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ANTI-ADRENAL CELLULAR HYPERSENSITIVITY IN ADDISON'S DISEASE

III. SPECIES-SPECIFICITY AND SUBCELLULAR LOCALIZATION OF THE ANTIGEN

J. NERUP AND G. BENDIXEN

Medical Department A and Medical Department P, Copenhagen University Hospital, Rigshospitalet, Copenhagen

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SUMMARY

In previous studies the existence of anti-adrenal, cellular hypersensitivity in idiopathic Addison's disease was demonstrated by means of the leucocyte migration test.

The antigenic activity of variously derived adrenocortical material was examined to evaluate the species-specificity of the reactivity and determine the subcellular localization of the antigen.

As with extract of foetal, human adrenals it could be demonstrated that extracts of adult human benign-hyperplastic adrenals, of pig adrenals and of monkey adrenals possessed the capacity to induce an inhibition of the *in vitro* migration of leucocytes from patients with idiopathic Addison's disease.

A similar reactivity was not seen with extracts of adrenocortical adenoma or adrenocortical carcinomas in parallel experiments. Out of the various subcellular fractions isolated from adult human benign-hyperplastic adrenals only the mitochondrial fraction was able to induce migration inhibition, indicating that the anti-adrenal cellular and the anti-adrenal humoral hypersensitivity in idiopathic Addison's disease are probably directed against different antigenic determinants at the subcellular level.

INTRODUCTION

The occurrence in patients with idiopathic Addison's disease of anti-adrenal, cellular hypersensitivity was demonstrated by means of the leucocyte migration test (LMT) in previous experiments (Nerup, Andersen & Bendixen, 1969; Nerup & Bendixen, 1969). These investigations indicated that the reactivity was organ-specific, and the correlation of anti-adrenal cellular hypersensitivity to clinical parameters and to the occurrence of anti-adrenal humoral hypersensitivity was examined.

The anti-adrenal cellular hypersensitivity was measured by means of the LMT, using an

Correspondence: Dr J. Nerup, Medical Department A, Rigshospitalet, Blegdamsvej, 2100 Copenhagen Φ, Denmark.

extract of pooled, lyophilized, human, foetal adrenal glands as antigen. This procedure was selected for the following reasons:

(a) Human foetal adrenal gland contains antigenic components which specifically react with the circulating, anti-adrenal antibody in serum from patients with idiopathic Addison's disease.

(b) Perinatally the foetal zone of the human adrenal gland normally undergoes a non-inflammatory haemorrhagic necrosis, which may be instrumental in inducing a state of 'enhanced immunological tolerance' to antigenic components of the adrenal tissue (Bech, Tygstrup & Nerup, 1969). It would consequently be of interest to know if a specifically altered reactivity of immunocompetent cells from patients with idiopathic Addison's disease could be directed against components of foetal, adrenal tissue.

(c) Foetal adrenal glands are easily available. On background of the investigations so far it was found of interest to examine whether adult, human adrenal gland contained components with the same antigenic specificity and to study if a similar antigenic activity could be demonstrated in adrenocortical tissue of different species. Further, it was interesting to attempt a clarification of the subcellular localization of the antigen(s). The results of experiments along these lines are reported below.

MATERIAL AND METHODS

Patients

The material comprised twenty-four patients, fifteen females and nine males, with idiopathic Addison's disease. The diagnosis was verified in the usual way, and clinical details are summarized in a previous report (Nerup & Bendixen, 1969). The group consisted of patients who were willing to have several examinations including collection of blood samples in the out-patient ward. The patients were not selected on the basis of other criteria.

Three patients suffering from diabetes mellitus, with circulating anti-adrenal antibody in serum, but without Addison's disease, were included in the study.

Controls

Twenty-nine subjects (sixteen healthy persons and thirteen patients with various medical diseases but without symptoms of endocrine or immunological disorders) were tested by means of the LMT with the same antigens as the patients. The results were used for calculation of normal ranges.

Leucocyte migration test (LMT)

The leucocyte migration test is described in detail by Søborg & Bendixen (1967), Bendixen & Søborg (1969) and Nerup & Bendixen (1969).

Immunofluorescence technique

Anti-adrenal antibody was detected by an indirect immunofluorescent technique described by Blizzard *et al.* (1962) using monkey adrenals (*Cercopithecus aetiops*) as antigen. The sera were titrated by doubling dilutions.

Antigens

(1) Extract from pooled lyophilized human foetal adrenal glands was prepared as described in our previous reports (Nerup *et al.*, 1969; Nerup & Bendixen, 1969).

