Involvement of PIT-1-Reactive Cytotoxic T Lymphocytes in Anti-PIT-1 Antibody Syndrome

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Context: Anti-pituitary-specific transcriptional factor 1 (PIT-1) antibody syndrome is characterized by acquired growth hormone (GH), prolactin (PRL), and thyroid-stimulating hormone (TSH) deficiencies associated with circulating anti-PIT-1 antibodies. Although autoimmunity to PIT-1 has been suggested as a pathogenesis, the precise mechanism of the syndrome remains unclarified.

Objective: To elucidate the involvement of antibody- or cell-mediated immunity in anti-PIT-1 antibody syndrome.

Materials and Methods: To investigate a direct effect of anti-PIT-1 antibody on pituitary cells, cell proliferation, and cytotoxicity detection assays were performed using patient serum. Enzyme-linked immunospot (ELISpot) assay was performed to evaluate the involvement of PIT-1-reactive cytotoxic T lymphocytes (CTLs). An immunohistochemical analysis using anti-CD4 or anti-CD8 antibody was performed to examine tissue infiltration by CTLs.

Results: Patient serum did not exhibit any inhibitory effect on cell proliferation and secretion of GH and PRL in GH3 cells. In addition, complement-dependent cytotoxicity was not detected in patient serum on GH3 cells or primary pituitary cells. The ELISpot assay revealed the presence of CTLs that specifically reacted to the recombinant PIT-1 protein in the patient’s peripheral lymphocytes. CD8⁺ cell infiltrations, which is the characteristic of CTLs, were observed in the pituitary gland, adrenal gland, stomach, thyroid gland, liver, and pancreas of the patient with anti-PIT-1 antibody syndrome.

Conclusions: These results suggest that the anti-PIT-1 antibody is not a cause but a marker of anti-PIT-1 antibody syndrome, in which CTLs play a pivotal role in the pathogenesis. (J Clin Endocrinol Metab 99: E1744–E1749, 2014)

Autoimmune polyglandular syndromes (APS) are characterized by insufficiency of several endocrine glands and nonendocrine organs associated with autoimmunity, such as autoimmune thyroid disease and type 1 diabetes mellitus (T1DM) (1). APS patients produce various autoantibodies against proteins, including glutamic acid decarboxylase (GAD), insulin, and insulinoma-associated protein–2 in T1DM, and calcium-sensing receptors in hypoparathyroidism (1).

Recently, anti-pituitary-specific transcriptional factor–1 (PIT-1, also known as POU1F1) antibody syndrome has been reported as a novel clinical entity related to APS (2). Anti-PIT-1 antibody syndrome is characterized by acquired combined pituitary hormone deficiency exhibiting...
growth hormone (GH), prolactin (PRL), and thyroid-stimulating hormone (TSH) deficiencies associated with circulating anti-PIT-1 antibodies (2). PIT-1 is a specific transcription factor for the differentiation and maintenance of GH-, PRL-, and TSH-producing cells in the pituitary gland (3), thus suggesting that autoimmunity to PIT-1 plays an essential role in the development of this disease. Patients with anti-PIT-1 antibody syndrome exhibit several autoantibodies, such as those against microsome, thyroglobulin, thyroid peroxidase, and GAD, along with autoimmune endocrinopathies, indicating that anti-PIT-1 antibody syndrome meets the definition of APS (1).

Antibodies are well-known causes of the pathogenesis of several diseases, including Graves’ disease (4), hypoparathyroidism (5), and myasthenia gravis (6). In general, antibodies are involved in several different mechanisms in immunity, including neutralization, blocking or stimulating the target protein, antibody-dependent-cellular cytotoxicity, and complement-dependent cytotoxicity (CDC) (7). Antibody-dependent-cellular cytotoxicity is a mechanism of cell-mediated immunity that is mediated by effector cells, such as natural killer cells, monocytes, and macrophages that lyse target cells, whose membrane-surface antigens have been bound by specific antibodies. In contrast, CDC is caused by the effect of the membrane attack complex comprised of the C5b, C6, C7, C8, and C9 complement proteins, which causes osmotic lysis of the target cells (8). This effect is predominantly enhanced by immunoglobulin G1 (IgG1), IgG3, and IgM. In either mechanism, the antigen generally resides on the cell surface. In contrast, intracellular antigens that are recognized by autoantibodies have also been reported. In some cases, the autoantibodies against transcription factors, SOX9 and SOX10, were detected in the serum of patients with autoimmune endocrinopathies, indicating that anti-PIT-1 antibody syndrome meets the definition of APS (1).

