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ANTI-ADRENAL CELLULAR HYPERSENSITIVITY IN ADDISON'S DISEASE

IV. *IN VIVO* AND *IN VITRO* INVESTIGATIONS ON THE MITOCHONDRIAL FRACTION

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SUMMARY

Nine patients with idiopathic Addison's disease and three patients with diabetes mellitus and circulating anti-adrenal antibody but without Addison's disease were studied.

The organ-specific, anti-adrenal cellular hypersensitivity previously demonstrated by means of the leucocyte migration test in patients with idiopathic Addison's disease was found to be directed against the mitochondrial fraction of normal, human, foetal adrenocortical cells as well as of human, benign hyperplastic adrenal glands. The mitochondrial fraction of human, foetal liver cells did not cause inhibition of migration.

In lymphocyte cultures from the patients with idiopathic Addison's disease the adrenocortical, mitochondrial fraction did not induce blast transformation. The two *in vitro* reactions therefore probably express different kinds of reactivity.

The adrenocortical mitochondrial fraction was able to elicit intracutaneous reactions of delayed type in patients with a positive leucocyte migration test to the same antigen. This observation confirms, that inhibition of leucocyte migration *in vitro* indicates a state of anti-adrenal cellular hypersensitivity in patients with idiopathic Addison's disease.

INTRODUCTION

By means of the leucocyte migration test (LMT) it was possible in a previous study (Nerup, Andersen & Bendixen, 1969) to demonstrate the existence of anti-adrenal cellular hypersensitivity in patients with idiopathic Addison's disease. Blood leucocytes from patients with idiopathic Addison's disease were found to possess a specifically altered reactivity against an organ-specific, species-non-specific antigen of the adrenal cortex. Experiments with various subcellular fractions of human benign hyperplastic adrenal cortex as antigen

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showed that the leucocyte migration of idiopathic Addison patients was inhibited only by the mitochondrial fraction of adrenocortical cells (Nerup & Bendixen, 1969a, b).

Antigen-induced blast transformation of lymphocytes *in vitro* has been considered an indication of hypersensitivity of the cellular type (Mills, 1966), but antigenic components of foetal, adrenocortical extract did not induce blast transformation of peripheral lymphocytes from patients with idiopathic Addison's disease although the same antigen induced a clearly positive reaction in the LMT (Nerup *et al.*, 1969).

In the present study we report the result of further studies of anti-adrenal cellular hypersensitivity, intended especially to illustrate:

(1) The organ-specificity of anti-adrenal cellular hypersensitivity at the subcellular level by experiments comparing the antigenic activity in the LMT of the mitochondrial fractions from (a) human, hyperplastic adrenal glands, (b) human foetal adrenal glands, (c) human foetal liver.

(2) If the lack in ability of adrenal extract to induce *in vitro* blast transformation in patients with anti-adrenal cellular hypersensitivity also exists when a mitochondrial fraction of adrenal cortex is used as antigen.

(3) If the anti-adrenal cellular hypersensitivity in patients with idiopathic Addison's disease as demonstrated *in vitro* by means of the LMT corresponds to anti-adrenal cellular hypersensitivity as demonstrated *in vivo* by means of the delayed type intracutaneous reaction.

MATERIAL

The study comprises *nine patients with idiopathic Addison's disease*. The diagnosis was made according to generally accepted criteria. Seven of the patients (Nos. 1, 4, 6, 20, 21, 22, 24) have been characterized in a previous study (Nerup & Bendixen, 1969a) using identical case numbers. Two cases have not been described previously. One of these (case No. 41) was a woman of 24 years. After 2 years with slight symptoms mainly as pigmentation, her adrenal insufficiency became clinically manifest during an episode of urinary infection in the 4th month of pregnancy. The diagnosis was verified by the corticotrophin test. She had no associated diseases and was examined for the present study at the time of diagnosis. The last patient in this group was a man of 24 years (case No. 42). A diagnosis of idiopathic Addison's disease was made 6 years before the study, the initial manifestation being an Addison crisis associated with severe hypoglycaemia. The diagnosis was verified by the corticotrophin test. Besides diabetes mellitus which became manifest 6 years before the idiopathic Addison's disease, the patient had developed a diffuse non-toxic goitre which was still present at the time of study.

The examination further comprises *three patients with diabetes mellitus* and circulating anti-adrenal serum antibodies, but without signs of idiopathic Addison's disease. Two of these cases (Nos. 38 and 39) were described in a previous study (Nerup & Bendixen, 1969a). The third patient in this group (case No. 43) was a man of 36 years. His diabetes mellitus was diagnosed $2\frac{1}{2}$ years before the examination, and he had no associated disorders.

METHODS

Leucocyte migration test

The leucocyte migration test (LMT) (Søborg & Bendixen, 1967; Bendixen & Søborg, 1969) was used for demonstration of organ-specific cellular hypersensitivity according to the technique previously described (Nerup *et al.*, 1969; Nerup & Bendixen, 1969a).