The same technique of antigen-preparation and standardization was applied to:

(2) Histologically verified human, benign, hyperplastic adrenal glands from two patients (a man aged 58 and a woman aged 57) undergoing bilateral adrenalectomy for Cushing's disease.

TABLE 1. Preparation of adrenal cell fractions (modified from Goudie *et al.*, 1968)

Preparation	Centrifugation		Products	
	Time (min)	Maximum RCF (*g*)	Deposit	Supernatant
Crude homogenate	10	600	Nuclei and coarse debris	A
Supernatant A	10	600	Nuclei and debris	B
Supernatant B	20	5,000	Mitochondria	C
Supernatant C	60	104,000	Microsomes	D

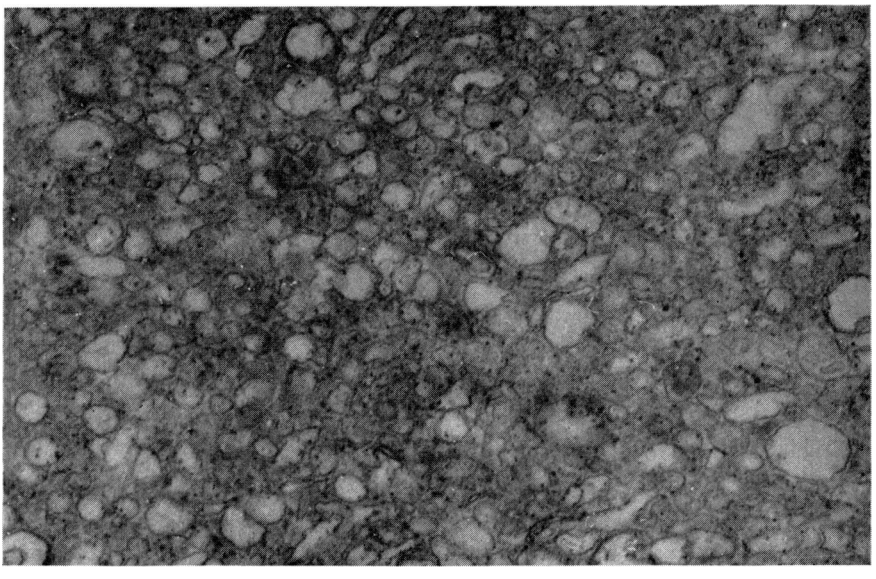

FIG. 1. Electron micrograph of microsomal fraction from human, hyperplastic adrenal gland. ×40,000.

(3) Thirty-two pooled adrenal glands from sixteen pigs (Danish Landrace).

(4) Fifteen pooled adrenal glands from eight monkeys (*Cercopithecus aetiops*).

(5) Two histopathologically verified adrenocortical carcinomas and one histopathologically verified benign adrenocortical adenoma.

(6) *Adrenocortical subcellular fractions* were prepared as described below.

The two contralateral benign hyperplastic adrenal glands from the two patients with Cushing's disease named above were used for preparation of subcellular fractions. Immediately after the operative removal the adrenals were cooled and during all following

procedures kept at temperatures between 0° and 4°C. After sieving, the tissue was homogenized in a Potter–Elvehjelm homogenizator in 10 volumes of 0·25 M sucrose solution. The cell fractions were obtained by differential centrifugation as shown in Table 1.

After obtaining representative samples for electron-microscopy, the pellets were resuspended in the original volumes of 0·25 M sucrose and frozen at −20°C and kept at this temperature until use. Tubes containing 1 ml suspension was used only once and then discarded.

The concentration of protein in the adrenal cell fractions was determined by the method of Lowry *et al.* (1951) and the RNA content was estimated by a micromethod described by Hess & Thalheimer (1965).

