Clin. exp. Immunol. (1972) **12**, 429–438.

THE EFFECT OF THYROID ANTIGENS ON THE *IN VITRO* MIGRATION OF LEUCOCYTES FROM PATIENTS WITH HASHIMOTO THYROIDITIS

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(*Received* 26 *June* 1972);

SUMMARY

A total of fifty-two patients with Hashimoto thyroiditis were tested for delayed hypersensitivity to thyroid antigens using the leucocyte migration test. The percentage of patients showing abnormal migration in the presence of crude thyroid extract, thyroglobulin, thyroid mitochondria and thyroid microsomes was 75, 44, 54 and 34% respectively. Fifty-three control patients were studied concurrently with the same antigens and the percentage showing abnormal migration was 4, 6, 6 and 6% respectively. The antigenic activity of the mitochondrial fraction was not organ specific; both liver and kidney mitochondria interfered with the migration of leucocytes from patients with Hashimoto thyroiditis.

INTRODUCTION

During the past 10–15 yr, an extensive literature has accumulated on autoimmune phenomena in Hashimoto thyroiditis. A variety of circulating autoantibodies directed against components of the thyroid gland have been demonstrated in the serum of patients with Hashimoto thyroiditis (Doniach & Roitt, 1969). The importance of these antibodies in the disease process is, however, uncertain. Doniach (1967) reported that the titre of thyroid autoantibodies correlated with the histological extent of focal thyroiditis, but attempts to passively transfer thyroiditis to experimental animals with Hashimoto serum have failed (Doniach, 1967).

Experimental allergic thyroiditis can be readily induced by the injection of thyroglobulin in Freund's adjuvant, but no correlation exists between the severity of thyroid damage and the titre of circulating antibody (Wasserman & Packalen, 1965; Ringertz *et al.*, 1971). Furthermore, thyroiditis may be induced in animals by the transfer of immunocompetent cells from immunized donors (McMaster & Lerner, 1967), but less readily by serum, although some successful passive transfer experiments have been reported (Nakamura & Weigle, 1969; Vladutiu & Rose, 1971). As a result, interest has centred upon the role of

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cellular immune mechanisms. The massive infiltration of lymphocytes and mononuclear cells lends some support to this view.

To date, however, relatively few observations of cellular hypersensitivity to constituents of the thyroid gland have been published. Using the leucocyte migration test as an *in vitro* correlate of delayed hypersensitivity, Søberg & Halberg (1968) demonstrated cellular hypersensitivity to crude thyroid extract in twelve out of fifteen patients. Brostoff (1970) later demonstrated hypersensitivity to thyroglobulin in sixteen out of twenty-six Hashimoto patients.

The purpose of this investigation was to extend knowledge of cell-mediated immune mechanisms in patients with Hashimoto thyroiditis by studying the effect of crude thyroid extract and also sub-cellular components of the thyroid gland on leucocyte migration. In addition, the organ specificity of the reaction with mitochondrial fractions from other tissues was investigated.

METHODS AND PATIENTS

Patients

Migration tests were performed on a total of fifty-two patients with Hashimoto thyroiditis, forty-eight females and four males. Diagnosis was on clinical and serological evidence and twenty-nine of the patients had histological confirmation. Migration tests were performed concurrently on fifty-three control subjects, forty-nine females and four males. These consisted of medical and laboratory personnel (forty) and hospital patients with miscellaneous conditions (thirteen). Only control patients with negative thyroid antibody levels, as determined by tanned red cell and complement fixation tests, were included in the results. Control and Hashimoto patients were not strictly age matched. However, no correlation existed between migration results and the age of the patients.

