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CELLULAR HYPERSENSITIVITY TO MITOCHONDRIAL ANTIGENS IN DIABETES MELLITUS

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SUMMARY

The occurrence of cellular hypersensitivity to rat liver mitochondria has been investigated in diabetic patients and control subjects by means of the leucocyte migration technique. Cellular hypersensitivity was demonstrated in 72% of insulin-dependent diabetics and 46% of non-insulin-dependent diabetics compared with 11% in the control group of normal individuals.

INTRODUCTION

There is a considerable amount of circumstantial evidence to suggest that autoimmunity may play a part in the development of some cases of diabetes mellitus. Thus there is an association between the occurrence of diabetes mellitus and other diseases such as Addison's disease which are thought to be autoimmune in origin (Turkington & Lebovitz, 1967). In addition, in cases of juvenile onset diabetes lymphocyte infiltration of the islet cells has often been seen (Warren, 1927; Gepts, 1965). Finally, patients, particularly with juvenile onset diabetes, show an abnormal incidence of circulating antibodies to gastric parietal cells, intrinsic factor and thyroid antigens (Irvine *et al.*, 1970).

Recently, using the leucocyte migration technique, Bendixen & Søborg (1969) and Nerup *et al.* (1971) found organ-specific cellular hypersensitivity against the microsomal fraction of islet cells in approximately two-thirds of a group of diabetics.

Brostoff (1970) has shown similar organ-specific cellular hypersensitivity in a proportion of patients with Hashimoto's disease, pernicious anaemia and primary biliary cirrhosis. He has also shown that approximately two-thirds of the patients in these groups showed migration inhibition when tested against rat liver mitochondria, which may be a general manifestation of autoallergic diseases.

In the current study we have, therefore, used the leucocyte migration technique to see

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whether peripheral leucocytes from diabetics show migration inhibition in the presence of preparations of mitochondria.

MATERIALS AND METHODS

Patients

Diabetic patients were divided into two groups for the purpose of this study. The first group contained the insulin-dependent diabetics, mainly under the age of thirty-five (mean age 27 years, range 15–47 years) and the second group the non-insulin-dependent diabetics (mean age 62 years, range 20–84 years). The mean duration of diabetes in the first group was 7 years (range 2 weeks to 15 years) and in the second group was 9 years (range 1 month to 25 years).

Migration tests were also carried out in a group of normal subjects and hospital patients with miscellaneous conditions (drug overdoses, broncho-pneumonia, congestive heart failure, duodenal ulcer).

Leucocyte migration test

This test depends on the fact that leucocytes from sensitized individuals do not migrate from a capillary in the presence of the specific antigen to the same extent as they do in its absence. The method of Bendixen & Søborg (1969) was used. 25 ml of heparinized venous blood was allowed to sediment in sterile containers. The supernatant containing the leucocytes was removed, centrifuged at 100 *g* for 10 min and the cell pellet washed three times in Eagles medium MEM (Wellcome Reagents Ltd). The washed leucocytes were resuspended in medium containing 20% foetal calf serum (Flow Laboratories, Irvine, Scotland), and taken up into capillary tubes which were recentrifuged. Capillaries were then broken at the fluid cell interface and the cell pellet placed in a chamber containing medium and different antigens. Control chambers were set up without antigen. After 20 hr incubation at 37°C the areas of migration were measured by projection and planimetry and a 'migration index' calculated by dividing the mean of three to five measurements in the chambers to which the antigen had been added by the mean of a similar number of measurements for the control chambers. A migration index of less than 0·8 was taken to indicate significant inhibition of migration and greater than 1·2, significant stimulation.

Antigens

Mitochondrial antigens were prepared from several sources using the method of Zamecnik & Keller (1954). They were resuspended after preparation in sufficient 0·25 M sucrose to give a final protein concentration of 1 mg/ml and were stored in 1-ml aliquots at −20°C. The majority of the experiments were carried out using mitochondria prepared from liver taken from 3-month-old Sprague-Dawley rats. To check the specificity of the reaction, additional experiments were performed using mitochondria similarly prepared from rat adrenal gland and kidney, and from human liver, obtained as soon as possible post-mortem.

The antigen concentration routinely used was 100 μg protein/ml tissue culture fluid, but additional concentrations over the range 100 ng–200 μg protein/ml tissue culture fluid were also sometimes used.

RESULTS

Using rat liver mitochondrial preparations at both 100 μg and 50 μg protein/ml tissue culture fluid, approximately 70% of insulin-dependent diabetics and 45% of non-insulin-dependent diabetics showed inhibition of migration compared to 11% of normal controls (Fig. 1). The mean values for each group with the significance of the difference of the means are shown in Table 1.