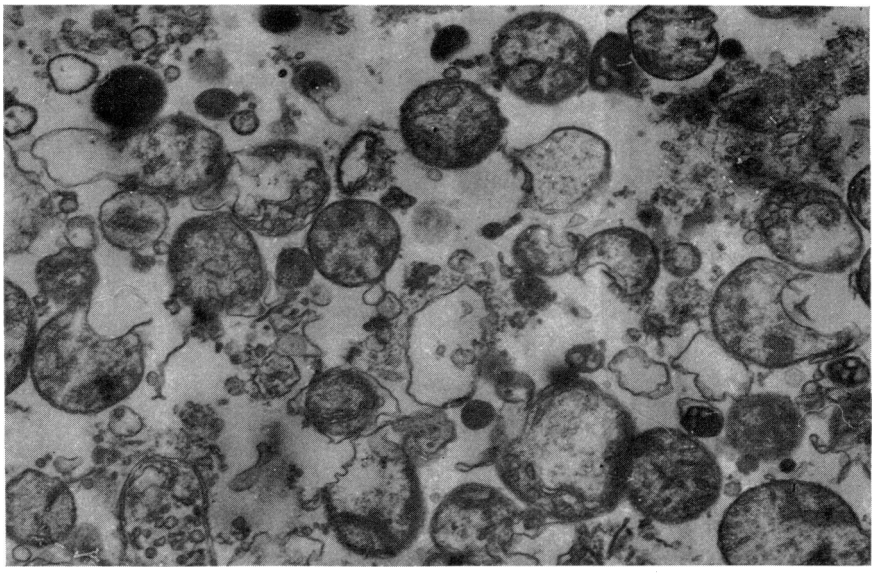

FIG. 2. Electron-micrograph of mitochondrial fraction from human, hyperplastic adrenal gland. ×6250.

TABLE 2. Biochemical characterization of adrenal cell fractions

Fraction	Protein (mg/ml)	Ribonucleic acid (μg/ml)
Nuclei and debris	0·41	31
Mitochondria	5·41	22
Microsomes	5·02	142
Supernatant D	8·61	8

The electron micrographs (Figs. 1 and 2) show that a remarkably good separation between microsomal and mitochondrial fractions has been achieved, although some microsomal vesicles are still present in the mitochondrial fraction (Fig. 2). Contamination of the microsomal fraction with mitochondria was not found. From Fig. 2 it can be seen that the mitochondrial fraction has a certain admixture of lysosomes.

Table 2 presents the results of the chemical analysis of the adrenal cell fractions confirming that a satisfactory separation has been obtained.

RESULTS

The highest concentration, which did not cause an unspecific, toxic inhibition of the migration in controls, was determined in pilot experiments with each antigen. The results are recorded in Table 3, which indicates the various normal ranges and the antigen concentrations employed.

TABLE 3. Summary of results from leucocyte migration tests with various antigens in different control materials

Antigen	No. of controls tested	Highest non-toxic concentration of antigen (μg protein/ml culture medium)	Mean MI	Normal range (mean $\pm$2 SD)
Extract: human, foetal adrenal glands	69	200	0·98	0·84–1·12
Extract: human, hyperplastic adrenal glands	29	200	0·94	0·74–1·14
Extract: pig adrenal glands	13	50	0·98	0·78–1·18
Extract: monkey adrenal glands	12	300	1·07	0·87–1·27
Mitochondrial fraction: human, hyperplastic adrenal glands	13	50	0·96	0·76–1·16

The outcome of the LMT in twenty-three patients with idiopathic Addison's disease, in three patients with diabetes mellitus and in twenty-nine controls is shown in Fig. 3.

Nine of the twenty-three patients with idiopathic Addison's disease and two of the three diabetics had MI-values below the normal range when extract from human, hyperplastic adrenal gland was used as antigen. This extract accordingly possesses the ability to reveal a specifically altered reactivity of migrating leucocytes from patients with idiopathic Addison's disease.

The results of the LMT with porcine adrenal extract and monkey adrenal extract in respectively, thirteen and twelve patients with idiopathic Addison's disease are shown in Fig. 4.

It appears that normal adrenal cortex from pig as well as monkey contains antigenic component(s) which can elicit a specific cellular response, quite similar to the response seen with human adrenocortical extract. The reactivity could not be demonstrated when patients with idiopathic Addison's disease were tested with extracts from adrenocortical carcinomas or from a benign adrenocortical adenoma, indicating that these tumours did not contain the antigenetically active component(s).

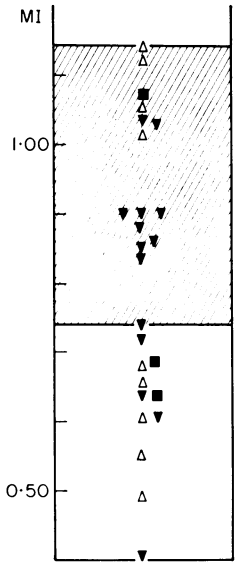

FIG. 3. Leucocyte migration test (LMT) with human hyperplastic adrenal gland extract in twenty-three patients with idiopathic Addison's disease. △, Males; ▼, females; ■, female diabetics with anti-adrenal antibody in serum, but without Addison's disease. Hatched area, normal range (mean ±2 SD). MI, Migration indices.