Leucocyte migration test

The method of Bendixen & Søberg (1969) was used with minor modification. The theoretical basis of the test depends on the fact that lymphocytes from a sensitized individual, on contact with specific antigen, produce a soluble factor which modifies cell migration. In the absence of antigen cell migration is unaffected. 50 ml venous blood was collected. 10 ml was allowed to clot and the serum stored for subsequent antibody determination. The remainder was heparinized (Evans preservative-free heparin, 10 units/ml) and the blood allowed to sediment at 37°C for 1–2 hr. The leucocyte-rich plasma was removed, centrifuged at 150 *g* for 10 minutes and the cell pellet washed three times in Tissue Culture Medium 199 (Wellcome Reagents Ltd). If red cell contamination was excessive, they were lysed with ammonium chloride (0·84%) for 5 min and the leucocyte pellet washed a further three times with Tc 199. The washed cells were then resuspended in Tc 199 supplemented with 10% foetal calf serum (Wellcome Reagents Ltd). Capillary tubes (Drummond 25 μl microcaps) were filled with the cell suspension, sealed at one end (Cristaseal; Hawksley) and centrifuged at 150 *g* for 5 min. The tubes were cut 1 mm below the cell-fluid interface and the cell pellet positioned, by means of silicone grease, in a leucocyte migration chamber (Sterilin Ltd). One series of at least three chambers was filled with culture medium alone and a second series with culture medium plus antigen. The chambers were sealed with glass coverslips (Chance No. 2) and incubated horizontally for 24 hr. The fan-like pattern of

migration was then projected (Projectina Microscope) and the area measured by planimetry. The effect of antigen on cell migration was expressed as a percentage of the migration without antigen as follows:

$$\frac{\text{Mean migration in antigen}}{\text{Mean migration in Tc 199}} \times 100\%.$$

Results were analysed statistically using Student's *t*-test to see if the presence of antigen had had a significant effect on cell migration. The average variation between replicate cultures was 10% and it was generally found that migrations of $\leqslant 80\%$ or $\geqslant 120\%$ were significant at $P \leqslant 0{\cdot}05$.

Antigens

Thyroglobulin: Three different batches of thyroglobulin were tested. One was supplied by Wellcome Reagents Ltd and was used at a concentration of 50 μg/ml. The second was prepared by ammonium sulphate precipitation of crude thyroid extract as described by Roitt & Doniach (1958) and was tested at a concentration of 50–200 μg/ml. The third batch was prepared by centrifuging crude thyroid extract at 105,000 *g* for 60 min to remove the mitochondria and microsomes. The remaining supernatant, which contained thyroglobulin (20 mg/ml as determined by gel-diffusion), was used without further purification at a concentration of 100–200 μg/ml protein. These three batches of thyroglobulin had similar physicochemical and serological properties in terms of Sephadex G-200 chromatography and gel-diffusion.

Crude thyroid extract was prepared as described by Goudie *et al.* (1957) with slight modification. Fresh human thyroid gland was frozen, cut finely, homogenized in ×4 volumes of saline and left to extract overnight at 4°C. The crude extract was then filtered through muslin, centrifuged at 600 *g* for 15 min to remove debris, dialysed overnight against water and stored in small aliquots at −20°C. It was used in the migration cultures at a concentration of 350 μg/ml protein.

Thyroid mitochondria and microsomes were prepared from the crude extract by differential centrifugation exactly as described by Nerup & Bendixen (1969). Subcellular preparations were washed three times in saline and stored in small aliquots at −20°C. Thyroid mitochondria and microsomes were used at a concentration of 200 and 500 μg/ml respectively.

Liver and kidney mitochondria were prepared from fresh human liver and kidney in an identical manner. The concentrations used were 10 and 100 μg/ml protein respectively. Antigen concentrations higher than those quoted inhibited the migration of leucocytes from control subjects.

Serology: All sera were tested and titred for thyroid and mitochondrial antibodies by indirect immunofluorescence using unfixed sections of human thyroid and rat kidney and by tanned red cell agglutination and complement fixation using purified thyroglobulin and thyroid extract respectively.

