PITUITARY-CELL AUTOANTIBODY DIVERSITY IN SERA FROM PATIENTS WITH UNTREATED GRAVES' DISEASE

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Sera from 22 untreated patients with recently diagnosed Graves' disease (GD) were screened in an immunocytochemical tissue assay for presumptive pituitary IgG autoantibodies, as defined by the presence of immunoreaction with rat and swine pituitary cell types. Forty four patients with Hashimoto's thyroiditis (HT) and 97 healthy subjects were also studied. Anti-pituitary antibodies were found in 14 of the 22 GD sera (64%). Of these, 6 sera reacted with cytoplasmic components of growth hormone (GH) cells, 3 with prolactin (PRL) cells, and 5 with both GH and PRL cells. Yet, none of the immunoreactive sera reacted with human GH, bovine PRL or TSH in dot-blot assays and absorption studies. Anti-pituitary antibodies also occurred in 4 of the 44 HT patients (9.1%) and in 9 of the 97 healthy subjects (9.2%). The frequency of sera revealing anti-pituitary antibodies was significantly higher in patients with GD compared to the groups of HT patients (P < 0.00005), and healthy subjects (P < 0.00005). Healthy subjects and patients with HT had a similar frequency of anti-pituitary antibodies (P = 1.0000). These data demonstrate that in thyroid autoimmune conditions antibodies reactive with cytoplasmic components of pituitary GH/PRL cells, may be present in sera from patients with GD. The pathological importance of this observation is at present unknown.

KEY WORDS: Graves' disease, Autoimmunity, Pituitary-cell autoantibodies.

INTRODUCTION

Graves' disease is an autoimmune thyroid disorder believed to be caused at least in part by the presence of antibodies directed against cell-surface receptors for thyroid-stimulating hormone (TSH). These antibodies mimic the effect of TSH binding to this receptor, stimulate cAMP, and result in enhanced production and secretion of thyroid hormones¹. Beside the classic thyroid autoantibodies antithyroglobulin (ATA), antimicrosomal (AMA) antibodies and thyroid growth stimulating immunoglobulin (TGSI) has been observed in Graves' patients¹. Additionally, antithyrotropin antibodies in sera from this group of patients has been reported². A more profound disturbance of the immune system may be anticipated from the observation of aberrant MHC class II expression in the diseased thyroid gland as distinct from the healthy gland (see Feldmann³ for references). Autoimmune thyroid disease may
further be associated with autoimmune syndromes involving other endocrine glands and other tissues\(^1\). The pituitary gland, however, has not been regularly mentioned in this context.

The present study was undertaken to determine by immunocytochemistry, absorption studies, and dot-blot assays, the frequency and antigenic specificity of pituitary-cell IgG autoantibodies in sera from patients with Graves’ disease in comparison with Hashimoto’s disease and with healthy controls.

**MATERIALS AND METHODS**

**Human Subjects**

Serum samples, collected at random from 22 patients (20 females) with Graves’ disease (GD), 44 (38 females) with Hashimoto’s thyroiditis (HT) and 97 (57 females) healthy subjects, were studied.

Graves’ disease was diagnosed on clinical and biochemical evidence of hyperthyroidism and on a diffuse thyroid scan with \(^{99m}\text{Tc}\). Median age was 43 years (range 13–63). Medium serum thyroxine (T\(_4\)) and triiodothyronine (T\(_3\)) level was 210 nMol (range 110–245) and 7.8 nMol (range 2.7–10.3) respectively. Median serum thyrotropin (TSH) level was < 0.03 U/l (range < 0.03–0.05). Serum concentrations of T\(_4\) (normal range 56–129 nMol) and T\(_3\) (normal range 1.6–2.8 nMol) were determined as previously described\(^4\). Serum TSH was determined by a sensitive immunoradiometric assay (Boots-Celltech, England). The inter-assay variation for TSH was 2.3–4.7% in the range of serum TSH measured. The detection limit was 0.03 U/l and the normal range 0.4–3.5 U/l. All sera from Graves’ patients were, by routine methods (State Serum Institute, Denmark), further examined for (1) IgA and IgM rheumatoid factors, (2) antinuclear antibodies (ANA) on Hep-2 cells, (3) cytoplasmic components of adrenals, thyroid, gastric parietal cells and pancreatic islands.