The results of the LMT using the microsomal fraction and supernatant D as antigens were identical in patient and control material. This was also the case in a few experiments with the nuclear fraction as antigen. Out of the adrenal subcellular fractions only the mitochondrial fraction contained an antigenic activity, which could clearly be demonstrated as a capability

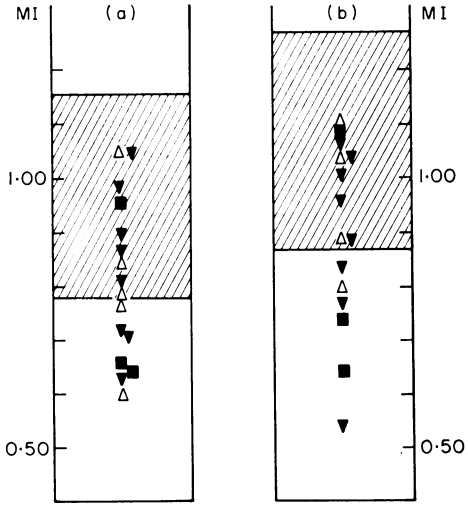

FIG. 4. Leucocyte migration test (LMT) with: (a) pig adrenal extract, and (b) monkey adrenal extract in thirteen and twelve patients, respectively, with idiopathic Addison's disease. △, Males; ▼, females; ■, female diabetics with anti-adrenal antibody in serum but without Addison's disease. Hatched area, normal range (mean ±2 SD).

of this fraction to elicit an inhibition of cell migration in cultures of leucocytes from patients with idiopathic Addison's disease (Fig. 5).

Results similar to those obtained in patients with idiopathic Addison's disease were found in two of the three patients investigated with diabetes mellitus.

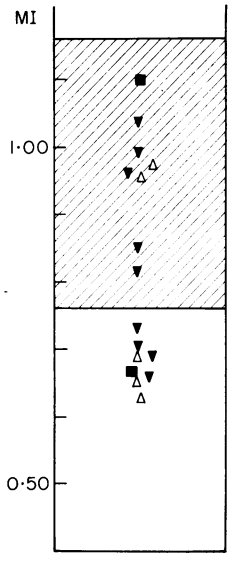

FIG. 5. Leucocyte migration test (LMT) with human hyperplastic adrenal cortex mitochondrial fraction in fourteen patients with idiopathic Addison's disease. △, Males; ▼, females; ■, female diabetics with anti-adrenal antibody in serum, but without Addison's disease. Hatched area, normal range (mean ±2 SD).

The results are summarized in Table 4. It appears that there is no correlation between the occurrence of low MI-values and high titres of circulating anti-adrenal antibody.

The migration indices found with extracts of human foetal and human adult hyperplastic adrenal glands are in good agreement. Three patients (cases Nos. 1, 6 and 29) who had low MI-values with foetal adrenal extract were within the normal range when the human hyperplastic adrenal extract was used as antigen. The reverse were found in two cases (Nos. 12 and 18).

All the patients, who had MI-values below normal range with extract from hyperplastic adrenal gland, also had sub-normal MI-values with the mitochondrial fraction of hyperplastic adrenal gland as antigen.

The LMT with human and animal derived antigen-preparations gave comparable results, although some patients, who showed specific reactivity against the human antigens did not react with porcine (cases Nos. 18, 21 and 22) and monkey (cases Nos. 18 and 22) adrenal extracts.

DISCUSSION

The existence of organ-specific anti-adrenal cellular hypersensitivity in patients with idiopathic Addison's disease has been demonstrated in a previous report (Nerup *et al.*, 1969). As it is the case with anti-adrenal humoral hypersensitivity (Irvine, Stewart & Scarth,

TABLE 4. Leucocyte migration test in patients with idiopathic Addison's disease (Case Nos. 1, 4, 6–16, 18–22, 24–26 and 28–30), and patients with diabetes mellitus and circulating anti-adrenal antibody in serum, but without Addison's disease (Case Nos. 38–40) (migration indices obtained with various adrenocortical fractions)