RESULTS

Fig. 1 shows the effect of crude thyroid extract on leucocyte migration. Thirty-eight out of fifty-one Hashimoto patients tested (75%) gave abnormal migration; four patients showed stimulation, the mean percentage migration being $133{\cdot}0 \pm 7{\cdot}5$ (SEM); thirty-four showed

inhibition, 63·4±2·1, and the remaining thirteen were unaffected, 98·3±3·4. Of the fifty-three control subjects tested, only two (4%) showed slightly abnormal migration, 95·8±1·3. Crude thyroid extract contains a number of antigenic components, one of which could be thyroglobulin. The effect of pure human thyroglobulin on leucocyte migration is shown in Fig. 2. With this purified antigen, only fourteen out of thirty-two Hashimoto patients (44%) gave abnormal responses. One patient was stimulated, 133·0; thirteen showed inhibition, 65·4±3·1, and the remaining eighteen responded normally, 92·5±1·9. Only two of the thirty-six control patients (6%) were affected 97·7±1·7.

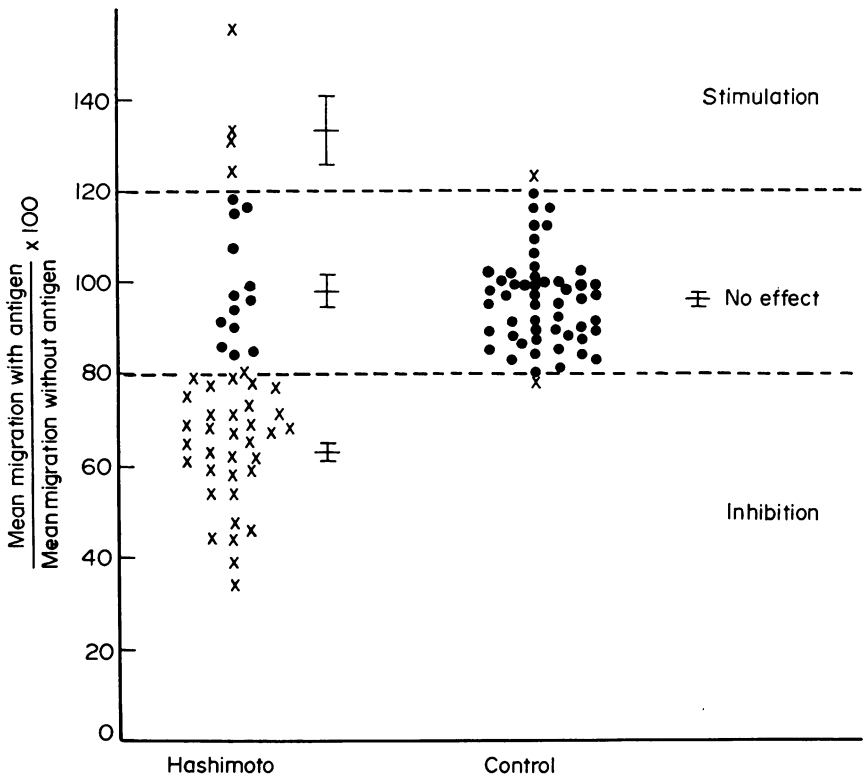

FIG. 1. The effect of crude thyroid extract on the migration of leucocytes from control subjects and patients with Hashimoto thyroiditis. ×, Significant at $P \leqslant 0{\cdot}05$; ●, not significant.

It would appear from these results that the antigenic activity of crude thyroid extract is not due entirely to its thyroglobulin content. In an attempt to localize the additional antigenic activity, sub-cellular components of thyroid gland were studied.

The effect of thyroid mitochondria on leucocyte migration is shown in Fig. 3; twenty-eight out of fifty-two patients (54%) showing abnormality; five of those being stimulated, 127·0±2·7; twenty-three being inhibited, 63·3±2·7 and the remaining twenty-four being unaffected, 93·5±1·6. Again, control patients gave a normal response, only three out of fifty-three (6%) giving a significant effect, 98·5±1·5.

