was reached with 250 μg of mitochondrial antigen per ml and no toxic effects were noted with 500 μg. At this concentration two of the controls showed some migration inhibition. One female aged 56 had antinuclear antibodies and a family history of autoimmune disorder.

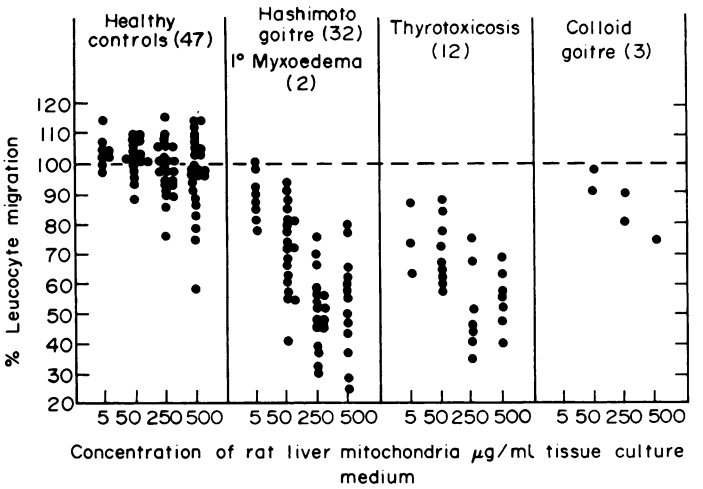

FIG. 1. Dose response curve of leucocyte migration inhibition with rat liver mitochondria in thyroid patients and controls. Each point represents the mean per cent migration obtained in a patient for the dose of antigen.

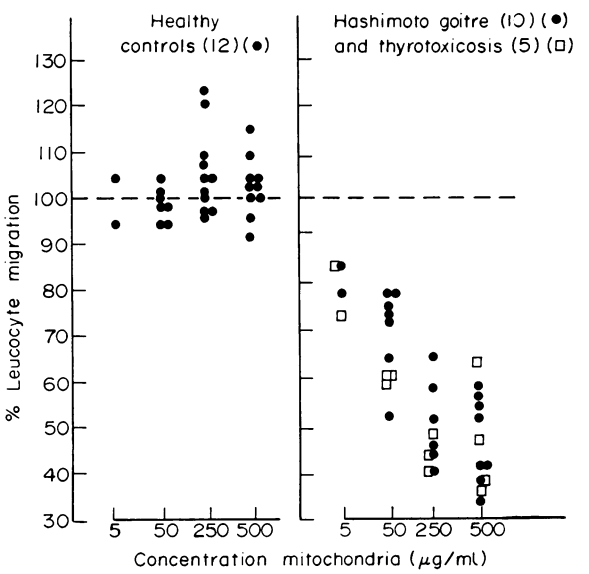

FIG. 2. Dose response curve of leucocyte migration inhibition with human liver mitochondria.

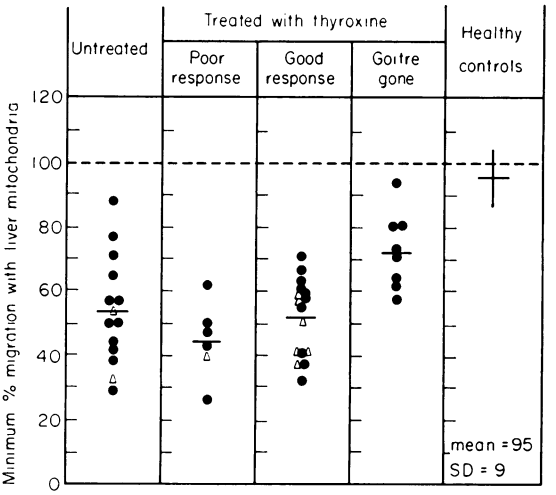

FIG. 3. Correlation of leucocyte migration inhibition using (△, human; ●, rat) liver mitochondrial antigen, with the response to prolonged thyroxine replacement therapy in autoimmune thyroiditis (thirty-two Hashimoto goitre, two primary myxoedema).

