Immune Checkpoint Inhibitor-Induced Hypoparathyroidism Associated With Calcium-Sensing Receptor-Activating Autoantibodies

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Context: Whereas therapy with immune checkpoint inhibitors (ICIs), such as nivolumab, have substantially improved survival in several types of cancer, increased attention has been given to adverse immune events associated with their use, including the development of endocrine autoimmunity.

Objectives: First, to describe a patient with a 2-year history of metastatic small cell lung cancer who had been treated with nivolumab a few months before presentation with the signs and symptoms of severe hypocalcemia and hypoparathyroidism. Second, to investigate the etiology of the patient's hypoparathyroidism, including the presence of activating autoantibodies against the calcium-sensing receptor (CaSR), as humoral and cellular immune responses against the CaSR have been reported in patients with autoimmune hypoparathyroidism.

Participants: A 61-year-old female was admitted with persistent nausea, vomiting, epigastric pain, constipation, and generalized weakness. Laboratory analyses showed low total serum calcium, ionized calcium, and parathyroid hormone (PTH). The patient was diagnosed with severe hypocalcemia as a result of autoimmune hypoparathyroidism after testing positive for CaSR-activating autoantibodies.

Interventions: She was treated with intravenous calcium gluconate infusions, followed by a transition to oral calcium carbonate, plus calcitriol, which normalized her serum calcium.

Results: Her serum PTH remained low during her hospitalization and initial outpatient follow-up, despite adequate repletion of magnesium.

Conclusions: This case illustrates autoimmune hypoparathyroidism induced by ICI blockade. As ICIs are now used to treat many cancers, clinicians should be aware of the potential risk for hypocalcemia that may be associated with their use. (*J Clin Endocrinol Metab* 104: 550–556, 2019)

Immunotherapies targeting the immune checkpoint molecules, such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death protein 1 (PD-1) and its ligand (PD-L1), are now commonly used in clinical practice to treat malignancies (1). For example, nivolumab is an anti-PD-1 monoclonal antibody that works as an immune checkpoint inhibitor (ICI). It blocks the interaction between PD-1 on the surface of activated T cells and PD-L1 produced by cancer cells—a signaling process that would normally prevent T lymphocytes from attacking tumors. Whereas therapy with ICIs, such as nivolumab, has substantially improved cancer survival, increased attention has been called to immune-related adverse events associated with their use, including endocrine autoimmunity (1, 2). ICI-induced autoimmune endocrinopathies involving the pituitary and thyroid are frequent being reported in up to 10% of patients treated with antibodies against CTLA-4 or PD-1/PD-L1 (1–5). In contrast, primary adrenal insufficiency and type 1 diabetes are uncommon (1–3), whereas ICI-induced autoimmune involvement of the parathyroid gland is exceedingly rare (4). To date, there have been two reported cases of ICI-induced hypocalcemia, but the etiology of their low calcium levels was not reported (6).

A common cause of hypocalcemia is hypoparathyroidism. Normally, low blood calcium concentrations are detected by the parathyroid-expressed calcium-sensing receptor (CaSR), which responds by stimulating parathyroid hormone (PTH) secretion from the parathyroid glands (7). There is a resulting normalization of serum calcium as it is reabsorbed by the kidneys, released by bone, and absorbed by the intestine. However, in circumstances of absent, reduced, or ineffective PTH, this regulatory function is lost, and hypocalcemia ensues. Hypoparathyroidism can be a result of surgical or autoimmune destruction of the parathyroid glands (8). In addition, autoantibodies that stimulate CaSR activity, even when blood calcium levels are lower than normal, can cause the inhibition of PTH secretion from the parathyroid, such that calcium remains below the critical concentration that would normally stimulate PTH secretion (9). Such CaSR-activating autoantibodies have been identified in patients with idiopathic hypoparathyroidism and autoimmune polyendocrine syndrome type 1 (APS1), in which hypoparathyroidism is a prominent manifestation (10, 11).

The aims of this study were to describe a patient with a 2-year history of metastatic small cell lung cancer (SCLC), who had been treated with nivolumab a few months before presentation and developed the signs and symptoms of severe hypocalcemia and hypoparathyroidism, and to investigate a possible autoimmune etiology of the patient's hypoparathyroidism by testing for the presence of CaSR autoantibodies. In addition, NACHT, LRR, and PYD domains-containing protein 5 (NALP5) and cytokine autoantibodies were evaluated, as these are indicative of APS1.

