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A human-specific mitochondrial antibody

ITS IMPORTANCE IN THE IDENTIFICATION OF ORGAN-SPECIFIC REACTIONS

G. T. SWANA, MARLENE R. SWANA, G. F. BOTTAZZO & DEBORAH DONIACH *Department of Immunology, Middlesex Hospital Medical School, London*

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SUMMARY

A previously unrecognized autoantibody, detected by immunofluorescence, reacted with all human organs but gave negative results on tissues from rat, mouse, rabbit, guinea-pig, calf and chicken. From its predilection for mitochondria-rich cells (oncocytes) and its selective absorption with human but not animal mitochondria, it was identified as an anti-human mitochondrial antibody and named AHMA.

The antibody is found in about 1% of normal subjects and is mostly of IgG class and of low titres. Its prevalence is increased in primary biliary cirrhosis where it may be associated with the standard non-species-specific AMA used for the differential diagnosis of this disease. The importance of AHMA is mainly in possible confusion with organ-specific reactions in submaxillary duct, parathyroid oxyphil cells and in trying to identify new endocrine cells such as those producing pancreatic polypeptide (HPP) in human tissues. Animals immunized with human hormones develop reactions to human mitochondria and thus produce misleading immunofluorescence reactions when used in low dilutions.

INTRODUCTION

Mitochondrial antibodies (AMA) detected by immunofluorescence (IFL) are important in the differential diagnosis of primary biliary cirrhosis (Walker *et al.*, 1965; Klatzkin & Kantor, 1972; Sherlock & Scheuer, 1974). These antibodies have been extensively studied (Berg *et al.*, 1969) and are known to be completely non-organ and non-species specific (see Doniach & Walker, 1974). Any organs rich in mitochondria are suitable as substrates and most laboratories employ rat or mouse kidney and stomach for diagnostic tests.

In the course of studies on organ-specific autoimmunity to submaxillary gland, pancreas, parathyroid and pituitary, difficulties were encountered in the interpretation of IFL patterns seen in glands of human origin. The normal pancreas is known to contain mitochondria-rich cells, also called oncocytes (Hamperl, 1962) scattered among the exocrine acini (Tasso & Sarles, 1973). These are difficult to distinguish from endocrine cells which exist in a similar location. The oxyphil cells of thyroid and parathyroid are rich in mitochondria. Here again it could be difficult to distinguish between organ-specific reactions and those caused by AMA.

It is known that submaxillary duct gives particularly clear IFL with mitochondrial antibodies. We therefore included a human salivary gland in all screening tests in the past 18 months. This revealed a number of sera which stained the duct cells with a mitochondrial pattern but which failed to react with rat kidney or liver. The same sera also stained oncocytes in pancreas and parathyroid. The characterization of these reactions will be described in the present paper.

Correspondence: Dr D. Doniach, 40 Tottenham Street, London W1P 9PG.

MATERIALS AND METHODS

Patients' sera. 9600 sera sent to the laboratory for antibody screening were automatically tested undiluted on human submaxillary gland and stomach in addition to the tissue block containing rat organs. In autoimmunity involving pancreas, pituitary and parathyroid the titre of organ-specific antibodies is generally low, making it essential to test undiluted serum. On the other hand, high-titre antinuclear, mitochondrial and smooth muscle antibodies tend to show prozones so that all sera were also tested at 1:10 dilutions. Positive reactions were titred to end point. Sera were tested fresh or after storage at 4°C and −20°C with and without prior inactivation at 56°C for 30 min.

Primary biliary cirrhosis sera. About 10% of the sera received in the laboratory were from patients with various liver disorders but in addition, we specifically investigated thirteen sera collected over several years from primary biliary cirrhosis (PBC) patients with negative AMA, in case a human-specific reaction might have been missed.

Controls. Sera of 196 healthy persons, mainly Swedish blood donors, were tested on pancreas in the course of a collaborative study and are included as controls.

