Substrate Specificity for the Detection of Autoantibodies to Anterior Pituitary Cells in Human Sera

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Summary

Using the indirect immunofluorescence test for the detection of pituitary autoantibodies in human serum, the results obtained with human fetal and non-human pituitary antigens were compared. Of the sera that were positive on human fetal substrate, 4% were recovered with adult baboon, 0% with fetal cynomologus, 20% with porcine, 11% with bovine, 11% with ovine, and 7% with rat tissue. The rate of heterophile antibodies to the above animal substrates was 5% to 14%. In contrast to human adult pituitaries, normal human sera did not bind to Fe receptors on ACTH-cells of human fetal pituitaries. This allowed the demonstration of ACTH-cell antibodies. The specificity of the reaction was proven by absorption studies with purified Fe fragments. These results suggest that human fetal tissue is the best source of antigen for the detection of pituitary autoantibodies. The use of animal tissue including non-human primate pituitary yields results that bear no clinical significance.

Key words

Autoantibodies – Anterior Pituitary – Substrate

Introduction

The right choice of tissue is a crucial premise for the determination of organ-specific autoantibodies in human sera (Scherbaum, Mirakian, Pujol-Borrell, Dean and Botazzo 1986). Islet cell antibodies were not detected until fresh human pancreas from kidney transplant donors was employed (Botazzo, Florin-Christensen and Doniach 1974), and post-mortal autolysis also restricted the use of adult hypothalami for the determination of vasopressin cell antibodies in central diabetes insipidus (Scherbaum and Botazzo 1983; Scherbaum, Botazzo, Czernichow, Wass and Doniach 1985). This is also true of anterior pituitary antigen that is, however, available from surgery specimens.

Up to now, there has been no standard test for the detection of autoantibodies to anterior pituitary cells in human sera. These antibodies have been determined on unfixed (Botazzo, Pouplard, Florin-Christensen and Doniach 1975) and fixed organs (Hansen, Hansen, Hagen and Brodersen 1983). Various species, including human adult (Mirakian, Cudworth, Botazzo Richardson and Doniach 1982), rat and bovine antigens (Botazzo et al. 1975; Pouplard 1982; Sugiyama, Hashimoto, Shizawa, Tsukada, Matsumoto, Ishido, Kasahara and Hirata 1986) have been used as tissue substrate donors.

The reactivity of normal human sera with Fe-receptors on ACTH cells of human adult pituitaries has produced major difficulties for the detection of pituitary cell antibodies (Pouplard, Botazzo, Doniach and Roit 1976). To exclude Fe-binding on ACTH cells, a double-fluorochrome staining technique had to be employed and it had also not been possible so far to detect specific autoantibodies reacting with pituitary ACTH cells. We have recently been able to avoid these difficulties by the use of human fetal pituitaries which were shown not to express Fe-receptors. It was thus possible for the first time to detect specific autoantibodies to ACTH cells in sera from patients with Cushing’s disease (Scherbaum, Schrell, Glück, Fahlbusch and Pfeiffer 1987).

Per definition, autoantibodies react with auto-

logous tissues, and therefore, a comparative study of antibody results on human and non-human substrates is required before any pathogenetic role may be attributed to the presence of an antibody in human sera. International conferences have been held to standardize immunofluorescence tests for the determination of non-organ-specific antibodies (Holborow and Johnson 1983; Bonifacio, Holingsworth and Dawkins 1986) and of islet cell antibodies (Botazzo and Gleichmann 1986; Gleichmann and Botazzo 1986). The aim of this paper was to systematically study anterior pituitary autoantibody tests on pituitary substrates from different donor species.