Antigenic activity of the sub-cellular components of thyroid tissue was not confined to the mitochondrial fraction. Thyroid microsomes also interfered with migration as can be

seen in Fig. 4 where fourteen out of forty Hashimoto patients (35%) gave abnormal migration; three showing stimulation, 151·3±21·1; eleven being inhibited, 63·6±2·9 and twenty-six migrating normally, 100·1±2·1. Only two of the thirty-five control patients were affected, 99·2±1·6.

The results shown in Fig. 5 indicated that the antigenic activity of the mitochondrial preparation was not organ-specific. Both kidney and liver mitochondria inhibited leucocyte migration in Hashimoto patients although to a lesser extent than did thyroid mitochondria.

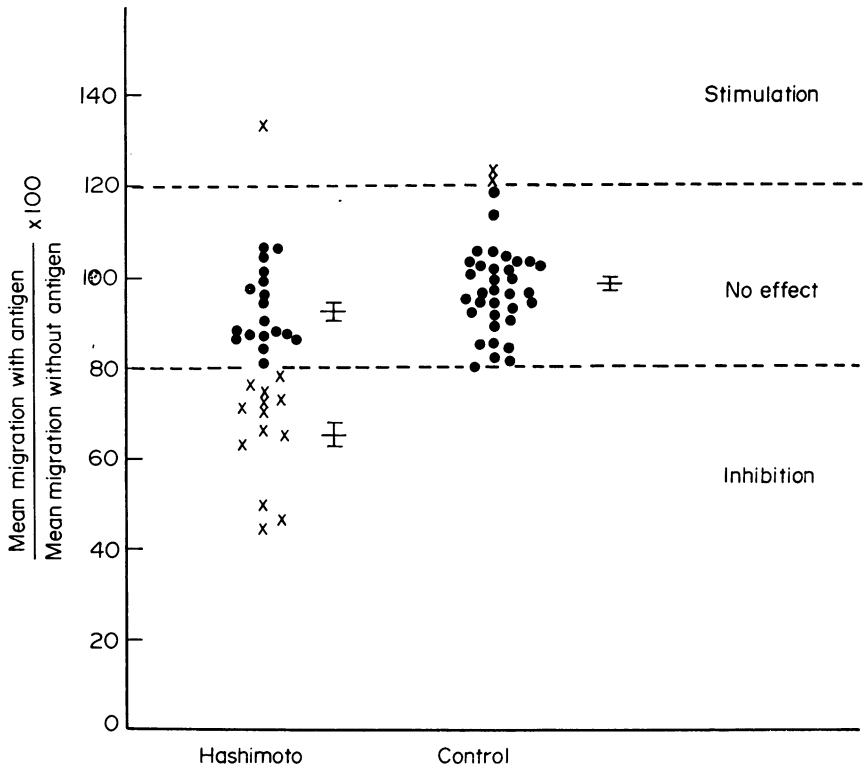

FIG. 2. The effect of thyroglobulin on the migration of leucocytes from control subjects and patients with Hashimoto thyroiditis. ×, Significant at $P \leqslant 0{\cdot}05$; ●, not significant.

With kidney mitochondria, five out of eleven patients (45%) showed inhibition, 72·0±3·6 while six patients responded normally, 94·5±3·8. With liver mitochondria, eight out of seventeen patients (47%) gave abnormal migrations, one stimulation of 126·0, seven inhibitions, 61·7±6·7 and nine unaffected 99·1±3·0. None of the control patients gave abnormal migrations, 99·2±1·4 and 97·6±1·3 respectively.

The effect of crude thyroid extract, centrifuged to remove the mitochondria and microsomes, on leucocyte migration is shown in Fig. 6. Of the twenty-five Hashimoto patients tested, only one gave a slightly abnormal response, the mean migration being 97·4±2·3. Twenty-one control subjects were tested but only three showed slight stimulation, the mean migration being 103·81±2·9.