Tissue substrates. Unfixed 5 μm cryostat sections of human submaxillary gland, stomach, thyrotoxic thyroid, kidney, pancreas and parathyroids, and rat liver and kidney were the major substrates used in immunofluorescence studies. The human parathyroids were oxyphil adenomas or hyperplastic glands. Experiments to establish species specificity were performed on sections of human liver, calf thymus, kidneys from mouse, rabbit and chicken, and salivary glands from rat and rabbit. Most of the human organs were obtained at operations or at post mortems from kidney donors.

Blood groups of human organs. To exclude the possibility of the human-specific reaction being due to differences in blood groups between sera and substrates, human organs were selected from donors of different blood groups. Two stomachs of group A Rh+ and group B Rh+ were tested. The thyroid was group A Rh+; the submaxillary gland and kidney were group O Rh+; and the pancreases were O Rh+ and O Rh−. The sera reacting with pancreas and submaxillary duct selected for blood group studies were of group A.

Immunofluorescence (IFL). The standard indirect technique was employed, using FITC-conjugated sheep anti-human Fab prepared in the laboratory and Wellcome anti-IgG, -IgM, -IgA and -beta 1C. The cell types reacting with positive sera in exocrine pancreas were identified using a three-layer IFL technique in which a direct rhodamine conjugate of PBC immunoglobulins containing AMA was added as a third layer. The sections were viewed in a Leitz Ortholux microscope with epi-illumination using selective excitation filters BG38/2KP 490 and suppression filter S525 for fluorescein; excitation filters BG38/S546 with suppression filter K570 for rhodamine. Comparison of red and green fluorescence was made visually and after double-exposure photomicrography using GAF colour film (ASA 500).

Preparation of mitochondria. Mitochondria were prepared from normal rat liver and from post mortem human liver, by differential centrifugation in 0·25 M sucrose using the method of Parsons *et al.* (1966). The nuclear fraction was removed by centrifuging the homogenate at 125 *g* prior to sedimenting the mitochondria at 9000 *g*. The mitochondrial pellets were washed by repeated resuspension and centrifugation in phosphate-buffered saline pH 7·2 (PBS). After the third wash the pellets were resuspended in PBS, and their protein concentration determined by the method of Lowry *et al.* (1951). Aliquots of mitochondrial suspensions were stored at −70°C until used.

Venereal diseases reference laboratory (VDRL) antigen. The Cardiolipin Fluorescent (CLF) antibody gives rise to a pattern of mitochondrial distribution (Wright *et al.*, 1970; Doniach, 1976), which differs from AMA in that it can easily be absorbed with cardiolipin. There might have been a possibility that the human reaction under study was of this nature. VDRL antigen was purchased from DADE division, American Hospital Supply Corporation, Miami, Florida. The floccules were prepared as for VD serology, centrifuged at 10,000 *g* and the pellet used for absorption.

Absorption experiments. A serum reacting only with human tissues to 1:80, a known PBC serum containing the usual AMA and a serum containing CLF antibody were diluted 1:20 and incubated with equal volumes of serial dilutions (1:1–1:16) of a suspension of human mitochondria containing 5·8 mg protein/ml. The sera were similarly absorbed with rat mitochondria (6·6 mg protein/ml) and with VDRL floccules. The tubes were kept at 37°C for 1 hr followed by 18 hr at 4°C with constant shaking. They were then centrifuged at 10,000 *g* for 30 min and the supernatants tested by IFL on sections of human submaxillary gland and stomach. In control tubes, the same sera were incubated with equal volumes of buffer only.

Fixation experiments. Composite blocks of rat kidney and human submaxillary gland were fixed for times ranging from 3–60 min in acetone, ethyl and methyl alcohol, 1% paraformaldehyde and 1% glutaraldehyde to compare the properties of the human antigen under study with that of AMA.