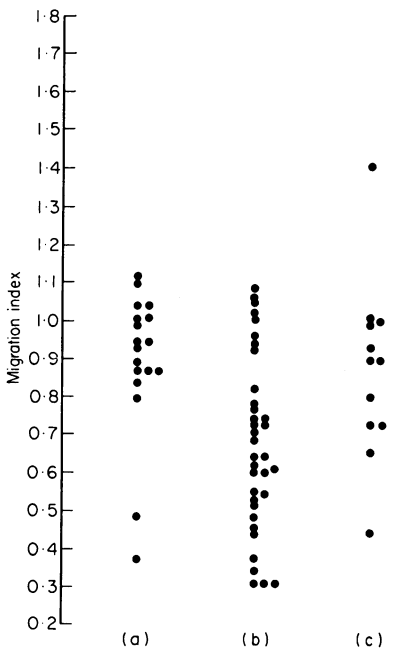

FIG. 1. Leucocyte migration test with rat liver mitochondria (100 μg protein/ml tissue culture fluid) in (a) control subjects, (b) insulin-dependent diabetics and (c) non-insulin-dependent diabetics.

One of the two subjects in the control group who gave low migration indices was an elderly man with haematemesis who had recently been transfused in whom it was not possible to repeat the test. The second subject was a healthy young man with no record of illness apart from jaundice 17 years previously, and who yielded a similar result on repeat testing 6 months later. Using complement fixation and immunofluorescence tests, his serum was found to be negative for circulating antibodies to mitochondria, and also to thyroid, gastric mucosa, DNA and smooth muscle antigens.

Repeat tests were carried out on some diabetic patients and control subjects at intervals of 3 and 6 months after the first measurement, to assess their reproducibility (Table 2).

The insulin-dependent diabetics were divided into those of fairly recent onset, i.e. less than 3 years' duration, and those with a longer history of the disease (Fig. 2). The mean migration indices obtained with rat liver mitochondria were not significantly different.

Further tests were carried out on groups of control subjects and insulin-dependent diabetics to compare the migration inhibition obtained with rat liver mitochondria with antigens from other sources. Human liver mitochondria and rat kidney and adrenal gland

TABLE 1. Leucocyte migration indices (mean ± S.D.) in diabetic and control subjects using rat liver mitochondria

Group	Antigen concentration (μg protein/ml tissue culture fluid)	
	100	50
(a) Control	0·89 ± 0·19 (18)*	0·92 ± 0·18 (19)
(b) Insulin-dependent diabetics	0·68 ± 0·23 (34)	0·74 ± 0·26 (25)
(c) Non-insulin-dependent diabetics	0·73 ± 0·27 (12)	0·77 ± 0·17 (6)

Significance of difference of means

Groups compared	*P* values of Student's *t*-test			
	100		50	
a–b	$P = 0{\cdot}001$	sig.	$0{\cdot}02 > P > 0{\cdot}01$	sig.
a–c	$0{\cdot}10 > P > 0{\cdot}05$	NS	$0{\cdot}10 > P > 0{\cdot}05$	NS
b–c	$P > 0{\cdot}10$	NS	$P > 0{\cdot}10$	NS

* Number in each group.

mitochondria were used. The results are shown in Table 3 and indicate that there was no difference between the effects of the rat and human liver mitochondria preparations. However, mitochondria prepared from other rat tissue did not inhibit leucocyte migration.

On one occasion it was noted that stimulation of migration resulted from contact with antigen at one concentration, while at a higher concentration inhibition was obtained. The leucocytes from two other insulin-dependent diabetic patients were therefore tested with a range of antigen concentration from 100 ng–200 μg rat mitochondrial protein/ml tissue

TABLE 2. Reproducibility of leucocyte migration test in several subjects tested on different occasions with rat liver mitochondria (100 μg protein/ml tissue culture fluid)

Subject		Migration index (MI)					
		Initial		3 months		6 months	
		M.I.	% of mean	M.I.	% of mean	M.I.	% of mean
Diabetics	1	0·73	112	0·60	93	0·62	97
	2	0·44	87	0·54	106	0·56	110
	3	0·58	113	0·46	90	0·49	96
Controls	1	1·03	103	1·03	103	0·94	94
	2	1·07	98	1·11	102	1·08	99

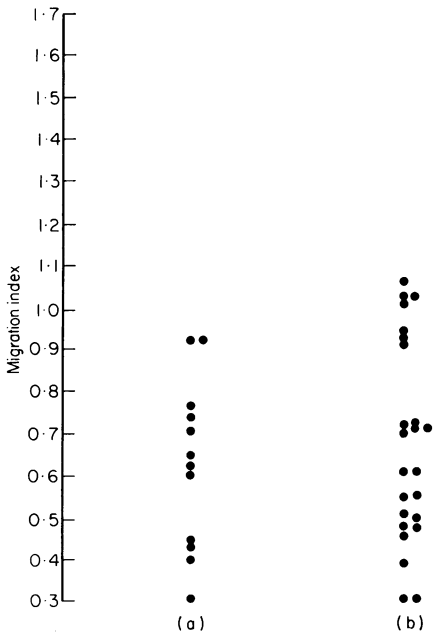

FIG. 2. Leucocyte migration test with rat liver mitochondria (100 μg protein/ml tissue culture fluid) in insulin-dependent diabetics of (a) less than 3 years' duration and (b) more than 3 years' duration.

culture fluid (Fig. 3). No stimulation was seen in these patients but the degree of inhibition is increased with increasing concentrations of antigen.

