Cell-mediated Immunity to Human Pancreas in Diabetes Mellitus

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SUMMARY

Cellular hypersensitivity to an extract of human pancreas, using the leucocyte migration test (LMT), was found in twenty-nine of 101 diabetic and eight of fifty normal control subjects. However, the difference in response between diabetics and controls was confined to young insulin-dependent patients, there being no distinction between normal subjects and older diabetics treated by diet or oral hypoglycemic agents. The use of rat liver mitochondria and bovine insulin as antigens in the LMT did not induce inhibition of leucocyte migration in diabetics or controls. DIABETES *23*:693-97, **August, 1974.**

Immune phenomena in diabetes mellitus have recently been studied by in vitro tests of cell-mediated immunologic function to detect an antigen-sensitized population of lymphocytes. Thus Federlin et al.[1,2] showed that lymphocytes from diabetics allergic to insulin were transformed to blast cells when exposed to purified bovine insulin antigen. Other investigators, using the leucocyte migration test (LMT),[3] have suggested that autoimmune mechanisms may be present in diabetics by demonstrating migration inhibition of diabetic leucocytes when exposed to antigens derived from duct-ligated porcine pancreas[4,6] and fetal calf pancreas.[5,6] Attempts to inhibit migration by using insulin alone as antigen have so far been unsuccessful.[4-6] Migration inhibition in diabetics has also been reported using human and rat liver mitochondria;[7] these antigens can, however, only be considered as general nonspecific "markers" of autoimmune disease since inhibition of leucocyte migration to liver mitochondria is commonly present in patients with Hashimoto's thyroiditis[8,9] and pernicious anemia[8,10] as well as in primary biliary cirrhosis.[8]

The present study extends these observations by (1) assessing the effect of an antigen derived from human pancreas on leucocyte migration in diabetics and controls and (2) comparing the results with those obtained using rat liver mitochondria and bovine insulin as antigens.

PATIENTS AND METHODS

Patients. One hundred and one diabetic and fifty-six control subjects were studied and were classified as follows:

Young diabetics. These were thirty-one insulin-dependent diabetics: eighteen women and thirteen men aged eighteen to forty-two years (mean 28.5) and insulin treated for between one and nineteen years (mean 7.4).

Young controls. These were twenty-seven healthy nondiabetic volunteers who were selected to allow statistical comparisons with the young diabetics. Fifteen were women and twelve men aged eighteen to forty-two years (mean 28.6).

Older diabetics on oral hypoglycemic agents (OHA). There were thirty-four diabetics diagnosed at age forty years or more who were controlled by dietary restriction plus a sulfonylurea. Twenty were women and fourteen men aged forty-five to seventy years (mean 59.7).

Older diet-treated diabetics. These thirty-six patients were controlled by simple carbohydrate restriction only; twenty-one were women and fifteen men aged forty-six to sixty-nine years (mean 60.5).

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Older controls. Twenty-three healthy nondiabetic volunteers were studied, selected by sex (thirteen women and ten men) and age (range forty-eight to sixty-nine, mean 57.4 years), to allow valid statistical comparison with the previous two groups.

Positive reactors. This small group, included to check the potency of the mitochondrial preparation, consisted of one patient with primary biliary cirrhosis and five with Hashimoto's thyroiditis, known to show inhibition of leucocyte migration when previously tested.[9]

All the diabetics regularly attended the Diabetic Department as outpatients and when blood was taken for study, were well controlled and free of infection. Control subjects were healthy volunteers, with the exception of the positive reactors.

Antigens

Human pancreas was obtained immediately after death from a previously healthy young man who sustained fatal head injuries in an automobile accident and whose kidneys were used for transplant. The fresh pancreas was cut into small pieces, homogenized in sterile phosphate buffered saline, filtered through fine gauze and then centrifuged at 700 g for twenty minutes. The supernatant was used as antigen, its protein content being adjusted to 10 mg./ml. and stored at −20° C. in small aliquots. When required these were thawed and further diluted with tissue culture fluid consisting of Eagle's Basal Medium (EBM, Wellcome) supplemented with 10 per cent fetal calf serum. Pilot experiments showed the highest nontoxic concentration of antigen to be 200 μg./ml., which was used for all experiments.

Mitochondrial preparations were obtained from the livers of young Wistar rats by the differential centrifugation technic of Nerup and Bendixen.[11] This mitochondrial antigen was used at a concentration of 200 μg./ml. culture medium.