RESULTS

Evidence for human mitochondrial specificity

Immunofluorescence patterns. Of the 9796 sera tested a total of 129 (1·3%) gave IFL patterns confined to human tissues and independent of blood groups as follows:

Submaxillary gland. Bright diffuse cytoplasmic staining on ducts (Fig. 1a), dull granular staining maximal towards base of cells on acini. The pattern on duct differed from the usual organ-specific salivary gland antibody which is more fibrillary or reticular (Fig. 2).

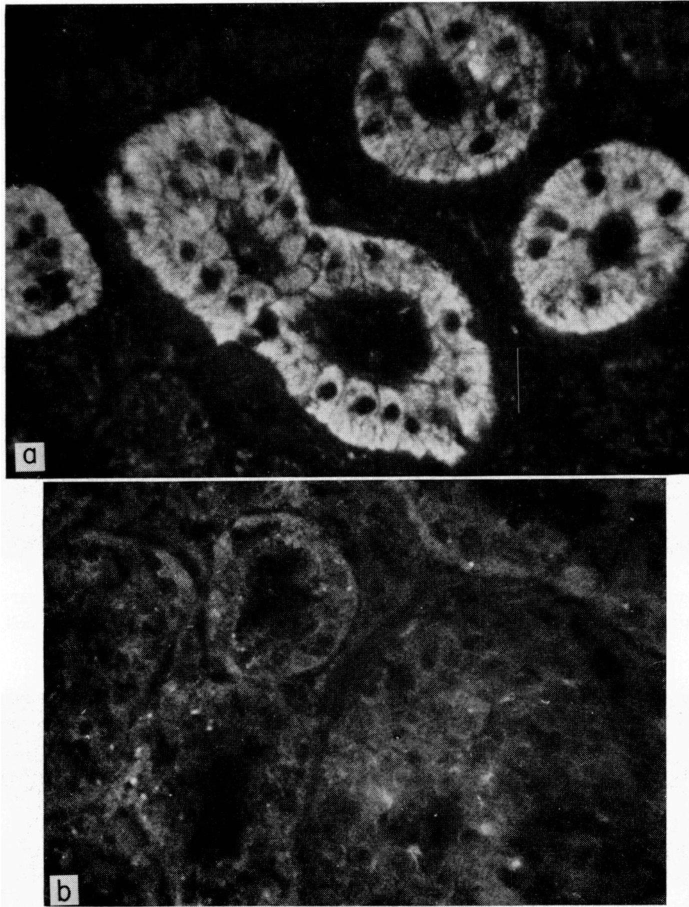

FIG. 1. (a) Cryostat section of human submaxillary gland stained by indirect IFL with serum containing human-specific mitochondrial antibodies (AHMA). The duct cells show a granular uniform cytoplasmic staining pattern. (b) Section of rat kidney stained with the same AHMA-positive serum showing absence of fluorescence on distal and proximal tubules. (Magnification approx. × 450.)

Pancreas. Diffuse cytoplasmic IFL on large polygonal cells seen among the exocrine acini with the distribution typical of oncocytes. These were arranged as single cells, in small clumps or in larger groups which could be mistaken for small islets (Fig. 3). Their occurrence in different parts of the pancreas was variable. They could be absent in one block and present in large numbers in another. They were never situated inside the islets but were occasionally quite near the edge of an islet.

The three-layer IFL with rhodamine-conjugated AMA showed that every cell which stained brightly with the test sera also reacted with the PBC mitochondrial antibody, i.e. the cells were simultaneously red and green with appropriate filters, and appeared yellow to orange in double-exposure photographs.

Thyrotoxic gland. In the thyroid mitochondria-rich cells are named Askenazy cells. These cells stained brightly with AMA and with the antibody described here (Fig. 4). Normal epithelial cells showed a dull granularity.