All patients with Hashimoto’s thyroiditis had an enlarged thyroid gland. All were euthyroid or hypothyroid and had serum antibodies against thyroglobulin and/or microsomal fraction measured by ELISA\(^5\) and immunofluorescence\(^6\) techniques, respectively. In these patients median age was 59 years (range 15–91). Median serum T\(_4\) level was 47 nMol (range 14–90) while median serum T\(_3\) concentration was 1.7 nMol. Median serum TSH level was 20.9 U/l (range 6.7–134.2).

All patients were untreated and gave informed consent to the present investigation.

Sera from 97 healthy blood donors, mean age 33 years (range, 20–63), in addition to two pooled sera (a serum from 75 healthy blood donors, sex and age unknown and a standard human serum: Behring, 041008H) were tested as controls.

All sera were stored at \(-20^\circ\text{C}\) until used.

**Immunocytochemistry**

Initially all serum samples (1:100) were screened for the presence of antibodies against anterior pituitary cells in a non-species specific tissue assay by means of an indirect immunocytochemical method, with 3,3’-diaminobenzidine–tetrahydrochloride (DAB; Fluka, 32750) as sequential substrate. Paraffin sections (6 \(\mu\m)\) of Bouin-sublimate fixed rat pituitaries and swine pituitaries prepared as previously described\(^7\) were used as tissue substrates. In brief, deparaffinized rehydrated sections were soaked for 5 min in 0.05 M Tris-HCl, pH 7.2, with 0.5 M NaCl (TBS) containing 0.1% Triton X-100 and then incubated for 30 min with 3% normal rabbit serum diluted in TBS for
1 h to block nonspecific binding of proteins, followed by rinsing in TBS containing 0.1% Triton X-100. Incubation with primary antibodies (human serum to be tested) in a moist chamber for 24 hrs at 4°C; re-equilibration at room temperature for 1 h followed by rinsing in TBS containing Triton X-100; incubation with horseradish peroxidase (HRP) conjugated rabbit anti-human IgG (Dakopatts, P214), diluted 1:100 followed by rinsing in TBS containing 0.1% Triton X-100. Peroxidase activity was demonstrated with DAB-H₂O₂. All sera were diluted 1:100 in TBS containing 0.25% crystalline bovine serum albumin (BSA). The initial screening of all sera included determination of optimal and end-point staining dilutions on tissue sections.

All serum samples revealing positive pituitary immunoreaction in the above mentioned tissue assay were further tested for specific identification of pituitary cell types harboring cytoplasmic antigenic determinants reactive with the human sera. For this purpose an indirect simultaneous two step immuno-enzyme staining procedure was used. In this procedure primary antibodies (raised in different animal species) as well as species-specific secondary antibodies were mixed. The primary reference antibodies raised in rabbits against mammalian pituitary hormones include: (i) antiovine growth hormone (bGH) IgG, anti-ovine prolactin (oPRL) IgG, and anti-ovine lutropin (oLH) IgG produced as described elsewhere; (ii) antisera against the β-subunits of human lutropin (hLH), follitropin (hFSH) and thyrotropin (hTSH) (NIAMDD). All antisera have been previously described and characterized. After pretreatment as above, sections were treated with a mixture (1:1) of two primary antisera. This included the human serum to be tested (in dilution 1:2) and reference antibodies (dilution varies for the different antibodies employed). The serum/antibodies were diluted in BSA-TBS. Incubation for 24 hrs at 4°C with this mixture of first-layer antibodies was followed by washing in TBS containing 0.1% Triton X-100. The sections were then incubated for 30 min at room temperature with a 1:1 mixture of biotinylated no-nonsense goat anti-human IgG (Kem-En-Tec, diluted 1:50) and swine anti-rabbit IgG (Dakopatts, Z196, diluted 1:10). Following washing in TBS containing 0.1% Triton X-100 sections were further applied with 1:1 mixture of the detector streptavidin-β-galactosidase complex (Amersham, RPN 1053, diluted 1:50) and rabbit PAP (Dakopatts, Z113, diluted 1:40). Reaction for HRP was accomplished with DAB prior to reaction for β-galactosidase. β-galactosidase was localized with BCG (Sigma, B-4252) substrate solution. The enzyme reaction was allowed to run over night at room temperature.