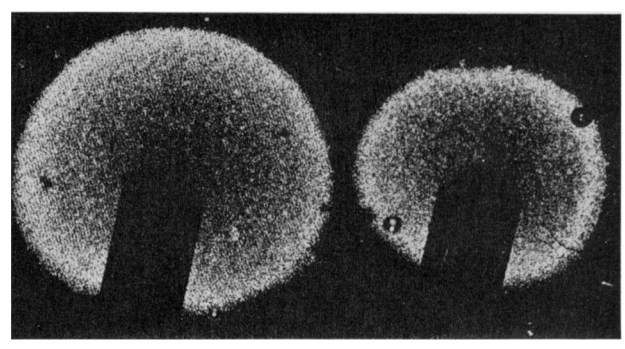
Highly purified bovine insulin was obtained from the Lilly Research Laboratories (lot no. 615-D63-5) in crystalline form and diluted in culture medium to give a working concentration of 116 μg./ml.

Leucocyte migration test (LMT)

The LMT was performed as described previously from this laboratory,[9,10] the method used being that of Bendixen and Soborg[3] with minor modifications. 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(s) which modifies cell migration. In the absence of antigen, cell migration is unaffected. Fifty milliliters of venous blood were collected from patients and controls, heparinized (preservative-free heparin 10 units/ml.) and allowed to sediment at 37° C. from one to two hours. The leucocyte-rich plasma was removed, centrifuged at 150 g for ten minutes and the cell pellet washed three times in EBM. Contaminating red cells were lysed with ammonium chloride (0.83 per cent) for five minutes and the leucocyte pellet washed a further three times with EBM. The washed cells were then resuspended in EBM with 10 per cent fetal calf serum. Capillary tubes (25 μl.) were filled with the cell suspension, sealed at one end and centrifuged at 150 g for five minutes. The tubes were cut 1 mm. below the cell-fluid interface and the cell pellet positioned, with a dab 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 and incubated on a flat surface at 37° C. for twenty-four hours. The fanlike pattern of migration was then projected (Projectina microscope) and the area measured by planimetry. The effect of antigen on cell migration (the "migration index") was expressed as a percentage of migration without antigen using the formula:

$$\text{Migration index} = \frac{\text{Mean migration with antigen} \times 100\%}{\text{Mean migration without antigen}}$$

A figure of less than 80 per cent was taken to indicate significant inhibition of migration and above 120 per cent, significant stimulation; this range is identical to that adopted by other investigators[4,7] and represents two standard deviations above and below the mean migration index shown by normal subjects to antigens in this and previous studies.[9,10]



Photograph: **Inhibition of leucocyte migration in the presence of pancreatic antigen. On the left, leucocytes have migrated normally from the capillary tube into culture medium; on the right, migration has been inhibited by the addition of pancreatic antigen to the culture medium.**

RESULTS

All diabetics and normal controls were tested with human pancreatic antigen (figures 1 and 2). Seventeen of the thirty-one young diabetics showed inhibition as compared with only four of the twenty-seven young controls; the mean migration index for the former (77.7 ± 2.3 S.E.M.) was significantly lower than in the latter (88.3 ± 2.1). In contrast only six older diabetics on OHA and six of those on diet alone showed migration inhibition compared with two of the corresponding controls; the respective mean migration indices were 93.4 ± 2.8, 91.5 ± 2.4 and 94.4 ± 2.5 and do not differ significantly. The young insulin-dependent diabetics differed significantly from all other groups, both individually and collectively ($p < 0.01$ by the Wilcoxon test) but there were otherwise no significant differences (table 1).

The results of tests using rat liver mitochondrial antigen are shown in figure 3. The mean migration