Parathyroid. In four operative specimens tested there were large polygonal cells among the chief cells which gave a bright granular fluorescence (Fig. 5a, b). Oxyphil adenomas contained sheets of bright cells. The chief cells showed a dull background.

Human kidney, liver and stomach. The pattern of staining could not be distinguished from that seen with the usual AMA, except that the human-specific sera were generally weaker.

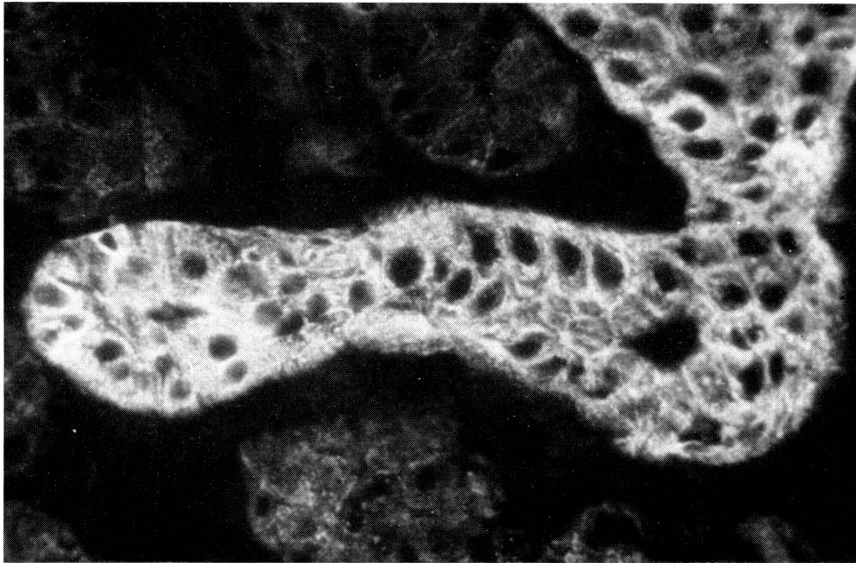

Fig. 2. Human submaxillary gland stained by IFL with serum containing organ-specific salivary-duct antibody. The staining pattern is filamentous with maximum staining around the nuclei and at the cell periphery.

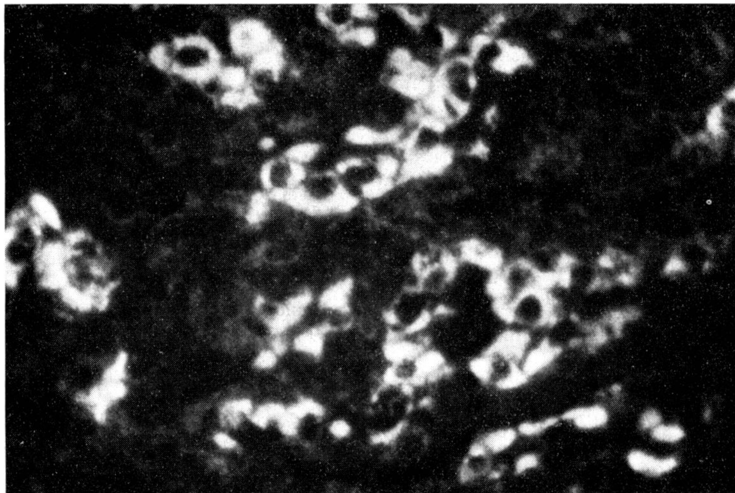

Fig. 3. Human group O pancreas stained with the same AHMA-positive serum as in Fig. 1a. Groups of large cells (oncocytes) in exocrine acini showing diffuse cytoplasmic fluorescence.

Anterior pituitary gland. This showed only a weak granular staining in all cell types. No oncocytes were encountered in the blocks tested.