The antigen-induced inhibition of the migration is an *in vitro* correlate to cellular (delayed type) hypersensitivity (Søborg, 1967, 1968). The influence of an antigen upon the migration is expressed as a migration index, *MI*, which is calculated in the following way:

$$MI = \frac{M_x}{M_0}$$

where M_x is the mean 24-hr migration area of a series of antigen-containing cultures, and M_0 the mean 24-hr migration area in a series of antigen-free cultures. Values for *MI* less than unity indicate an inhibition, and values higher than unity a stimulation.

As antigens were used the following subcellular fractions, prepared as previously described (Nerup & Bendixen, 1969b):

(a) Mitochondrial fraction of two histopathologically verified, benign hyperplastic, human adrenal glands.

(b) Mitochondrial fraction from the adrenal glands of a normal, human embryo 16 weeks of gestation.

(c) Mitochondrial fraction of the liver of the same human embryo.

With the mitochondrial fraction of the benign hyperplastic adrenal glands 50 μg protein per ml culture medium was used, in accordance with a previous study (Nerup & Bendixen, 1969b). With the mitochondrial fraction of foetal adrenal tissue the highest non-toxic dose was found in pilot experiments with normal persons to be 100 μg per ml culture medium, determined as protein. This dose was used throughout the experiment, and the same concentration was employed in experiments with the mitochondrial fraction of foetal liver.

Lymphocyte transformation test

The lymphocyte transformation test was carried out as described by Nerup *et al.* (1969). The mitochondrial fraction of human, benign hyperplastic adrenal glands was used as antigen in concentrations of 2, 10, 50 and 200 μg protein per 2 ml culture medium.

The cultures were harvested after 96 hr. The viability of the cultures was ensured by means of parallel cultures, in which a normal transformation response to phytohaemagglutinin (PHA) could be induced when PHA was added after a culture period of 48 hr.

Intracutaneous reaction

As another criterion of specific anti-adrenal cellular hypersensitivity, the intracutaneous reaction of the delayed type was studied. The microsomal as well as the mitochondrial fractions of human, benign hyperplastic adrenal glands were used as antigens. The subcellular fractions were stored at −20°C for 6 months. During this period of time none of the two tissue donors had developed symptoms or signs of hepatitis. The sterility of the preparations was ensured through aerobic and anaerobic cultivations. Both subcellular fractions were dissolved in 0·25 M sucrose and administered in doses of 0·1 ml containing 10 μg and 50 μg protein. The intracutaneous injections were applied upon the volar surface of the forearm, and the skin reactions were examined after 10 min and after 24, 48 and 72 hr. An infiltration of 5 × 5 mm or more at 24 hr was considered positive. The intracutaneous tests were undertaken in four normal controls (two of the authors and two colleagues) and later in six patients, who all agreed to participate.

RESULTS

Leucocyte migration test

Six patients with idiopathic Addison's disease (cases Nos. 4, 24, 20, 21, 22, 41) and three patients with diabetes mellitus and circulating anti-adrenal antibody but without Addison's disease (cases Nos. 38, 39, 43) were examined.

Fig. 1 presents the migration indices found in the LMT using (1) the mitochondrial fractions of human, benign hyperplastic adrenocortical cells, (2) human foetal adrenocortical cells, and (3) human foetal liver as antigens.

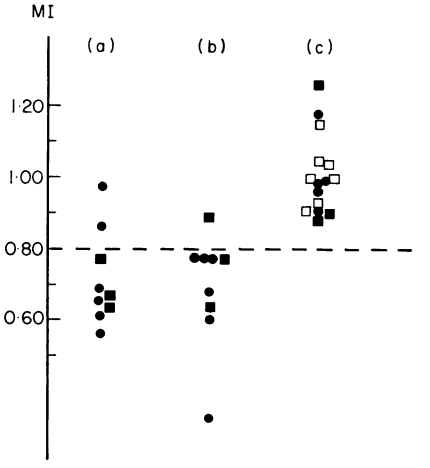

FIG. 1. Leucocyte migration test (LMT) in patients with idiopathic Addison's disease ●, patients with diabetes mellitus and circulating anti-adrenal antibody but without Addison's disease ■ and controls □. Mitochondrial fractions from (a) human hyperplastic adrenal glands, (b) human foetal adrenal glands and (c) human foetal liver were used as antigens.

It appears that in patients with idiopathic Addison's disease low migration indices could be demonstrated with the mitochondrial fractions of hyperplastic as well as foetal adrenocortical cells as antigen, the reaction to the foetal, subcellular fraction being somewhat less pronounced.

One patient (case No. 41) had an extremely low migration index with the human, foetal, mitochondrial fraction as antigen. This patient was a woman of 24 years, whose idiopathic Addison's disease became manifest in the 4th month of pregnancy.

It appears that in none of the patients with idiopathic Addison's disease was the leucocyte migration inhibited by the mitochondrial fraction of foetal, human liver. The migration indices obtained with this subcellular fraction are within the same range as the indices of the controls (Fig. 1).

In two of the patients with diabetes mellitus and circulating, anti-adrenal antibody but without idiopathic Addison's disease the LMT with human, foetal, adrenocortical, mitochondrial fraction showed low migration indices, whereas the mitochondrial fraction of foetal, human liver did not cause any demonstrable alteration of the leucocyte migration.