Materials and Methods

Sera

188 sera were tested for autoantibodies to human fetal anterior pituitary cells. The sera came from patients with Cushing’s disease (n = 24), acromegaly (n = 30), prolactinoma (n = 36), Nelson tumour (n = 7), empty sella syndrome (n = 2), inactive pituitary adenoma (n = 39), craniopharyngioma (n = 7), secondary diabetes insipidus (n = 2), germinoma (n = 2), pinealectomy (n = 2), and 37 mixed hospital controls without evidence of endocrine or autoimmun e disease. All the sera were stored at –20 °C until used.

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To determine the cross-reactivity of autoantibodies to anterior pituitary cells, 46 sera that were positive on human fetal tissue were tested on cryostat sections of pituitaries from other species.

**Anti-hormone sera and monoclonals**

The following anti-hormone reagents were used:
- rabbit anti-human ACTH (DAKOPAP kit)
- rabbit anti-human prolactin (NIH, Bethesda, U. S. A.)
- guinea-pig anti-human growth hormone (Dr. Eitzroth, Ulm)
- rabbit anti-human LH (NIH, Bethesda, U. S. A.)
- mouse anti-human TSH, mel. (Dr. Siddle, Cambridge, U. K.).

**Source of tissues**

**Human fetal tissues**

Human fetal pituitaries were gained from prostaglandin-induced hysterotomies at the 19th to 22nd week of gestation. Formal consent for these tests was given by the local Ethical Committee.

**Animal tissues**

Pituitaries from adult baboons were obtained from Robert Harris, Ph. D., Institutes of Neurological Science, London, U. K. They were dissected and snap-frozen immediately after pentobarbitone narcisization of animals. Tissues from fetal cynomolous were kindly donated by Dr. Weimann, Behringwerke Marburg, W. Germany, and they were snap-frozen immediately after delivery by caesarean section. Rat pituitaries were dissected immediately after decapitation under CO$_2$ anesthesia. Specimens from swine, beef and sheep were obtained from the local slaughterhouse.

All tissue substrates were initially kept on ice and within one hour of collection they were “snap-frozen” in isopentane cooled to $-70$ $^\circ$C on dry ice. The tissues were stored in liquid nitrogen until 4 micron cryostat sections were cut. The sections were then air-dried for 20 min and immediately used for antibody testing.

**Immunofluorescence tests**

The indirect immunofluorescence test was performed on unfixed cryostat sections of tissues. Fluoresceinated goat-anti-human IgG (Dianova, Hamburg) was used at 1:60; and TRITC-labelled goat-anti-human IgA (Dianova, Hamburg) at 1:60. Incubation times of sera and conjugates were 30 minutes each. Intermediate washings were done for 30 minutes, and a final washing for 60 minutes, each with PBS pH 7.2 without shaking. For comparison, some of the tests were also performed on cryostat sections after a 5 min fixation with acetone (Scherbaum et al. 1986).

IgG and IgA antibodies were initially investigated using both conjugates in separate assays. The tests with all the sera were later performed using a 2-layer double-fluorochrome method where both, fluoresceinated anti-human IgG and rhodaminated anti-human IgA were employed in one step. The reaction obtained with each of the conjugates was visualized under the fluorescent microscope (Leitz) using alternatively a red and a green filter.

**4-layer double-fluorochrome IFL test**

To identify the reactive cells, the 4-layer double-fluorochrome IFL test was performed on acetone-fixed sections. In this assay, the patient’s serum and fluoresceinated goat anti-human conjugate were applied in the first sandwich, and specific anti-hormone sera and rhodaminated anti-human (or anti-mouse) sera were used in the second. On ultraviolet microscopy this technique shows red and green staining on separate or identical cells according to whether or not the patient’s antibodies and the anti-hormone sera react with the same or different cells. The identity of the reaction to the same cells was confirmed by double-exposure colour photography (Scherbaum et al. 1986).