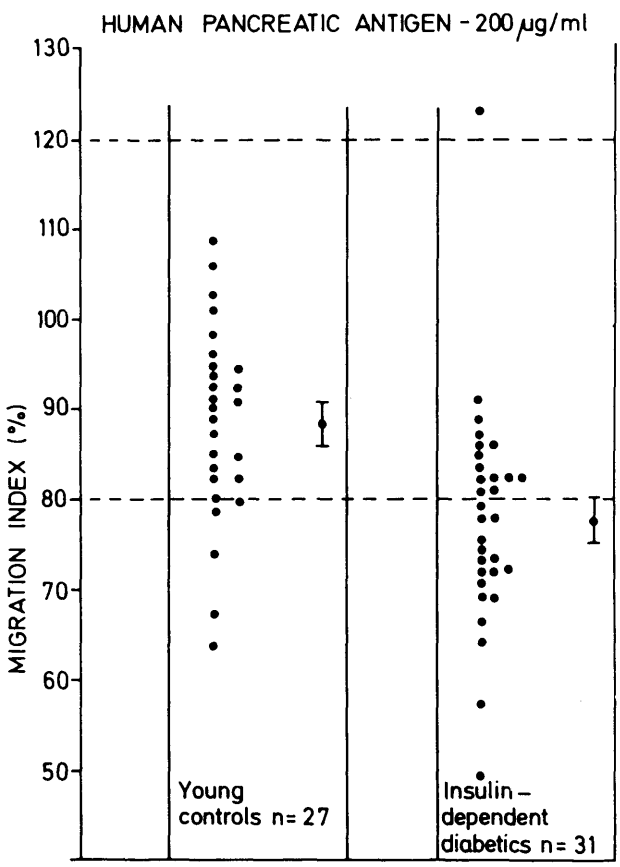


FIG. 1. Leucocyte migration tests with human pancreatic antigen in young controls and insulin-dependent diabetics. Inhibition of migration is shown by seventeen (55 per cent) diabetics compared with four (15 per cent) controls.

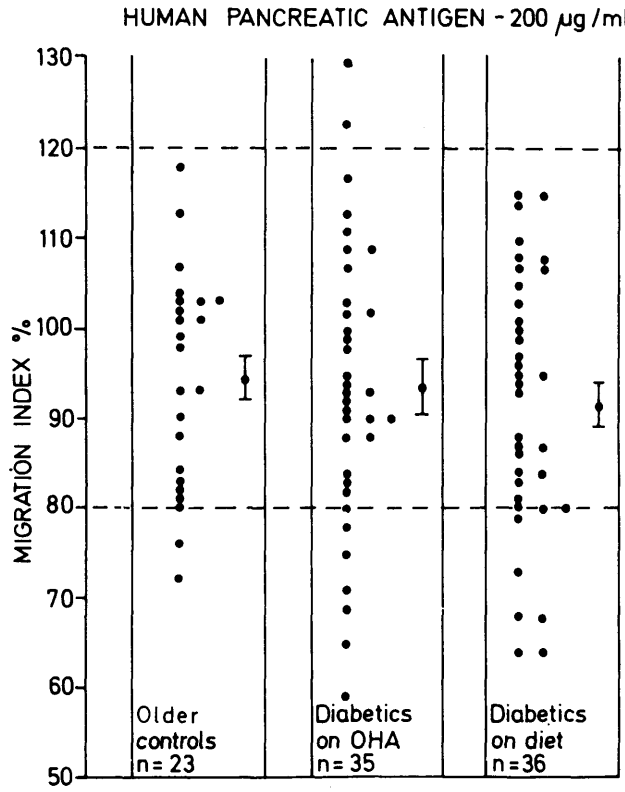


FIG. 2. Leucocyte migration tests with human pancreatic antigen in older controls and insulin-independent diabetics. Inhibition of migration is shown by six (17 per cent) each of diabetics on OHA and diet respectively, compared with two (9 per cent) controls.

indices ± S.E.M. for young diabetics, normal controls and older diabetics were 88.5 ± 3.2, 92.7 ± 2.3 and 91.9 ± 2.3, respectively, these groups not differing significantly. In contrast all patients with Hashimoto's thyroiditis or primary biliary cirrhosis, included as positive reactors, showed marked migration inhibition (mean 69.5 ± 2.4), confirming the potency of the mitochondrial antigen; this group differed significantly from all other groups, both collectively and individually ($p < 0.01$ by Wilcoxon's test, table 2).

Bovine insulin was used as antigen to test leucocyte

TABLE 1

Leucocyte migration tests in diabetics and controls
Human pancreatic antigen—200 µg./ml.

Groups compared	Difference between groups
Young diabetics—all other groups	$p < 0.01$ sig
Young controls—older controls	$p > 0.10$ NS
Young controls—older diabetics (Diet & OHA)	$p > 0.10$ NS
Older controls—older diabetics (Diet & OHA)	$p > 0.10$ NS