DISCUSSION

The altered *in vitro* reactivity of leucocytes from diabetics demonstrated in this study presumably reflects a cellular hypersensitivity against some antigenic component present in liver mitochondria. The reaction appears to be specific to mitochondria prepared from liver since comparable preparations from rat kidney and rat adrenal gland did not induce inhibition, and species non-specific since human liver mitochondria elicited a similar response.

Thus, the results suggest that a species non-specific anti-liver mitochondrial hypersensitivity of the cellular type is demonstrable in patients with diabetes mellitus. The phenomenon was most clearly seen in the insulin-dependent group of diabetics. Within this group there was no significant difference between those patients with a recent onset of the disease and those with a longer duration since onset.

The finding of cellular hypersensitivity to mitochondria in diabetics supports the hypothesis that this disease is related in some way to other conditions considered to be of autoimmune origin. In Addison's disease (Nerup & Bendixen, 1969) and primary biliary cirrhosis (Hardt *et al.*, 1969) migration inhibition with mitochondria is considered to be organ-specific, but the effect shown in diabetics, and in pernicious anaemia and Hashimoto's thyroiditis, indicate that liver mitochondria may be active against a variety of tissues. Two

TABLE 3. Leucocyte migration indices (mean ± S.D.) in (a) diabetic and (b) control subjects using liver mitochondria (rat and human) and rat mitochondria (liver, kidney and adrenal gland)

Group	Mitochondrial antigen (100 μg protein/ml tissue culture fluid)				
	(1) Rat liver	(2) Human liver	(3) Rat liver	(4) Rat kidney	(5) Rat adrenal gland
(a) Diabetics	0·62 ± 0·13 (12)*	0·68 ± 0·15 (12)	0·57 ± 0·20 (5)	1·11 ± 0·28 (5)	1·03 ± 0·10 (5)
(b) Controls	0·89 ± 0·19 (18)	0·98 ± 0·13 (7)	1·06 ± 0·10 (5)	0·98 ± 0·10 (5)	0·98 ± 0·04 (5)

Significance of difference of means

Groups compared	*P* values of Student's *t*-test	Significance
a1–b1	$P < 0{\cdot}001$	sig.
a2–b2	$P < 0{\cdot}001$	sig.
a1–a2	$P > 0{\cdot}10$	NS
b1–b2	$P > 0{\cdot}10$	NS
a3–b3	$0{\cdot}01 > P > 0{\cdot}001$	sig.
a4–b4	$P > 0{\cdot}10$	NS
a5–b5	$P > 0{\cdot}10$	NS
a3–a4	$0{\cdot}02 > P > 0{\cdot}01$	sig.
a3–a5	$0{\cdot}01 > P > 0{\cdot}001$	sig.
b3–b4	$P > 0{\cdot}10$	NS
b3–b5	$P > 0{\cdot}10$	NS

* Number in each group.

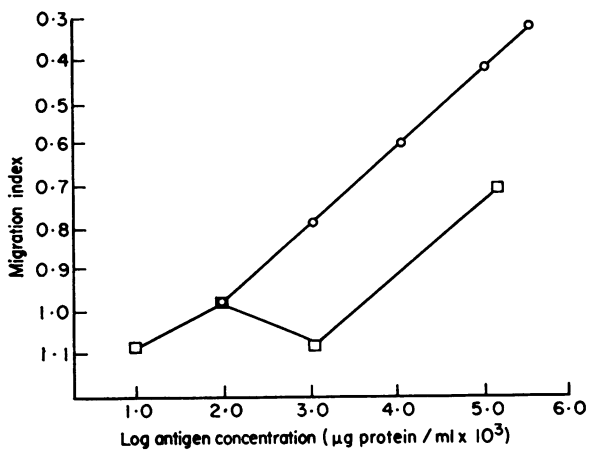

FIG. 3. The effect of antigen concentration on leucocyte migration in diabetic subjects.

possible explanations for the development of hypersensitivity to mitochondria may be: (1) The postulated failure of the body to develop tolerance to particular subcellular components (Pinckard & Weir, 1966). (2) The 'foreign' nature of mitochondria, which have many characteristics in common with protokaryotic organisms (Nass, 1971).

In either of these cases the malfunction of immunological surveillance manifested in autoimmune disease may exacerbate a predisposition for hypersensitivity to mitochondria.

The occasional stimulation of migration which was noted has been reported with weak sensitization to an antigen (Søborg, 1967; Smith *et al.*, 1971) as compared with inhibition which is indicative of strong sensitization. Alternatively, a low antigen concentration may give rise to stimulation of migration whereas a higher concentration may give either a normal (i.e. transition) index or inhibition of migration. In the cases where an antigen titration was performed, apparently transitional indices were obtained at low antigen dilution.

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