In T1DM, T cells, not autoantibodies, have been shown to play a causal role in the development of the disease (10) and CD8+ cytotoxic T lymphocytes (CTLs) recognize intracellular antigens expressed in β cells in association with specific major histocompatibility complex (MHC) class I molecules (11). Interestingly, studies have reported CTLs that react to GAD (12) and zinc transporter 8 (13) demonstrated by enzyme-linked immunospot (ELISpot) assays and the infiltrates of CD8+ T cell in islets. Similarly, alopecia areata, an autoimmune hair loss disease, is considered to be a cell-mediated autoimmune disease in which autoreactive CD8+ T cells that recognize autoantigens are expressed by melanin-producing anagen hair follicles (14).

Although autoimmunity to PIT-1 has been suggested as the pathogenesis of anti-PIT-1 antibody syndrome, whether anti-PIT-1 antibody is a marker or cause in this condition remains unknown. In this study, we investigated the role of anti-PIT-1 antibody and the presence of CTLs that react to PIT-1 protein in patients with anti-PIT-1 antibody syndrome.

Materials and Methods

Patients

This study was approved by the ethics committee of Kobe University Graduate School of Medicine. The patients provided written informed consent for the analysis.

Patient 1, a 44-year-old man showed acquired central hypothyroidism. Endocrinological examinations revealed a specific defect in GH, PRL, and TSH secretion while ACTH, LH, and FSH secretion was not impaired. Autoantibodies against thyroglobulin, parietal cells, and PIT-1 were detected in the serum. ELISpot assay was performed in this patient.

Patient 2, a 75-year-old man with slowly progressive insulin-dependent diabetes mellitus showed acquired central hypothyroidism. Endocrinological examinations revealed a specific defect in GH, PRL, and TSH secretion, primary adrenal insufficiency, and primary hypogonadism. Autoantibodies against GAD, parietal cells, and PIT-1 were detected in the serum. Immunohistological analysis was performed in this patient.

Patient 3, a 78-year-old man showed acquired central hypothyroidism. Endocrinological examinations revealed a specific defect in GH, PRL, and TSH secretion. Autoantibodies against thyroid peroxidase and PIT-1 were detected in the serum. More detailed information on these patients was previously described (2).

Cell cultures, cell proliferation assay, GH and PRL assay, and cytotoxicity detection assay

GH3 cells and pituitary primary cells were maintained in Dulbecco’s Modified Eagle’s medium containing 10% fetal calf serum. Pituitary primary culture cells were prepared as previously reported (15). In cell proliferation assay, GH3 cells were cultured in DMEM with the sera of the patient or control subjects, and cell numbers were evaluated. The supernatant GH and PRL concentrations were measured using enzyme-linked immunosorbent assay. In cytotoxicity detection assay (16), cell viability of GH3 cells and the pituitary primary cells was evaluated with patient serum with or without heat inactivation.

Enzyme-linked immunospot (ELISpot) assay

ELISpot assays were performed as previously described (12). Briefly, 96-well microtiter plates precoated with antihuman interferon (IFN)-γ monoclonal antibody (mAb; ELISpotPRO for Human IFN-γ, Mabtech, Stockholm, Sweden) were washed with phosphate-buffered solution and aliquots of 2.0 × 105 lymphocytes per well were incubated in mAb-coated plates with the antigens at 10 μg/ml under 37°C and a 5% CO2 condition for 48 hours. After washing, biotin-conjugated anticytokine mAb (7-B6-1) was added and incubated followed by streptavidin conjugated with alkaline phosphatase. Finally, 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium substrate solution was added and incubated. Homogeneously stained spots were evaluated as positive (12). All data are expressed as means of quadruplicate determinations. Recombinant human PIT-1 protein (Santa Cruz Biotechnology, Santa Cruz, CA) and PROP-1
Figure 1. Cell proliferation assay using GH3 cells cultured in Dulbecco’s modified eagle medium containing 10% patient and control sera (A). (B) The effect of patient serum on secretion ability of GH and PRL in GH3 cells. The concentrations of GH and PRL in the supernatant were measured. Cytotoxicity detection assay using patient serum with or without heat inactivation using (C) GH3 cells and (D) mouse pituitary cells.
protein (Origene, Rockville, MD) were used as the antigen, and Ab-mCD3-2 was used as a positive control in this study.

**Immunohistochemistry**

Immunostaining was performed as previously described (2). The tissue specimens from patient 2 (2) were fixed in 10% buffered formaldehyde, dehydrated in graded ethanol, and embedded in paraffin. Anti-CD8 (Roche Diagnostics) and anti-CD4 (Leica Biosystems, Newcastle, UK) were used as primary antibodies. Images were obtained with a BZ-8100 microscope (Keyence, Osaka, Japan).

**Statistical analysis**

Statistical analysis was performed using analysis of variance (ANOVA). A \( P < .05 \) was considered as statistically significant.