The purified batch of human thyroglobulin (Wellcome Reagents Ltd) inhibited leucocyte migration in seven of the same twenty-five Hashimoto patients. Thyroglobulin prepared by ammonium sulphate precipitation of crude thyroid extract gave very variable results in the migration test and after 2–3 weeks storage at −20°C was inactive.

Serology: An attempt was made to correlate thyroid antibody titres, both tanned red cell and complement fixing, with migration index but no correlation was found with any of the antigens tested.

The sera of all the patients studied were negative for mitochondrial antibody.

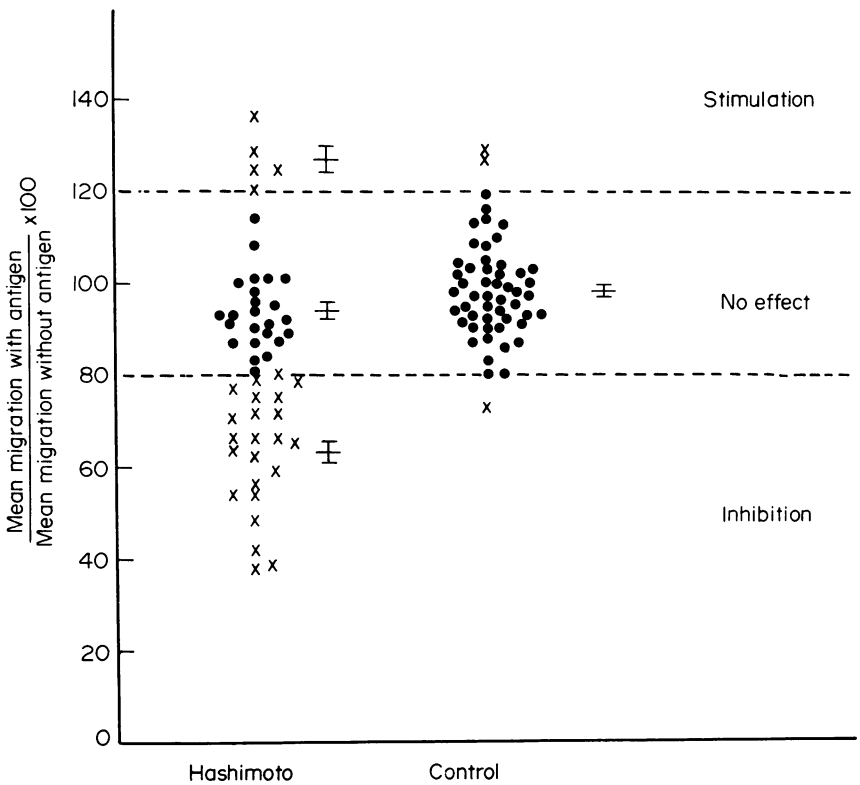

FIG. 3. The effect of thyroid mitochondria on the migration of leucocytes from control subjects and patients with Hashimoto thyroiditis. ×, Significant at $P \leqslant 0{\cdot}5$; ●, not significant.

DISCUSSION

The leucocyte migration test is now well established as an *in vitro* correlate of delayed hypersensitivity and has been used frequently to study cell-mediated immune mechanisms in a variety of diseases of a suspected autoimmune origin; thyroiditis (Søberg & Halberg, 1968), idiopathic Addison's disease (Nerup, Anderson & Bendixen, 1969), pernicious anaemia (Brostoff, 1970) and Crohn's disease (Willoughby & Mitchell, 1971). The results presented here indicate that a high proportion of patients with Hashimoto thyroiditis

75%) have circulating lymphocytes which are sensitized to constituents of the thyroid gland. Attempts to identify the components of thyroid extract responsible for the inhibition of migration, show that at least three are involved; thyroglobulin, mitochondria and microsomes. A higher percentage of patients showed inhibition with thyroid mitochondria (54%) than with either thyroglobulin (44%) or microsomes (35%).