Materials and Methods

Case description

A 61-year-old woman with metastatic SCLC was admitted to our hospital with persistent nausea, vomiting, epigastric pain, constipation, and generalized weakness in July 2017. In addition to these symptoms, she also reported bilateral distal lower limbs paresthesias but no muscle spasms or cramps. She had three prior admissions at other institutions within the last 2 months for similar symptoms. Low serum calcium levels were the most notable laboratory finding during these prior hospital admissions. The patient's hypocalcemia was treated during these hospitalizations, and the patient was instructed to take over-the-counter calcium and vitamin D3 supplementation at discharge. She was not taking the recommended calcium and vitamin D supplementation at the time of admission to our hospital.

The patient was diagnosed with SCLC in July 2015, which was initially treated with multiple cycles of platinum-based chemotherapy and radiation therapy. Given the progression of the disease, despite the initial treatment, nivolumab therapy was begun in March 2017. The patient received six intravenous infusions of nivolumab, which led to partial remission of her SCLC. The last infusion of nivolumab was 2 months before the current hospitalization.

At our hospital, the patient reported no family history of autoimmune endocrinopathies, neck irradiation or neck surgery. Her physical examination was unremarkable except for mild gait ataxia. Chvostek and Trousseau signs were negative. Laboratory findings upon admission are summarized in Table 1 and showed low total serum calcium, ionized calcium, and PTH levels. A CT scan of her neck, chest, and abdomen revealed a primary left lung mass with extensive lymph nodes and bone metastases without any identifiable lesions in the parathyroid glands.

During the current hospitalization, her serum calcium level normalized after she received intravenous calcium gluconate infusions. The patient experienced marked improvement in her symptoms following normalization of serum calcium.

Table 1.   Laboratory Test Results Upon Admission to Our Hospital

Test	Results	Normal Range
Calcium, mg/dL	5.8 (low)	8.3–10
Albumin, g/dL	4.2 (normal)	3.6–4.8
Ionized calcium, mM	0.85 (low)	1.12–1.32
PTH, pg/mL	7.77 (low)	12–65
Magnesium, mg/dL	1.4 (low)	1.6–2.6
Potassium, mEg/L	3.4 (low)	3.6–5.6
Phosphate, mg/dL	4.3 (normal)	2.5–4.5
25-Hydroxy vitamin D, ng/mL	23.8 (low)	30–200
1,25-Dihydroxy vitamin D, pg/mL	10.6 (low)	19.9–79.3
TSH, mIU/mL	0.99 (normal)	0.45–4.5
ECG-QTc, s	492 (high)	431–470 (women)

Abbreviation: ECG-QTc, ECG-corrected QT.
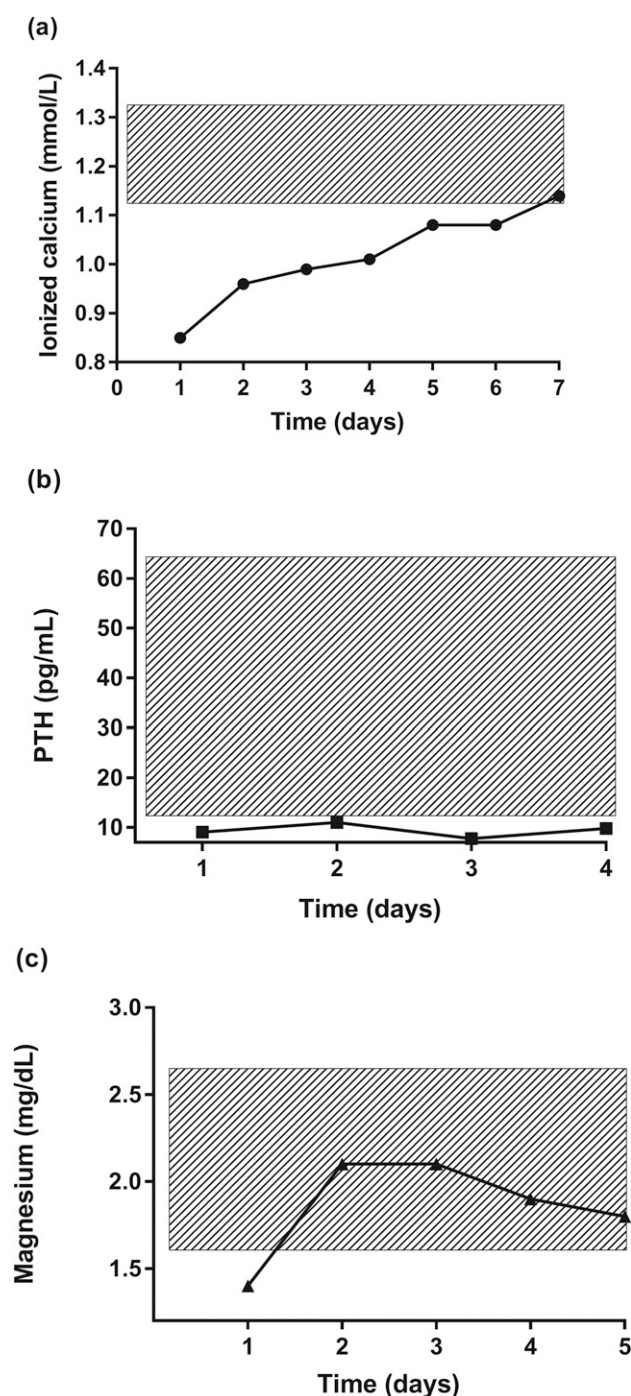
(a)