The details of these experiments were described in analysis of variance (ANOVA). The details of the methods were described in Supplementary data.

**Results**

**Patient serum did not exhibit cytotoxicity or complement-dependent cytotoxicity**

To investigate whether anti-PIT-1 antibody exerts cytotoxicity on GH3 cells, which express endogenous PIT-1 and secrete GH and PRL, we performed cell proliferation and cytotoxicity detection assays. In the cell proliferation assay, GH3 cells were cultured in media containing 10% serum of the patient or healthy control subjects. No significant differences in cell proliferation were observed between the patient and the control subjects, indicating that the patient serum did not show any toxic effect (Figure 1A). We further analyzed the effect of patient serum on the secretion ability of GH and PRL. We could not detect any antisecretory effect of patient serum (Figure 1B).

In the cytotoxicity detection assay, we could not observe any differences between patient serum with and that without heat inactivation (Figure 1C). In addition, the cytotoxicity detection assay using mouse pituitary primary cells showed similar results (Figure 1D), indicating the absence of CDC activity in patient serum.

**CTL reaction to PIT-1 in the patient**

To clarify the involvement of CTL to PIT-1 protein, we performed an ELISpot assay using the lymphocytes of the patient and healthy control subjects. As shown in Figures 2, A and B, the mean number of spots toward human recombinant PIT-1 protein in the patient was significantly increased compared with that in the healthy controls. In contrast, no spots were detected in the lymphocytes with human PROP-1 protein, indicating that the patient’s lymphocytes specifically reacted to PIT-1 protein.

**Immunostaining**

Immunohistochemical analysis revealed that infiltration of CD8\(^+\) cells were observed in the pituitary gland, thyroid gland, adrenal gland, liver, stomach, and pancreas (Figure 2C). Meanwhile, only a few CD4\(^+\) cell infiltrates were observed in these tissues.

**Discussion**

A novel cause of hypopituitarism, called anti-PIT-1 antibody syndrome, characterized by acquired combined pituitary hormone deficiency exhibiting GH, PRL, and TSH deficiencies associated with circulating anti-PIT-1 antibodies has recently been reported (2). Although the presence of anti-PIT-1 antibody
demonstrates autoimmunity to PIT-1, whether the anti-PIT-1 antibody itself plays a causal role in the pathogenesis of the syndrome remained elusive. First, we analyzed the effect of patient serum on cell proliferation and secretion of GH and PRL using GH3 cells and pituitary cells. There were no antiproliferative and -secretory effect. The anti-PIT-1 antibody belongs to IgG1 and IgG3 isotypes (2) that are capable of activating the complement system (7). Therefore, we performed a cytotoxicity detection assay using serum with or without heat inactivation. However, even in the presence of a complement component, patient serum did not show any cytotoxicity to GH3 or primary pituitary cells, indicating that the antibody does not exert CDC activity. In contrast, we showed the presence of evident CTL activity to PIT-1 protein, strongly suggesting that CTL-mediated autoimmunity plays an important role in the development of anti-PIT-1 antibody syndrome.

In the immunohistochemical analysis, we observed marked infiltration of CD8+ cells in various tissues, indicating that CTLs are involved in tissue impairment, compatible with the results of the ELISpot assay. Compared with the other tissues, the pituitary gland had a few CD8+ cells. This may be explained by the results of severe impairment and fibrotic changes in the pituitary (2). Consequently, PIT-1-positive cells might have been almost absent in the pituitary when autopsy was performed. The CD8+ cells infiltrates in the other tissues also suggest the presence of tissue-specific CTLs, which recognize tissue-specific antigens and destroy these tissues. Indeed, several autoantibodies against GAD, microsome, and parietal cells were detected in the patients’ sera (2). CD8+ cells play a causal role in tissue damage in human autoimmune disorders (17). For example, T1DM is a prototypic organ-specific autoimmune disease in which CD8+ cells play a critical role in pancreatic β-cell destruction (18).

Generally, CTL recognizes an antigen epitope presented by MHC class I, which is ubiquitously expressed, including the pituitary gland (19). It is possible that the fragments of PIT-1 protein were presented by MHC class I. However, it remains unclarified whether the presentation occurs in a normal or specific condition. How the immune tolerance to PIT-1 was impaired also remains elusive. One of the speculated pathways is molecular mimicry, a mechanism that encompasses cross-reactive immunity against epitopes shared between exogenous pathogens and autologous peptides. For example, the nonstructural protein 2C of Coxsackie virus B4 that demonstrates cross-reactivity between the viral protease and the human GAD has been speculated in T1DM (20).

In conclusion, although further study is necessary to clarify the pathophysiological mechanism of anti-PIT1 syndrome, these results strongly suggest the involvement of CTLs in the development of the anti-PIT-1 antibody syndrome.

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References