Lymphocyte transformation test

Blood samples from five patients with idiopathic Addison's disease (cases Nos. 1, 6, 21, 41, 42) and from two diabetics (cases Nos. 39, 43) were examined.

The mitochondrial fraction of human, benign hyperplastic adrenocortical cells was not able to induce blast transformation of the lymphocytes of these patients as estimated on the basis of ^{14}C-thymidine incorporation and morphological examination.

Intracutaneous reactions

Table 1 shows the results of the intracutaneous testing with the microsomal and mitochondrial fractions of human, benign hyperplastic adrenal cortex in three patients with idiopathic Addison's disease (Nos. 22, 24, 41), three diabetics with circulating anti-adrenal antibody but without Addison's disease (Nos. 38, 39, 43) and four healthy controls.

In all persons examined, including the controls, a typical wheal and flare reaction developed immediately after injection of both mitochondrial and microsomal fractions. The reaction was considered non-specific and was not further investigated. A delayed type intracutaneous reaction was induced only by the mitochondrial fraction, which caused this type

TABLE 1. Intracutaneous tests with subcellular fractions from a hyperplastic adrenal gland in three patients with idiopathic Addison's disease (cases Nos. 22, 24 and 41), in three patients with diabetes mellitus with circulating anti-adrenal antibody but without Addison's disease and in four healthy controls

Case No.	Name	Sex	Human hyperplastic adrenal gland. Mitochondrial fraction		Human hyperplastic adrenal gland. Microsomal fraction
			LMT (MI)	Infiltration 24 hr (mm)	Infiltration 24 hr
22	FPF	M	0·65	5×5	Negative
24	MK	M	0·97	Negative	Negative
41	IMG	F	0·61	7×7	Negative
38	AKJ	F	0·67	6×6	Negative
39	IJ	F	0·64	4×5	Negative
43	PEJ	M	0·77	6×6	Negative
Four controls		M	All normal	All negative	All negative

of reaction to develop in two of the three idiopathic Addison patients examined. Two of the three diabetic patients likewise developed a positive delayed type intracutaneous reaction to the adrenocortical mitochondrial fraction whereas a similar reactivity was found in none of four healthy controls. It is interesting to observe that the one patient with idiopathic Addison's disease, in whom the mitochondrial fraction did not induce a positive intracutaneous reaction of the delayed type, had an *MI*-value within the normal range in the LMT.

DISCUSSION

Previous studies using extracts from human and animal organs have indicated that anti-adrenal cellular hypersensitivity in idiopathic Addison's disease is directed against an organ-specific, species-non-specific antigenic component of adrenal cortex (Nerup *et al.*,

1969; Nerup & Bendixen, 1969a, b). It has further been shown that this antigenic activity is present in the mitochondrial fraction of human, benign hyperplastic adrenocortical cells (Nerup & Bendixen, 1969b).

The present investigations demonstrate that the mitochondrial fraction of normal, human, foetal, adrenocortical cells contains a component with similar specific properties. This finding indicates that the antigenic component is not unique for mitochondria of benign hyperplastic adrenal glands in Cushing's syndrome and suggests that the antigen is a component of normal adrenocortical mitochondria as well. Recent investigations in our laboratory have shown that the mitochondrial fraction of pig adrenals (Danish land race) possesses the same antigenic activity.

The mitochondrial fraction of human, foetal liver did not induce migration inhibition in leucocyte cultures from patients with idiopathic Addison's disease when added in doses equivalent to the doses of mitochondrial fraction of human, foetal adrenal gland that caused inhibition in parallel experiments. This observation seems to confirm that the antigen as studied in the present experimental system is organ-specific, also at the subcellular level. Brostoff, Roitt & Doniach (1969) presented observations suggesting that an *in vitro* inhibition of leucocyte migration induced by the mitochondrial fraction of rat liver and not demonstrable in normal controls might be a feature common to various autoimmune diseases in the human. The present findings are at variance with this suggestion.

The lack in ability of extracts of human, foetal adrenal glands to induce blast transformation in lymphocyte cultures from patients with idiopathic Addison's disease, in spite of the capacity of the same extracts to cause inhibition of the leucocyte migration (Nerup *et al.*, 1969), might be attributed to the extreme heterogeneity of the glandular extract. The observation, however, has now been confirmed with the more well-defined mitochondrial fraction as antigen. This finding supports the assumption previously made (Nerup *et al.*, 1969) that the specific reactivities expressed in the LMT and in the lymphocyte transformation test are not identical. Investigations by Søborg, Andersen & Sørensen (1969) confirm this assumption and demonstrate that of these two *in vitro* tests only the LMT is indicative of cellular hypersensitivity.

The demonstrated ability of the human, adrenocortical, mitochondrial fraction to induce an intracutaneous reaction of delayed type in LMT-positive patients with idiopathic Addison's disease confirms that the present modification of the LMT expresses a state of organ-specific cellular hypersensitivity.

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