The results with the particulate-free crude thyroid extract are of interest. This antigen contained a high concentration of thyroglobulin, as determined by gel-diffusion, but was

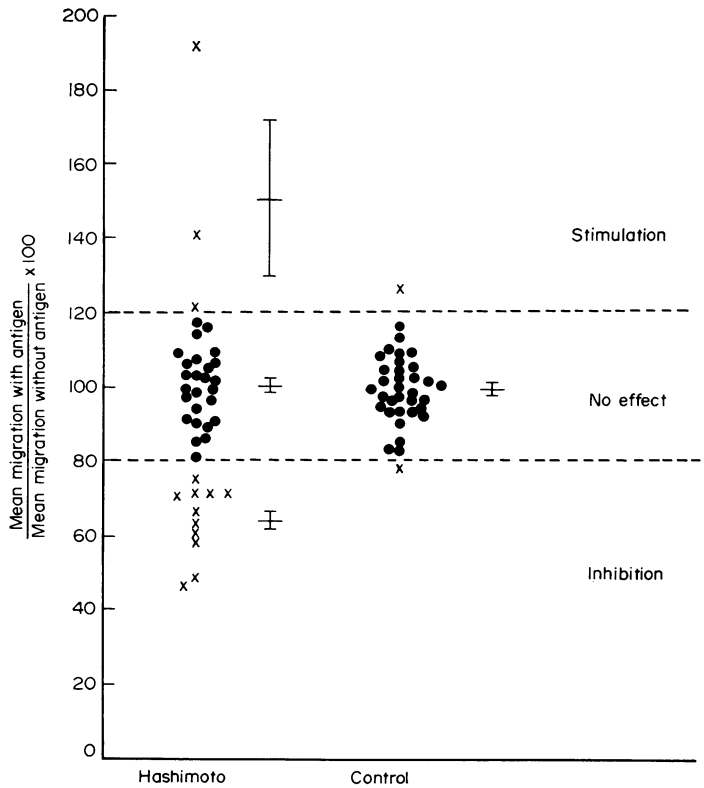

FIG. 4. The effect of thyroid microsomes on the migration of leucocytes from control subjects and patients with Hashimoto thyroiditis. ×, Significant at $P \leqslant 0{\cdot}05$; ●, not significant.

without effect on leucocyte migration in any of the Hashimoto patients tested, although some of these patients were inhibited with the commercially obtained human thyroglobulin. These results suggest that the thyroglobulin present in the crude thyroid extract is in some way different from the purified substance, perhaps complexed or partially denatured. Preliminary investigation suggested that thyroglobulin was not very stable; different batches did not have the same activity on a weight basis and one batch was found to be completely inactive. Furthermore, thyroglobulin prepared by ammonium sulphate precipitation of crude thyroid extract (Roitt & Doniach, 1958) gave variable results in the migration test and after 2–3 weeks storage at −20°C was inactive. It would seem therefore

that the molecular component of human thyroglobulin which is antigenically active in the leucocyte migration test is not stable. As regards the organ specificity of mitochondria in the test, the antigenic activity was found to be non-organ-specific, inhibition being observed with both liver and kidney mitochondria thus indicating some degree of cross-reactivity. This agrees with the results of Brostoff (1970) who showed that leucocyte migration in Hashimoto patients was inhibited by rat liver mitochondria. Nerup *et al.* (1969) reported, however, that the antigenic activity of mitochondria in Addison's disease was organ-specific.