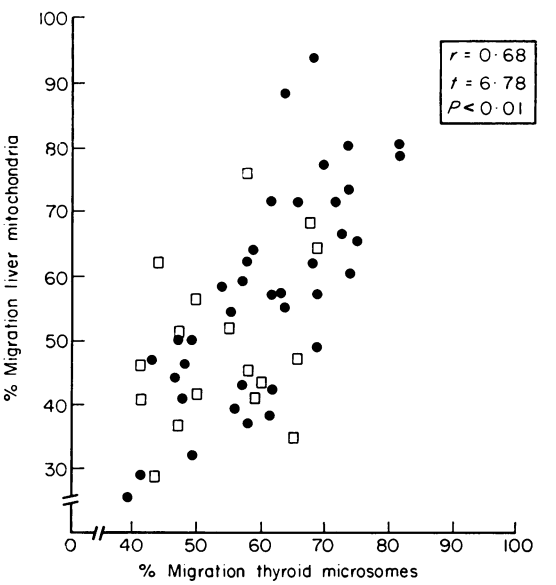

FIG. 4. Correlation of leucocyte migration inhibition obtained with human thyroid microsomal and rat liver mitochondrial antigens in thyroid autoimmune disease. Each point represents the minimum per cent migration obtained in a patient with antigen doses of 250 or 500 μg/ml culture medium. (●) Hashimoto thyroiditis; (□) thyrotoxicosis.

The other was a male laboratory worker who had continuous contact with these and several other antigens, with which he also showed reactivity in the LMT. The three patients with non-toxic colloid goitre did not react significantly.

The results obtained with human liver mitochondria in some of the patients and controls are shown in Fig. 2. The cellular reactivity of Hashimoto and thyrotoxic patients is equally evident with human as with rat mitochondria using the same antigen dose range. The human antigen may be preferable since it gave a narrow range in healthy subjects which did not overlap with that of the patients even at low antigen dilutions.

Hashimoto patients with persistent goitre and poor response to thyroxine had the lowest migration values with liver mitochondria (mean 44%) while patients in whom the thyroid gland had become impalpable showed the least response (mean 71%). Patients with good regression of the goitre but who still had a palpable gland showed intermediate migration

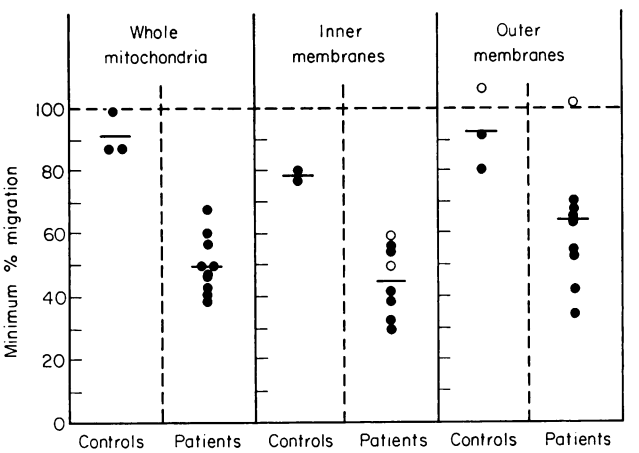

FIG. 5. Relative activities of inner and outer rat liver mitochondrial membranes in the leuco cyte migration inhibition test on ten selected patients and three controls. Each point represents the lowest per cent migration obtained for a subject with doses of submitochondrial fractions varying from 25 to 500 μg/ml culture medium. (●) LMT with first preparation of outer and inner membrane fractions. (○) LMT with second membrane preparation in which outer membranes were further purified on sucrose gradient.

values and untreated patients displayed a wide scatter of results (Fig. 3). The same correlation was obtained with thyroid microsomal antigen, whereas liver microsomes were inactive in LMT (Wartenberg *et al.*, 1973). A scattergram was made comparing liver mitochondrial with thyroid microsomal LMT in all available thyroid patients, taking the maximum inhibition value in each test (Fig. 4). There was a significant correlation between results obtained in LMT with these two antigens ($r = 0{\cdot}68$; $P<0{\cdot}01$)

Experiments with outer and inner mitochondrial membranes

Three doses of antigen, 50, 250 and 500 μg/ml were used in the tests done with the first membrane preparation. The more highly purified second batch of membranes yielded less material and the tests had to be done with 25 and 50 μg doses. In each subject the LMT was

concurrently done with 50, 250 and 500 μg of whole mitochondria. The results are summarized in Fig. 5, each point represents the maximum inhibition for a given subject. The more purified membrane fractions obtained in the second preparation seemed to give better discrimination in the LMT, suggesting that the active antigen may be in the inner membrane. Overall the inhibitions observed with inner and outer membranes differed significantly ($0{\cdot}025 > P > 0{\cdot}0125$). This experiment is, however, not conclusive and will be repeated with larger doses of highly purified subfractions.