**Immunocytochemical Controls**

Conventional methodological controls were carried out as indicated by Sternberger. Specificity controls: (i) single enzyme staining method: consisted of omitment, and stepwise dilution of primary antisera; (ii) double enzyme staining method: the noncoincident staining of dual antigens in the same tissue section served as an internal specificity control.

All positive results were confirmed on different days. Some sera were thawed more than 10 times during a period of 1.5 years without loss of immunoreactivity, as demonstrated in the immunocytochemical staining.

**Specificity Tests of Sera**

Sera, shown to be reactive with a specific pituitary cell-type, were tested in dot-blot assays and liquid phase absorptions for specificity against the principal hormone
Figure 1–6 (see Color Plates I–VI at the back of this publication). Sections of rat pituitary double-labeled (a) for reference hormones (brown color) and (b) with sera from patients with Graves' disease (blue color).
known to be produced in this cell-type. In addition, all sera were tested for reactivity against TSH. As specific test antigens were used: genetic engineered hGH (a kind gift from Novo-Nordisk A/S), oPRL and bTSH (NIAMDD), the β-subunit of hTSH (ucb bioproducts).

In the dot-blot assay the hormone/peptide was immobilized on nitrocellulose filter strips (Schleicher and Schüll, BA 85). The patient serum to be tested was then applied to the filters, and human IgG binding detected with no-nonsense biotinylated goat anti-human IgG (Kem-En-Tec) followed by streptavidin-Alkaline phosphatase (AP) complex (Amersham, RPN. 1052). The AP was demonstrated with a substrate solution containing BCIP (Sigma, B-8503) and NBT (Sigma, N-6876)\textsuperscript{11}. As methodological control was used rabbit anti-hormone as primary antibodies bridged to the streptavidin-AP complex with biotinylated swine anti-rabbit IgG (Dakopatts, E 353).

The absorption assays were conducted by allowing the optimal diluted sera to react with the above mentioned hormones/peptides at varying concentrations for 48 hrs at 4°C, prior to staining.

Statistics

Comparison between groups was by Fisher’s exact test.

RESULTS

Specificity of the immunocytochemical staining concerning reference antibodies was confirmed by absorption and staining controls. Only preabsorption with homologous antigens inhibited staining for each one of the reference antibodies. All staining controls were negative and no immunostaining was obtained when the reference antibodies were replaced by nonimmune serum.

The specific binding of the human IgG was confirmed by the decreasing staining intensity revealed in parallel with increasing dilution of the sera. Conventional staining controls were negative.

Figure 1 Staining of different cell-types produced by anti-oPRL and a patient serum (also illustrated in Figures 2 and 6).

Figure 2 Correspondence in staining pattern (mixed color) produced by anti-bGH and patient serum.

Figure 3 Cells reactive with anti-bGH not reactive with a patient serum (same patient as illustrated in Figure 4).

Figure 4 Correspondence in staining pattern produced by anti-oPRL and patient serum.

Figure 5 Enlargement of section shown in Figure 4. Cells revealing differential intracellular localization of reaction products, (arrows).

Figure 6 Staining of different cell-types produced by anti-bTSH and patient serum.
All peptide antigens could be immobilized on dot-blot filter strips as documented by positive immunohistochemical reaction with the reference antibodies. No non-specific staining of peptides was observed and all staining controls were negative. Antibodies preabsorbed with homologous antigens failed to stain in the dot-blot assay.

Patients with Graves’ Disease

Of the 22 patients with GD 14 (63.6%, with 95% confidence limits of 40.7–82.8%) demonstrated serum antibodies against adenohypophyseal cells when initially screened in the indirect immunocytochemical assay. The mean titer was 380 (range 50–3,500). The staining results obtained with these 14 sera in the double staining technique revealed that 6 sera reacted with GH cells (Figures 1, 2 and 6); 3 sera showed reaction with PRL cells (Figures 3 and 4); and 5 sera showed reaction with both GH and PRL cells. In all cases, although difficult to ascertain with the method employed, the impression was that patients IgG and reference antibodies localize different cytoplasmic structures (Figure 5). None of the immunoreactive sera showed reaction in the dot-blot assays and the absorption studies. None of the sera revealed IgG binding to TSH cells (Figure 6).