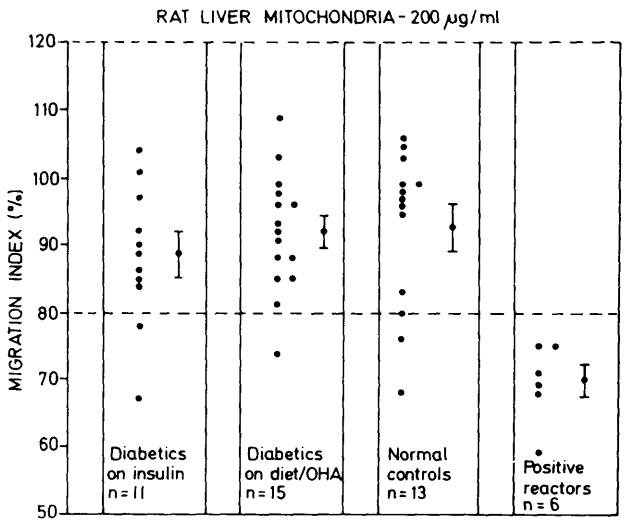


FIG. 3. Leucocyte migration tests with rat liver mitochondrial antigen in insulin-dependent and independent diabetics, normal controls and "positive reactors" (see text). Only the group of positive reactors shows significant inhibition of migration.

migration in all the young insulin-treated diabetics and corresponding controls; none showed migration inhibition to insulin at a concentration of 116 µg./ml. culture medium (equivalent to 3.2 i.u./ml. culture medium). Above this level a toxic effect was observed. The mean migration was virtually identical in diabetics and controls (94.8 ± 4.1 and 93.9 ± 3.7, respectively).

DISCUSSION

Nerup et al.[4] first described inhibition of leucocyte migration to a pancreatic antigen in fifteen of twenty-two diabetics, all but four of whom were insulin-dependent, and most were under forty-five years of age. The antigen used was derived from pooled porcine pancreas in which atrophy of exocrine tissue had been induced by prolonged ligation of the pancreatic duct. The same investigators[5,6] later used an antigen of homogenated fetal calf pancreas to demonstrate migration inhibition in thirty-one (28 per cent) of 112 diabetics. Inhibition was shown in both juvenile- and maturity-onset diabetics, occurring as often in insulin-dependent as in noninsulin-dependent patients; however, the phenomenon was most commonly found in young recently diagnosed diabetics, irrespective of therapy. The present studies using human pancreas have demonstrated migration inhibition in a comparable number of diabetics (29 per cent of 101 patients) and confirm that cell-mediated immunity as judged by the LMT is found most often in insulin-dependent patients with juvenile-onset diabetes. As yet we have not tested a sufficient number of young insulin-dependent patients, or newly diagnosed untreated diabetics, to draw any further conclusions. Nerup et al. did not find inhibition of migration in any type of diabetic by using porcine or bovine insulin antigens,[4-6] and this has been our experience using bovine insulin. It would seem that the results of the present and previous studies indicate the existence in diabetes of a state of cell-mediated immunity to an antigen which is present in the pancreas, species-nonspecific (demonstrable with porcine, bovine and human pancreas), and different from insulin. The phenomenon is found almost exclusively in juvenile-onset diabetics.

In diabetics the position regarding migration inhibition to antigens from other organs is more controversial. Richens et al.[7] showed inhibition in twenty-six of thirty-five young insulin-dependent diabetics and in four of twelve elderly insulin-independent diabetics tested with an antigen consisting of liver mitochondria from young Sprague-Dawley rats; subsequent studies by the same investigators have suggested that the antigenic component is localized to the inner mitochondrial membrane.[12] Richens et al. also described migration inhibition in a small number of insulin-dependent diabetics tested with human liver mitochondria but found no inhibition to antigens of mitochondria from rat kidney and adrenal.[7] On the other hand Nerup et al.[4-6] used antigens of porcine kidney, porcine liver, fetal calf liver and thymus in their series of diabetics and did not demonstrate migration inhibition to any of these antigens. Our own results, using rat liver mitochondria of proven potency, are in agreement with the latter investigators. The reason for the differences between the various studies are not clear but may in part reflect different methods of antigen preparation:

TABLE 2

Leucocyte migration tests in diabetics and controls
Rat liver mitochondrial antigen—200 µg./ml.

Groups compared	Difference between groups
Young diabetics—controls	$p > 0.10$ NS
Young diabetics—older diabetics (Diet & OHA)	$p > 0.10$ NS
Older diabetics—controls (Diet & OHA)	$p > 0.10$ NS
Positive reactors—all other groups. (Hashimoto, primary biliary)	$p < 0.01$ sig

Richens et al. used the method of Zamecnik and Keller[13] and employed as antigen the fraction obtained by ultracentrifugation at 5,000 g, while the earliest study of Nerup et al.[4] used a different method of antigen preparation[11] and employed as antigen the fraction obtained by centrifugation at 104,000 g, which has a high content of microsomes.

Further studies are required to clarify this problem and also to attempt to define which component of pancreatic tissue acts as antigen toward the lymphocyte population in patients with diabetes mellitus.

ACKNOWLEDGMENT

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