Correlation of LMT with serum autoantibodies

Four of the forty-nine patients had circulating mitochondrial antibodies in the absence of liver disease. These antibodies occur in less than 2% of Hashimoto patients but the positive reactors are followed assiduously in the clinic and so happened to be available at the time of these studies. One of these was a thyrotoxic in remission for many years following subtotal thyroidectomy. Of the three Hashimoto cases with mitochondrial antibodies one had a mother with primary biliary cirrhosis and was as yet untreated, another had undergone thyroidectomy and was on thyroxine for 10 yr and the third had an unresponsive goitre. Their migration inhibition was relatively small, both with thyroid microsomes (mean LMT 68%, range 58–75) and with liver mitochondria (mean LMT 65%, range 62–68). With respect to the relationship between LMT and thyroid antibodies in the serum, it was noted that the five patients with the best migration inhibition and a poor response to thyroxine treatment all had low or negative thyroglobulin antibody tests with moderate titres of microsomal antibodies. Therefore the Hashimoto group was divided into cases with low or negative TRC (titre up to 320) with positive microsomal CFT, and those with high TRC (640–5 million) and high *or* low microsomal antibodies. The results are shown in Table 1.

TABLE 1. Comparison of leucocyte migration tests in two groups of patients with Hashimoto thyroiditis

Antigen in LMT	Mean percentage migration in patients with following serum antibodies:		
	Low or negative TRC* CFT positive	High TRC ± CFT	*P*
Rat liver mitochondria	49 ± 3·8†	61 ± 5·3	$0{\cdot}05 > P > 0{\cdot}025$
Human thyroid microsomes	57 ± 3·2	62 ± 2·1	n.s.

* Titres ⩽ 320. † ± Standard error of the mean.

The first group of patients showed a more marked degree of cellular reactivity when tested with liver mitochondria ($T = 1{\cdot}95$; $0{\cdot}05 > P > 0{\cdot}025$). Surprisingly perhaps, no statistical difference could be demonstrated between these two groups in the LMT done with thyroid microsomes. This may be due to the heterogeneity of the group regarding duration of treatment and changes of antibody titres with time. Since patients with high TRC titres

showed little inhibition in LMT a negative correlation was looked for between these two parameters for all thirty-four thyroiditis cases. The results were significant for mitochondrial LMT-versus-$\log_{10}$ TRC titre ($r = -0{\cdot}40$; $P<0{\cdot}01$). Microsomal LMT did not correlate with TRC ($r = -0{\cdot}25$; $P>0{\cdot}1$).

DISCUSSION

The results of the present study confirm and extend the previous report by Brostoff (1970) and a more recent one by Calder *et al.* (1972) of leucocyte migration inhibition by liver mitochondria in patients with autoimmune thyroid disease. Normal subjects and cases of non-toxic colloid goitre were non-reactive in this test. There was a striking correlation between the organ-specific thyroid microsomal LMT and the non-organ specific mitochondrial reactivity. This was not due to contamination of the microsomes with mitochondrial fragments since comparable responses were obtained with equal weights of the two subcellular fractions. Furthermore, mitochondrial antigens could not be detected in thyroid microsomal preparations by complement fixation with the specific anti-mitochondrial antibodies found in the serum in primary biliary cirrhosis (Doniach *et al.*, 1966). It seems unlikely that the LMT results could be attributed to a common membrane component in view of the inactivity of liver microsomes when tested with the leucocytes of Hashimoto patients.