Evidence for species specificity

The positive sera reacted with submaxillary duct and gave negative results on rat kidney (Fig. 1b) and liver. Selected sera with titres of 10–160 and reacting with all human organs, were further tested on mouse, rabbit, calf and chick organs as shown in Table 1. No reactions were seen with these species while PBC sera and a serum positive for CLF included as controls, reacted well and gave the usual AMA and CLF patterns. The human-reacting sera could be absorbed with human mitochondria but were un-

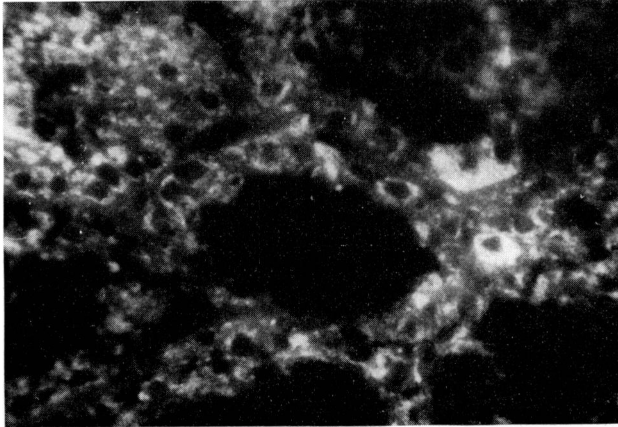

FIG. 4. Human thyrotoxic thyroid stained with AHMA-positive serum, showing bright diffuse IFL on two mitochondria-rich (Askenazy) cells, and dull granular staining of other epithelial cells.

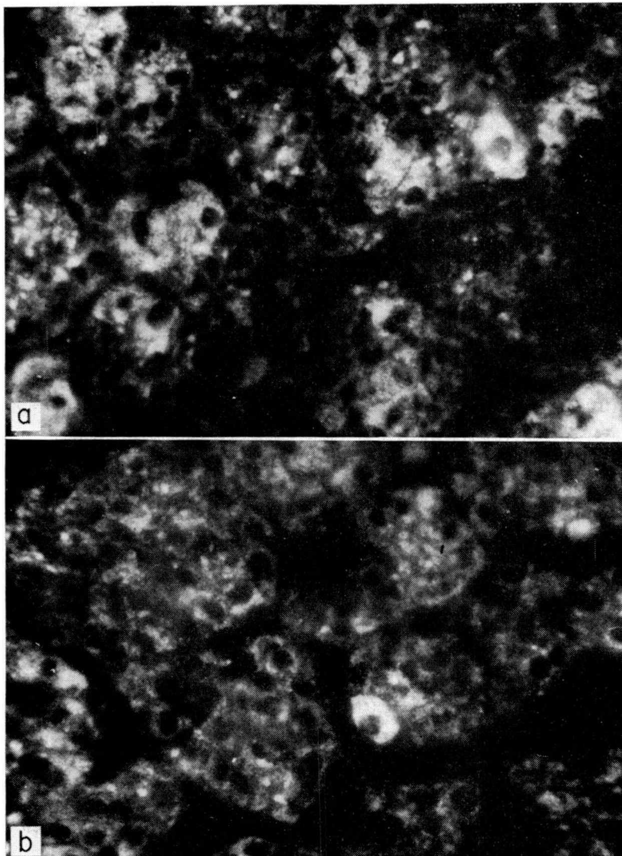

FIG. 5. (a) Human parathyroid oxyphil adenoma stained with AHMA-positive serum showing bright granular IFL on a large group of oxyphil cells. (b) Different field of the same preparation showing isolated cells.

TABLE 1. Species reactivity of mitochondrial antibodies

Species	Organs	AHMA	AMA
Human	Various	+	+
Rat	Various	−	+
Mouse	Kidney	−	+
Rabbit	Kidney and submax	−	+
Calf	Thymus	−	+
Chick	Kidney	−	+

affected by an equivalent amount of rat mitochondria while AMA could be absorbed with both human and animal mitochondria. VDRL reagent did not affect the human-specific reaction or the AMA while it absorbed the cardiolipin IFL.