**Specificity tests**

To exclude non-specific binding of positive sera to Fe-receptors on human fetal pituitary ACTH cells, sections were preincubated with purified rabbit Fe (Dianova, Code No. 011–0008) for 40 minutes. Pretreated sections were then submitted to IFL tests with positive patient’s sera. To exclude binding to the pituitary hormones, positive sera were preincubated with the respective hormones and their reactivity was subsequently assessed by IFL on unfixed cryostat sections of human fetal pituitaries.

**Results**

The IFL pattern of non-specific binding of normal human sera to Fe receptors on ACTH cells of human adult pituitaries is shown in Figure 1. The appearance of LH cell antibodies detected on human fetal pituitary is given in Figure 2.
Table 1 Comparison of pituitary antibody results obtained by the indirect immunofluorescence test on human fetal tissues and various non-human substrates.

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<th>antibody results on non-human pituitaries</th>
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The overall pituitary antibody results obtained on tissues from different species are given in Table 1. The upper two rows of figures indicate the rate of cross-reactivity of pituitary antibodies with various animal tissues. Out of 46 sera that had been positive on human fetal pituitary, none was recovered with fetal cymological, and a maximum of 9 (20%) were found on porcine tissue.

The lower two rows of figures in Table 1 indicate the rate of heterophile antibodies (Figure 3) seen when pituitary tissues from various non-human species were used for the detection of pituitary antibodies. Out of 37 normal human sera that had been negative for pituitary antibodies when tested on human fetal tissue, up to 5 (14%) gave a positive result when non-human antigens were used. Individual sera reacted with e.g. porcine, others with ovine or rat pituitary cells. The endocrine nature of cells reacting with heterophile antibodies was variable.

Comparison of cross-reactivity of pituitary antibodies with non-human tissues on the one hand, and heterophile antibodies present in normal sera on the other, clearly shows that non-human tissue is obsolet for the detection of pituitary antibodies in human sera. 18 (39%) of the 46 pituitary antibody-positive sera were detected when a combination of all the above animal substrates was applied, but also 10 (27%) of 37 normal human sera were falsely positive on one of the animal pituitaries used in these studies.

The endocrine nature of reacting cells and the immunoglobulin class of pituitary antibodies detected on human fetal anterior pituitary is given in Table 2. All the 16 positive sera that were randomly selected reacted with ACTH cells. 4 of them exclusively stained ACTH cells, and the other 12 showed a multiple staining pattern (ACTH + LH or ACTH + hGH or ACTH + LH + hGH). TSH cell and prolactin cell immunoreactivity was not observed in our series that mostly consisted of patients with Cushing’s disease.

Preincubation of unfixed cryostat sections with purified rabbit Fe for 40 minutes at room temperature had no effect on the antibody reaction after subsequent incubation with ACTH-cell antibody-positive sera. However, Fe-sticking of immunoglobulins to macrophages was removed after pretreatment of cells with purified rabbit Fe thus indicating that Fe receptors were sufficiently blocked by these methods. The reactivity of positive sera was not abolished by preabsorption with the respective hormones.

The antigenicity of human fetal pituitary was not changed when cryostat sections were pretreated with acetone for 5 minutes. The differences of titres on unfixed and on acetone-fixed sections were within the limits of interassay variations and indirect immunofluorescence tests (data not given). 20 normal human control sera were also tested on both, unfixed and acetone-fixed pituitary. Falsely-positive results were not obtained after acetone pretreatment of sections.

IgG and IgA antibody titres of positive sera were identical, irrespective of whether or not the anti-human conjugates were applied separately in different assays or at once in the same test. Thus, the 2-layer double-fluorochrome test yielded the same results as the conventional assay, with the advantage of saving time and tissue substrate with the new procedure.