The thyroid microsomal LMT in Hashimoto thyroiditis was previously shown to correlate with a certain variant of the disease (Wartenberg *et al.*, 1973) and inhibition was maximal in the few individuals whose goitre failed to respond to thyroxine replacement therapy. This variant (hypercellular, including oxyphil and lymphocytic thyroiditis subvariants) tends to present a characteristic serological pattern in that the thyroglobulin antibodies are low or absent, while microsomal antibodies are usually of moderate or high titre (CFT). When the Hashimoto patients in our study, regardless of their responsiveness to thyroxine, were considered together, those with low thyroglobulin antibodies (TRC titre up to 1:320) tended to have a more pronounced LMT with liver mitochondria than the group with high TRC titres, while no statistically significant difference was found between these two groups in the thyroid microsomal LMT. Furthermore by comparing the TRC with the mitochondrial LMT for each of the thirty-four thyroiditis cases a significant negative correlation was established between these two parameters. The more destructive (fibrous) variant of Hashimoto's struma, which is characterized by high titres of both thyroglobulin precipitins and microsomal antibodies also occasionally fail to regress in response to thyroxine but these cases are unusual, since severe Hashimoto thyroiditis is generally rare, and the goitres normally get smaller. Thyroxine-resistant fibrous cases were not available for study but it would be interesting to see if a strong cellular hypersensitivity might accompany poor response to treatment in this situation as it does in the milder forms of the disease. It is noteworthy that the leucocytes of the rare thyroid patients having mitochondrial antibodies in their serum reacted feebly in the LMT with liver mitochondria.

Mitochondrial leucocyte migration inhibition does not appear to be species dependent and preliminary results suggest that the active site is in the inner membrane as are the other two autoantigens known to be involved in human disease, i.e. cardiolipin, the Wassermann antigen, and the lipoprotein reacting with primary biliary cirrhosis sera.

The LMT phenomenon with mitochondria seems to be widespread in human disease

and to be independent of circulating antibodies to these particles. So far, in addition to autoimmune liver disease (Brostoff, 1970; Smith *et al.*, 1972) positive LMT results have been found in the three organ-specific autoimmune disorders affecting the thyroid gland, the gastric mucosa and adrenal cortex respectively (Nerup *et al.*, 1969; 1970) and in insulin-dependent diabetes mellitus (Richens *et al.*, 1972) which has some serological and clinical connections with these conditions. Calder *et al.* (1972) found thyroid mitochondria more active than liver and kidney mitochondria in thyroiditis patients and Nerup *et al.* (1970) considered the adrenal mitochondria as having an organ-specific effect in Addison's disease. Differences between mitochondria from various tissues have not yet been extensively investigated and further LMT work with standardized subcellular fractions checked by enzyme tests for contamination with microsomes and organ-specific cell surface antigens may be required before this aspect can be settled.

Mitochondrial leucocyte migration inhibition has also been found in patients with coronary ischaemia after obvious thrombosis as well as in angina pectoris without massive cardiac infarction (Wartenberg & Brostoff, 1973, in preparation). Preliminary findings also suggest that operative trauma is sufficient stimulus for this cellular 'stress reaction' which is at present poorly understood. The experimental organ-specific autoimmune diseases are produced most readily by the use of Freund's complete adjuvant which is thought to evoke a heightened T-cell reactivity (Allison, 1972). It has often been postulated that genetically determined factors simulating Freund's adjuvant are also operative in the human counterpart. Thus the mitochondrial LMT could be an expression of altered T-cell reactivity. The fact that the mitochondrial LMT parallels the organ-specific microsomal immunity so closely in thyroiditis certainly suggests that the two might be linked at some point.

Clearly no genetic abnormality of the immune systems can account for the post-operative LMT results, so the cellular response to mitochondria must have wider implications. On the other hand, cell death cannot be entirely responsible for the phenomenon since many cases of Graves' disease show no more tissue destruction than is seen in non-toxic colloid goitre. The positive findings in guinea-pigs injected with oil (Weir & Suckling, 1971) rather suggest that mitochondrial LMT is an expression of a response to the inflammatory reaction in general. The findings in autoimmune disorders would perhaps be compatible with such a hypothesis although there must be very little active inflammation in thyroid cases who have been operated upon years before and whose thyroid gland remnant must be small as judged by absent thyroid function, though evidently there is still some immunological stimulus, since antibodies and cellular hypersensitivity to thyroid persist. Perhaps a wider evolutionary explanation must be sought in the bacteria-like character of mitochondria (Thomas, 1972) and their possible foreignness in relation to the rest of the organism.

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