In this group of patients, the frequency of antibodies against thyroglobulin, rheumatoid factors, and cytoplasmic components of adrenals, gastric parietal cells and pancreatic islets as well as ANAs did not differ from the frequency observed in healthy populations. Thyroid microsomal antibodies of low titer were observed in 14 of the 22 patients, without correlation to anti-pituitary antibodies.

Patients with Hashimoto’s Thyroiditis

Four of the 44 sera from patients with HT, (9.1%, with 95% confidence limits of 2.5–21.7%) reacted with pituitary cells in the immunocytochemical tissue section assay. Mean titer was 350 (range 100–600). As shown by the double staining technique three sera showed reaction with cytoplasmic components of GH cells and one serum with PRL and TSH cells. None of the sera revealed reaction in the dot-blot assays and the absorption studies.

Normal Subjects

Sera from 9 of the 97 blood donors (9.2%, with 95% confidence limits of 4.3–16.6%) yielded immunoreactive deposits in GH cells only. Mean titer was 333 (range 100–800). The pooled human sera yielded no immunoreaction with anterior pituitary cells. None of the sera revealed reaction in the dot-blot assays or absorption studies.

Statistics

Normal subjects and patients with HT had a similar frequency of pituitary autoantibodies (P = 1.0000). In contrast, patients with GD had a significantly higher frequency of anti-pituitary antibodies compared to patients with HT (P < 0.00005) and to healthy subjects (P < 0.00005). The difference between GD and HT patients was 54.6% (95% confidence limits of difference: 32.7%–76.4%) and between GD patients and healthy subjects 54.4% (95% confidence limits of difference: 33.4%–75.3%).
DISCUSSION

The results demonstrate a significantly higher frequency of IgG antibodies reactive with cytoplasmic components of anterior pituitary cells in sera from untreated patients with recently diagnosed Graves' disease than in sera from patients with Hashimoto's thyroiditis and normal controls. The results were identical and independent of the light microscopic immunocytochemical techniques used. The immunoreactive sera from patients with Graves' disease reacted exclusively with GH or PRL or both these cell-types. However, immunocytochemical results obtained in dot-blot assays and conventional liquid phase absorption controls showed that the immunoreactivity revealed in the tissue sections may not be related at least to the mammalian GH's and PRL's tested. Our evidence therefore support the conclusion that the antibodies are not directed against the principal hormone produced in the reactive cell-type. This conclusion is strengthened by the observation that patient and reference antibodies revealed different intracellular epitopes in the double stained preparations.

The human sera were screened for reactivity against animal antigens, which might be regarded as unrepresentative as markers for human pathological conditions. It could be argued that sections of human pituitary were preferable as antigen substrates. However, aside ethical problems and problems related to criteria of death and supply of material the use of human pituitaries as screening substrate might also be questioned (cause of death?, pre-mortem conditions?, sex?, age?, expression of mediators?). Alternatively, material from sex, age and nutrition defined healthy animals, collected for the purpose immediately postmortem may be used. Identical immunocytochemical results obtained with human sera as primary antibodies on pituitary sections from different animal species as antigen substrates allow the interpretation that the human autoantibodies possess specificity against antigens which are evolutionally well-preserved. Thus, cross-species immunocytochemical reactions may be valuable and relevant, especially when concerning regulatory peptides as presented in endocrine organs.