Discussion

The cross-reactivity between species differs from one antigen-antibody system to another. Rat tissue may be used instead of human substrate for the detection of autoantibodies to skeletal muscle (Strauss, Seegel, Hsu, Burkholder, Nastuk and Osserman 1960). For the determination of islet-cell antibodies human tissue is required as an antigen (Botazzo and Gleichmann 1986; Gleichmann and Botazzo 1986). In the latter system, also organs from non-human primates may serve as a useful substitute when human tissue is not avail-
able (Scherbaum, Trischler and Pfeiffer 1989). However, our results clearly show that this is not the case for autoantibodies to anterior pituitary cells, because they do not usually cross-react with non-human substrates. Only two out of 46 sera which were positive on human substrate, gave a positive result on baboon anterior pituitary. Also with the substrate from other species, the recovery rate of pituitary antibodies was no more than 20 per cent.

Heterophilic antibodies may be a major pitfall that can result from the use of non-human tissues for autonaticity determination. When testing parietal cells antibodies on rat stomach, such non-specific reactivity occurs with about 5% of normal human sera. Heterophilic antibodies are even rarer when baboon tissue is used for the detection of gastric parietal cell, pancreatic islet cell or adrenal antibodies (own unpublished observations). In the present study, three out of 37 normal human sera gave heterophilic reactions with adult baboon pituitary. With bovine and ovine tissue, such non-specific binding even exceeded 10 per cent.

In a previous series of 100 sera from patients with polyendocrine diseases, human adult and baboon pituitaries were compared and found to give comparable results (Philipot, Colgan, Levy, Holland, Mirakian, Richardson and Bottazzo 1985). However, using rhesus monkey pituitary as a substrate for antibody determination the authors found a much higher number of positive reactions. The latter series was randomly selected from patients affected by several endocrinopathies, whereas ours consisted of patients with pituitary diseases which may well account for the different results obtained. Variable results have also been demonstrated when sera were tested on a variety of non-human tissues (Bottazzo et al. 1975; Mirakian et al. 1982; Pouplard, Bigorgne, Gérard, Rohmer and Poron 1980; Pouplard 1982; Hansen et al. 1983; Sugiura et al. 1986).

For the detection of autoantibodies unfixed tissue should always be used in the first instance. Some fixatives may destroy the putative autoantigens or reveal new antigenic sites which can give false positive results. This is now well illustrated in the use of Bouin’s-fixed pancreas for the detection of islet-cell antibodies (Bottazzo, Scherbaum and Hanafusa 1983). It was shown in the present study that pituitary cryostat sections may be fixed with acetone for 5 minutes without any alteration of the antigenic characteristics of the tissue. Acetone fixation increases the resistance of tissue sections when several washing procedures have to be applied in the 4-layer double-fluorochrome test.

The non-specific reaction of immunoglobulin via Fe part to Fe receptor-like structures in ACTH cells was avoided by the use of human fetal pituitary tissue which lacks Fe receptors (Glick and Scherbaum, in preparation). None out of 37 control sera gave a positive staining on this substrate. Antibody binding to ACTH cells was not abolished by pretreatment of sections with purified rabbit Fe fragments. The specificity of ACTH-cell antibodies in such sera was also proven by a F(ab)2-positive reaction of pepsin-digested pituitary antibody-positive patients’ immunoglobulins, thus proving the specificity of ACTH cell antibodies (Glick et al., in preparation). Furthermore, the binding of pituitary antibody-positive sera was not abolished by incubation with purified Fe. The specificity of other pituitary antibodies on human tissues has already been described by Bottazzo’s group (Bottazzo et al. 1975).

It appears from our studies that only human pituitaries can be applied to test for anterior pituitary autoantibodies. Specific ACTH-cell antibodies can be demonstrated when human fetal antigens are used (Scherbaum et al. 1987; Glick et al., in preparation), which unlike adult pituitaries (Pouplard et al. 1976) do not express Fe receptors on ACTH cells. It can also be concluded from our investigations that heterophilic pituitary antibodies are not unusual and there is a poor cross-reactivity with tissue from non-human species. Therefore, statements as to the prevalence of pituitary antibodies have to be considered with caution when non-human substrates are used in the tests.

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References


Anterior Pituitary Antibodies


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