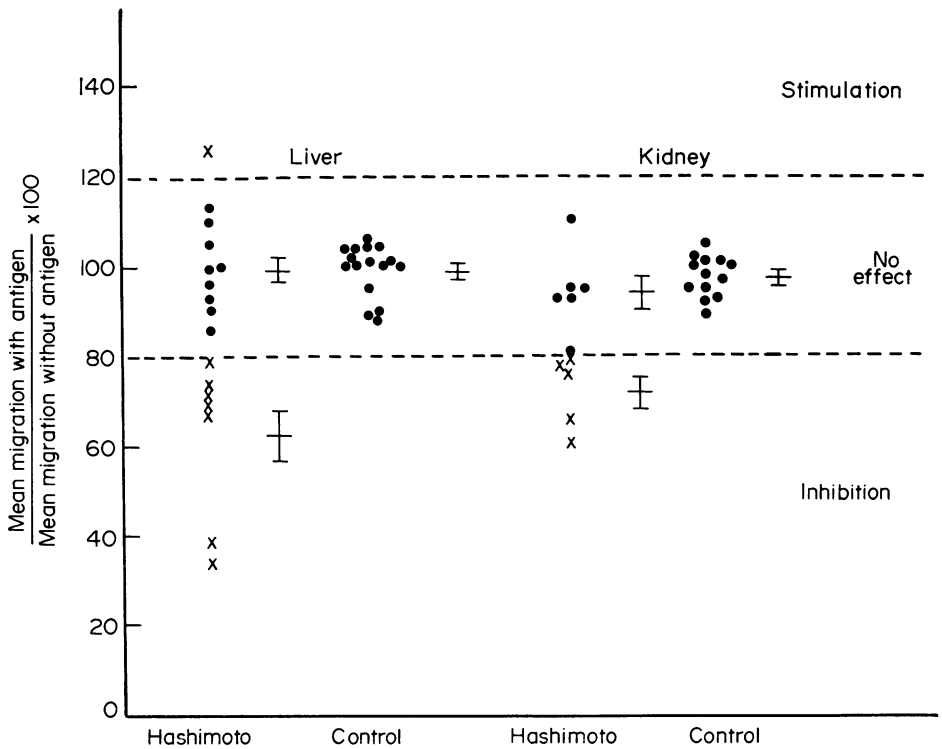

FIG. 5. The effect of liver and kidney mitochondria on the migration of leucocytes from control subjects and patients with Hashimoto thyroiditis. ×, Significant at $P \leqslant 0{\cdot}05$; ●, not significant.

It is somewhat surprising that delayed hypersensitivity to non-organ-specific mitochondria should be associated with an organ-specific disease such as Hashimoto thyroiditis. It is interesting that Richens *et al.* (1972) have recently reported similar findings with diabetes mellitis, which is known to be associated with the organ-specific group of autoimmune disorders (Irvine *et al.*, 1970). The possible pathogenic role of lymphocytes specifically sensitized to mitochondria is uncertain; they may be a sequel to the destruction of the thyroid gland, or, alternatively, simply be part of a general manifestation of autoimmune disease.

If in fact cell-mediated immune mechanisms play a part in the production of autoimmune lesions it is likely that the initiating reaction is one involving a cell-surface component.