It may be concluded from these results that the antibodies investigated in this study are human-specific mitochondrial antibodies (AHMA).

Characteristics of AHMA

AHMA were mostly of IgG class and the titres were generally low: in 112 of the 129 positive cases the reaction could be seen only with undiluted serum. Many of these (seventy-eight) showed a mere trace of staining on stomach and none on thyroid or human liver though they were positive on submaxillary ducts, pancreatic oncocytes and distal human renal tubules where mitochondria are more abundant. Ten sera gave titres of 4–10, eight reacted to between 20 and 40 and only two sera reached titres of 80 and 160. Ten high-titre sera were retested with specific conjugates; only two showed IFL of IgM class, and one also reacted with anti-IgA and -beta 1C conjugates.

Fixation experiments

The human mitochondrial antigen behaved similarly to that of AMA. The antigens were active in sections fixed for up to 5 min in glutaraldehyde and 30 min in formaldehyde.

Acetone fixation did not affect the antigens but methyl and ethyl alcohol destroyed them after 3 min.

Clinical correlations

Normal subjects. Of the 196 healthy subjects tested, three gave positive reactions with undiluted serum on mitochondria-rich human cells. No AMA were detected in this group: correlation with age and sex or hereditary factors has not yet been attempted.

Liver diseases. Of the thirteen PBC cases who had repeatedly given negative results in the standard AMA test, only 2 were found to have the human-specific AHMA in titres of 80 and 10. Therefore this antibody would be of limited additional diagnostic help in the 5% of patients with this disease who fail

TABLE 2. Distribution of positive AHMA in clinical groups

Clinical groups	No. of cases
Liver diseases	20
Thyroid or gastric autoimmunity	38
Other endocrine diseases	14
Collagen disorders	16
Miscellaneous	41
Total	129

to react with animal mitochondria. However we have evidence that AHMA can coexist with the more usual AMA in PBC sera. AHMA were found in twenty cases of liver disease, but the general frequency was not greater than in other disease groups.

Polyendocrine autoimmune disorders. Although it was the difficulty experienced when studying islet-cell antibodies in this rare group of patients that alerted our attention to the true nature of oncocyte IFL, overall, AHMA do not appear to be any more frequent in sera containing organ-specific antibodies than in the general population.

Collagen disorders and miscellaneous diseases. AHMA were found with about the same frequency in RA as in other mixed hospital patients. The disease categories of the 129 positive reactors are shown in Table 2. By extrapolation of the incidence among the first 1000 sera tested, it is estimated that roughly 1% in all categories were positive on human mitochondria.

DISCUSSION

The antibody described was shown to react specifically with human mitochondria. In the IFL test it stained various organs with the patterns expected for AMA, in particular it reacted strongly with oncocytes and other mitochondria-rich cells. It could be absorbed with human mitochondria and was unaffected by similar preparations from rat liver. Cardiolipin did not remove the IFL but perhaps to exclude this class of antigen, the absorption should be repeated with diphospholipids extracted from human mitochondria, since VDRL reagent is prepared from ox heart. The AHMA fluorescence is qualitatively quite similar to AMA and more granular than CLF. The three-layer IFL experiment which demonstrated that AMA and AHMA did not block one another suggests separate epitopes on the mitochondria. The human reactive antibody is found in about 1% of normal subjects and the same proportion of patients in other categories while the prevalence of regular AMA is definitely higher than normal in organ-specific endocrine disorders and in the collagenoses (3–8% compared with 0·4–0·7% in controls).

In primary biliary cirrhosis where AMA are present in over 90% of cases, the AHMA could not account for all the rare AMA-negative instances though 2/13 showed the reaction. Preliminary cross-absorption experiments with rat and human mitochondria have shown that almost all PBC sera contain the two antibodies (in preparation). In view of their rare occurrence, AHMA cannot be associated in any way with the unexplained leucocyte-migration inhibition obtained equally with rat and human mitochondrial inner membranes in patients with organ-specific autoimmune disorders (Brostoff, 1970; Wartenberg *et al.*, 1973) including insulin-dependent diabetes mellitus (Richens *et al.*, 1974).