Case No.	Sex	Anti-adrenal antibody titre	Extract: human foetal adrenal glands	Extract: human hyperplastic adrenal glands	Human hyperplastic adrenal mitochondrial fraction	Human hyperplastic adrenal microsomal fraction	Human hyperplastic adrenal post-microsomal supernatant	Extract: pig adrenal glands	Extract: monkey adrenal glands
1	F	4	0·62	0·84	0·85	–	1·05	1·05	1·07
4	F	16	0·56	0·40	0·69	0·98	1·06	–	–
6	F	32	0·67	0·86	0·82	0·97	1·14	0·86	1·04
7	F	16	0·90	0·85	0·99	–	–	–	–
8	F	64	1·00	0·90	0·96	–	–	0·90	1·01
9	F	–	0·95	0·90	1·04	1·06	1·04	–	–
10	F	64	0·98	1·04	–	–	–	0·98	0·96
11	F	–	0·62	0·61	–	–	–	0·63	0·54
12	F	4	0·87	0·72	0·66	0·97	1·04	0·72	0·84
13	F	8	0·96	0·90	–	–	–	–	–
14	F	–	0·96	0·88	–	–	–	–	–
15	F	32	1·02	1·03	–	–	–	–	–
16	F	NI	1·20	–	–	0·92	–	–	–
18	F	16	0·87	0·74	0·70	–	–	0·81	0·88
19	F	–	0·65	0·64	0·73	–	–	0·71	0·77
20	M	8	0·65	0·49	0·63	1·03	1·18	0·60	–
21	M	8	0·67	0·68	0·68	1·05	–	0·79	0·80
22	M	16	0·69	0·61	0·65	0·96	1·01	0·85	0·89
24	M	32	0·93	1·14	0·97	–	–	–	–
25	M	8	0·56	0·65	–	0·86	0·96	–	–
26	M	8	1·05	1·05	0·96	1·05	1·13	1·05	1·10
28	M	–	0·68	0·55	–	–	–	–	–
29	M	–	0·68	1·02	–	0·92	–	–	–
30	M	–	1·01	1·13	–	–	–	0·77	1·04
38	F	64	0·81	0·69	0·67	1·17	0·98	0·66	0·74
39	F	8	0·79	0·64	–	–	–	0·64	0·64
40	F	8	0·98	1·07	1·10	1·10	1·08	0·93	1·08

NI, Not investigated.

1967) anti-adrenal cellular hypersensitivity was not correlated to clinical parameters, and no correlation was found between the occurrence of circulating anti-adrenal antibody and anti-adrenal cellular hypersensitivity (Nerup & Bendixen, 1969).

The findings reported in the present study indicate that the antigen(s) against which the anti-adrenal cellular hypersensitivity is directed, is present in normal porcine and monkey adrenal cortex. The anti-adrenal cellular hypersensitivity in idiopathic Addison's disease, therefore, seems to be specific to the organ, but not to the species, which is the case also with anti-adrenal hypersensitivity of the humoral type (Blizzard & Kyle, 1963).

The experiments further show that the specific anti-adrenal cellular hypersensitivity response can be released by similar antigenic components of adult human hyperplastic adrenal gland, and that the antigen at the subcellular level is localized in the mitochondrial fraction.

Morphologically the mitochondrial fraction of hyperplastic adrenocortical tissue differs considerably from that of normal adrenals, and it might be argued, that a major structural difference could expectedly be reflected as a difference in antigenic structure. However, the morphology of the mitochondria of hyperplastic adrenocortical tissue found in the present study is similar to that seen in animal adrenals following treatment with corticotrophin (Luse, 1967) and the morphological changes may accordingly more probably be regarded as a correlate to an altered state of activity than to a more profound, structural alteration with development of new antigenic determinants.

The function of the benign, hyperplastic adrenal gland is qualitatively not different from that of the normal adrenal gland. Adrenocortical tumours, however, synthetizise steroids of a different pattern (Binder, 1968). This might very well be a reflexion of a difference in protein structure, corresponding with a difference of antigenic properties.

This assumption is in agreement with the present finding that adrenocortical neoplastic tissue in contradistinction to normal adrenocortical tissue lacks the capacity for inducing an inhibition of the white cell migration in cultures from patients with idiopathic Addison's disease.

In the experiments with subcellular fractions it was demonstrated that antigenic activity was associated with the mitochondrial fraction only. This fraction (Fig. 2) consists mainly of mitochondria, but a certain slight contamination with lysosomes is present. For this reason it cannot be concluded with absolute certainty whether the mitochondria *per se* or the lysosomes are the antigen-containing subcellular components and further studies are needed to give an answer to this question.

In our previous report (Nerup & Bendixen, 1969) it was found that organ-specific anti-adrenal hypersensitivity of either the humoral or the cellular type could be demonstrated in 90% of patients with idiopathic Addison's disease and it was further demonstrated that there was no correlation between the occurrence of the two types of hypersensitivity in the patients.

Goudie *et al.* (1968) demonstrated that the circulating anti-adrenal antibody is directed against an antigenic component in the adrenocortical microsomes.

On this background the present findings indicate that anti-adrenal hypersensitivity of the humoral and the cellular type in idiopathic Addison's disease are not directed against the same antigen, but against antigenic components belonging to different subcellular adrenocortical fractions.

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