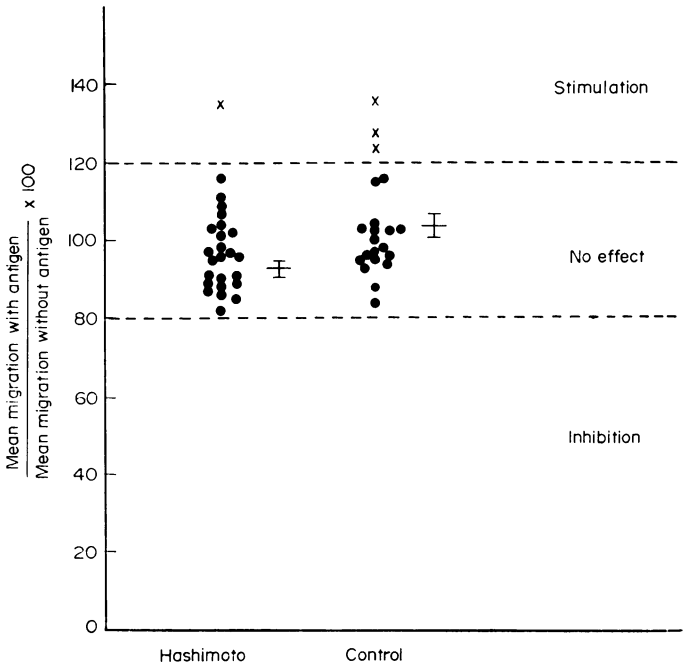

FIG. 6. The effect of particulate-free thyroid extract on the migration of leucocytes from control subjects and patients with Hashimoto thyroiditis. ×, Significant at $P \geqslant 0{\cdot}005$; ●, not significant.

REFERENCES

BENDIXEN, G. & SØBERG, M. (1969) A leukocyte migration technique for the *in vitro* detection of cellular hypersensitivity in man. *Dan. med. Bull.* **16,** 1.

BROSTOFF, J. (1970) Migration inhibition studies in human disease. *Proc. roy. Soc. Med.* **63,** 905.

DONIACH, D. & ROITT, I.M. (1969) Autoimmune thyroid disease. *Text Book of Immunopathology* (Ed. by P. A. Miescher and H. J. Müller-Eberhard) p. 516. Grune and Stratton, New York.

DONIACH, D. (1967) Thyroid autoimmune disease. *J. clin. Path.* **20,** 385.

GOUDIE, R.B., ANDERSON, J.R., GRAY, K.G., CLARK, D.H., MURRAY, I.P. & NICOL, G.P. (1957) Precipitin tests in thyroid disease. *Lancet*, **i,** 976.

IRVINE, W.J., CLARKE, B.F., SCARTH, L., CULLEN, D.R. & DUNCAN, L.J.P. (1970) Thyroid and gastric autoimmunity in patients with diabetes mellitis. *Lancet*, **ii,** 163.

MCMASTER, P.R.B. & LERNER, E.M. (1967) The transfer of allergic thyroiditis in histocompatible guinea-pigs by lymph node cells. *J. Immunol.* **99,** 208.

NAKAMURA, R.M. & WEIGLE, W.O. (1969) The transfer of experimental autoimmune thyroiditis by the serum from thymectomised donors. *J. exp. Med.* **130,** 263.

NERUP, J., ANDERSON, V. & BENDIXEN, G. (1969) Anti-adrenal cellular hypersensitivity in Addison's disease. *Clin. exp. Immunol.* **4,** 355.

NERUP, J. & BENDIXEN, G. (1969) Anti-adrenal cellular hypersensitivity in Addison's disease. *Clin. exp. Immunol.* **5,** 357.

RICHENS, E.R., ANCILL, I.J., GOUGH, K.R. & HARTOG, M. (1972) Cellular hypersensitivity to mitochondrial antigens in diabetes mellitis. *Clin. exp. Immunol.* **13,** (In press).

RINGERTZ, B., WASSERMAN, J., PACKALEN, TH. & PERLMAN, P. (1971) Cellular and humoral responses in experimental autoimmune thyroiditis. *Int. Arch. Allergy*, **40,** 917.

ROITT, I.M. & DONIACH, D. (1958) Human autoimmune thyroiditis. *Lancet*, **ii,** 1027.

SØBERG, M. & HALBERG, P. (1968) Cellular hypersensitivity in Hashimoto's thyroiditis. *Acta med. scand.* **183,** 101.

VLADUTIU, A.O. & ROSE, N.R. (1971) Transfer of experimental autoimmune thyroiditis in the mouse by serum. *J. Immunol.* **106,** 1139.

WASSERMAN, J. & PACKALEN, TH. (1965) Immune responses to thyroglobulin in experimental thyroiditis. *Immunology*, **9,** 1.

WILLOUGHBY, J.M.T. & MITCHELL, D.N. (1971) *In vitro* inhibition of leukocyte migration in Crohn's disease by sarcoid spleen suspension. *Brit. med. J.* **3,** 155.