At present the importance of these antibodies is to be aware of their existence so as to avoid misinterpretation when investigating immunofluorescent appearances in human tissues. The organs in which the greatest chance of confusion with organ-specific reactions might be expected are the salivary gland, the parathyroid and the pancreas. False reactions may also be anticipated in looking for new endocrine cells throughout the gastrointestinal tract.

Salivary duct antibodies have been described repeatedly in sera from patients with chronic sialoadenitis, Sjögren's syndrome and rheumatoid arthritis. In the studies by Bertram & Halberg (1964), McSween *et al.* (1967) and Feltkamp & Van Rossum (1968), the exact IFL pattern was not described. In our own experience the organ-specific duct staining is not uniform but filamentous and showing whorls around the nucleus going through to the periphery of the cells (Fig. 2). When sera give a uniform staining in these ducts, the majority turn out to contain regular AMA. We have now shown that a proportion of these reactions are due to human-specific mitochondrial antibodies.

In parathyroid and pancreas confusion with organ-specific reactions may arise due to the frequent occurrence of oxyphil cells or oncocytes. Parathyroid antibodies were described in idiopathic hypoparathyroidism by Blizzard, Chee & Davis (1966) and confirmed by Irvine & Scarth (1969). We have found it difficult to detect these antibodies, possibly owing to the rarity of suitable antigen. Using oxyphil adenomas and hyperplastic glands, a few positive reactions were obtained in cases of hypoparathyroidism

but since becoming aware of AHMA we retested the sera on human submaxillary duct and regrettably they were all positive.

In pancreas the difficulty has been of a different kind. The organ-specific autoantibodies described so far are the islet-cell antibodies (ICA) (Bottazzo, Florin-Christensen & Doniach, 1974; MacCuish *et al.*, 1974; Lendrum, Walker & Gamble, 1975), which react with at least four cell types in the islets of Langerhans i.e. those secreting insulin, glucagon, somatostatin and human pancreatic polypeptide (HPP) (Bottazzo & Doniach, in preparation). Recently, some human sera containing separate antibodies to glucagon or somatostatin-secreting cells have been described (Bottazzo & Lendrum, 1976). Several workers reported the presence of HPP cells scattered among the exocrine acini in addition to those seen inside the islets (Larsson, Sundler & Hakanson, 1975; Polak *et al.*, 1976). Since the distribution of oncocytes resembled that of the HPP cells, the double-immunofluorescence technique was applied to identify the two cell populations, as was done in the case of pituitary autoimmunity (Bottazzo *et al.*, 1975). When rabbit anti-HPP serum was used at a high dilution (1:250) the AHMA-positive sera stained an entirely different population of cells from those reacting with pancreatic polypeptide. Occasionally, isolated cells staining with an anti-hormone are due to tangential section through the edge of an islet but these cells did not react with AHMA.

In the course of these experiments it was found that some rabbit anti-hormone sera also reacted with human mitochondria, mast cells or smooth muscle fibres when used for IFL in low dilutions (less than 1:32) (Bottazzo & Doniach, 1977). SMA is often seen in normal rabbit sera but the mitochondrial antibodies are more likely due to impurities in the human hormone preparation employed for immunization since they did not react with rat tissues. These hormone antisera are raised primarily for radioimmunoassay work where extraneous antibodies do not interfere. IFL is less sensitive and for the double technique the antisera may have to be used at dilutions where cross-reactive antibodies are still visible. If with certain hormones it proves difficult to raise sufficient titres, then absorption with human mitochondria is advisable when trying to identify new types of endocrine cells by IFL or immunoperoxidase techniques.

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