(b)

(c)

Figure 1. Time course for changes in biochemical laboratory tests. The patient's (a) ionized calcium, (b) PTH, and (c) magnesium levels during admission to our hospital are shown. The normal range is indicated by the hatched boxes: ionized calcium, 1.12 to 1.31 mM; magnesium, 1.62 to 2.62 mg/dL; and PTH, 12 to 65 pg/mL.

Magnesium sulfate was infused to treat hypomagnesaemia. Serum PTH levels remained persistently suppressed despite normalization of serum calcium and magnesium (Fig. 1). Treatment with oral calcium carbonate and calcitriol was started as maintenance therapy for hypocalcemia. At 2 weeks following discharge from the hospital and continued treatment with calcium and calcitriol, her serum calcium was normal. However, her serum PTH level remained persistently low.

As a result of the terminal nature of her disease and the inconvenience of additional medical appointments, the patient decided on regularly scheduled follow-ups only with her oncologist at another institution. We called the patient and her oncologist, learning that nivolumab therapy was replaced with a different chemotherapy regimen in March 2018 as a result of progression of mesenteric nodal metastatic disease. The oncologist reported that the patient's serum calcium was low in March 2018 after the patient stopped taking calcitriol for a few days. Given the nature of her illness, the patient was reluctant to obtain regular laboratory testing.

Written consent was obtained from the patient for blood tests and publication.

Measurement of CaSR-binding antibodies

CaSR immunoprecipitation assays for detecting CaSR antibodies were carried out as before (12). The patient's serum and healthy control sera (n = 10) were stored at −80°C. Human embryonic kidney 293 (HEK293) cells were transiently transfected with plasmid pcCaSR-FLAG, and cell extracts were prepared containing expressed CaSR-FLAG protein (11). Aliquots (50 μl) of GammaBind® Sepharose beads (Amersham Biosciences, Little Chalfont, UK) were mixed with patient serum, control sera, or positive control anti-CaSR antiserum (Alpha Diagnostic International, San Antonio, TX) in duplicate at a 1:100 dilution in 1 ml immunoprecipitation buffer and incubated for 1 hour at 4°C. The bead/IgG complexes were collected by centrifugation and incubated with cell extract containing CaSR-FLAG protein at 4°C for 16 hours. The bead/IgG/protein complexes were collected by centrifugation, washed, denatured, and subjected to SDS-PAGE in 7.5% polyacrylamide gels. The separated proteins were transferred onto Trans-Blot® Transfer Membranes (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) using standard protocols. Immunoprecipitated CaSR-FLAG protein was detected using anti-FLAG® M2-Peroxidase Conjugate (Sigma-Aldrich, Poole, UK) and an ECL™ Western Blotting Analysis System (Amersham Biosciences) with a final exposure to preflashed x-ray film for 5 minutes. Densitometry of the developed films was performed in Bio-Rad GS 690 Scanning Densitometer with Multi-Analyst Version 1.1 Software (Bio-Rad Laboratories Ltd.), which produced a densitometry value for each individual band. A CaSR antibody index for each serum sample in the immunoprecipitation assay was calculated as the densitometry value of the tested serum/mean densitometry value of 10 control sera. The upper-normal limit for the assay was calculated using the mean CaSR antibody index + 3 SD of these control individuals. A CaSR antibody index above the upper-normal limit was designated as positive for CaSR antibody reactivity. All assays were run blinded to avoid operator bias.