Autoantibodies directed against cytoplasmic components of adenohypophysial cells in conjunction with extrapituitary autoimmune disorders has earlier been demonstrated in sera from patients with insulin-dependent diabetes (IDDM)\textsuperscript{12} and multiple sclerosis (MS)\textsuperscript{13}. Anti-TSH antibodies has been demonstrated in sera from patients with Graves' disease\textsuperscript{2}. In this single report the presence of antibodies were demonstrated by use of ELISA assay and inhibition studies. Our results are not in keeping with this report as neither dot-blot assays nor immunocytochemistry revealed any reaction with TSH. Although negative immunocytochemical observations are not confirmatory the parallel negative outcome of the dot-blots support our conclusion. In a recent publication Kobayashi et al.\textsuperscript{14}, using unfixed cryostat sections of rat pituitaries, report considerably higher prevalence of anterior pituitary cell antibodies, judged by immunofluorescence, in sera from patients with Hashimoto's disease than with Graves' disease. These authors do not identify the reactive cell-type. The apparently contradiction between this observation and our observations may be explained by the use of unfixed cryostat sections versus Bouin-fixed tissue material, resulting in preservation of different antigens. A possible support for a connection between the pituitary gland and Graves' disease was obtained from nude mice transplantation studies\textsuperscript{15}. Twelve days after transplantation of thyroid fragments from 7 patients with Graves' disease to nude mice, all immunologic disorders found on the operative samples had disappeared, and the tissue had lost its hyperfunctioning
characteristics. Transplants from normal thyroid tissue remained unchanged, as if under the influence of the hypothalamic and pituitary regulation of the mice. Transplants of toxic adenoma remained autonomous in this experimental model.

The regularly observed anti-TSH receptor antibodies in sera from Graves’ disease patients may be theoretically explained by the fact that some common human pathogens (including *Escherichia coli* and *Yersinia enterocolitica*) bind hTSH i.e. they possess a TSH receptor-like surface structure. Additionally, sera from patients with Graves’ disease, but not sera from normal subjects has been reported to inhibit TSH binding to the bacteria. Thus, one might expect that a proportion of patients with Graves’ disease reveal anti-TSH antibodies in the anti-idiotype response. In this context it is surprising that specific TSH-cell binding IgG in sera of the Graves’ patients studied was not recognized. A likely hypothesis for this absent recognition is that only a minor proportion of the polyclonal antibody response against the bacterial TSH receptor is directed against the TSH-binding epitope. The anti-idiotype response revealing anti-TSH reactivity will be further weakened and thereby elude recognition.

In recent time (see ref. for review) it has become increasingly experimentally verified that products of the immune system, such as interferon-gamma (IFN-γ) induce expression of HLA class II antigens in thyrocytes. These cells can then act as antigen-presenting cells for autoreactive T cells, which during the response produce mediators augmenting class II expression in a self-perpetuating interaction. A gradient of activation was found, depending on the type of class II antigen studied – DR being expressed most readily at low concentrations of IFN γ. Interestingly, it has been shown that IFN γ interact with other mediators. In case of Graves’ disease TSH and tumor necrosis factor (TNF) synergized with IFN γ, whereas epidermal growth factor (EGF) as an antagonist, inhibited class II expression. The concept of interaction between mediators regulating class II expression is important. At first, it suggests that regulation of HLA class II expression is complex and influenced by many ligands. Secondly, IFN γ, the most potent class II inducer act in synergy with different ligands in various target tissues, thereby explaining the selectiveness of class II expression and disease (assuming target-class II/disease interconnection). As to a possible interpretation of the importance of anterior pituitary-cell reactive immunoglobulins in sera from patients with untreated Graves’ disease described in the present paper, these may be considered in the above context. One hypothesis could be that specific pituitary cells express class II inhibiting mediators, the function of which is decreased by specific anti-mediator antibodies.

During the last three decades it has become increasingly realized that natural autoantibodies constitute a substantial part of normal circulating immunoglobulins. As partly discussed above, autoantibodies may in addition be induced by environmental factors, such as infectious agents, through different mechanisms: (i) molecular mimicry; (ii) increased HLA expression; (iii) polyclonal B-cell activation; (iv) idiotype crossreaction. Whether the presence/induction of autoantibodies eventually settles to autoimmune disease is further associated with several complicated conditions including genetic and hormonal factors, and inherited or acquired defects in regulatory circuits of the immune response. In the light of this, a possible conclusion concerning the pathological importance of the observed autoantibodies in relation to onset of Graves’ disease would be unrealistic.

Presently, the exact specificities of the anti-pituitary antibodies revealed in Graves’ sera, are not known. To determine whether this observation is important in the pathogenesis of the signs and symptoms of Graves’ disease requires further studies.
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References