Measurement of CaSR-activating antibodies

As detailed elsewhere (11), the response of the CaSR to Ca^{2+} was assessed in HEK293 cells stably expressing the receptor (HEK293-CaSR) by measuring intracellular inositol-1-phosphate (IP1) accumulation using an IP-One ELISA Kit (CIS Bio International, Gif-sur-Yvette, France). Monolayer HEK293-CaSR cells were cultured in 24-well plates. The cells were washed with serum-free medium and then Ca^{2+}-free assay buffer containing 10 mM lithium chloride. For investigating

antibody effects, cells were preincubated for 10 minutes at 37°C with duplicate IgG samples (1:100 in assay buffer), which had been prepared from the patient's and control sera (n = 10) by standard methods (11). Assay buffer containing varying concentrations of calcium chloride (0 to 5 mM final concentration) was added to the cells, followed by incubation for 60 minutes at 37°C. HEK293-CaSR cells without preincubation with IgG were also included in experiments as controls. Following incubation, cells were lysed for 30 minutes at 37°C with 50 μl of 2.5% IP-One ELISA Kit Lysis Reagent (CIS Bio International). The accumulation of intracellular IP1 was measured according to an IP-One ELISA Kit, an immunoassay based on competition between free IP1 and IP1-horseradish peroxidase (HRP) conjugate for binding to anti-IP1 monoclonal antibody. The results for IP1 accumulation were expressed as the following: percentage inhibition of IP1-HRP binding = $(1 - $ IP1-HRP binding in stimulated cells/IP1-HRP binding in unstimulated cells) $\times$ 100. Increasing IP1 accumulation in the HEK293-CaSR cells is reflected by an increase in the percentage inhibition of IP1-HRP binding.

Measurement of NALP5 antibodies

A TnT® T7-Coupled Reticulocyte Lysate System (Promega, Southampton, UK) was used to produce [^{35}S]-labeled NALP5 protein *in vitro* from the transcription-translation of NALP5 cDNA in plasmid pCMV6-XL5-NALP5 (a gift from Professor Olle Kampe, University Hospital, Uppsala University, Uppsala, Sweden), as detailed in the manufacturer's protocol. Radioligand binding assays (RLBAs) were carried out as detailed elsewhere (13). In brief, for each RLBA, an aliquot of NALP5 *in vitro* transcription-translation reaction containing 100,000 cpm trichloroacetic acid-precipitable material, as determined per the manufacturer's instructions, was suspended in 50 μl immunoprecipitation buffer [20 mM Tris-hydrochloride, pH 8.0, 150 mM sodium chloride, 1% Triton X-100, and 10 mg/ml aprotinin (Bayer, Newbury, UK)]. A sample of patient or healthy control serum was then added to a final dilution of 1:100. Anti-NALP5 polyclonal goat antibody (Santa Cruz Biotechnology Inc., Dallas, TX) was used at a 1:200 dilution as a positive control. All serum and antibody samples were tested in duplicate. After overnight incubation at 4°C, 50 μl protein G Sepharose™ 4 Fast Flow (GE Healthcare Life Sciences, Little Chalfont, UK), prepared according to the manufacturer, was added, followed by incubation for 1 hour at 4°C. Subsequently, the protein G Sepharose-antibody-antigen complexes were collected by centrifugation and washed six times for 15 minutes in immunoprecipitation buffer at 4°C. The complexes were then transferred to 1 ml Ultima-Gold® XR scintillation fluid (Packard Bioscience, Groningen, Netherlands) and immunoprecipitated counts per minute evaluated in a Beckman LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Inc., Fullerton, CA). A NALP5 antibody index for each serum was calculated as the following: counts per minute immunoprecipitated by tested serum/mean counts per minute immunoprecipitated by 10 healthy control sera. Each serum was tested in duplicate in two experiments and the mean NALP5 antibody index calculated. The upper limit of normal for the NALP5 antibody RLBA was calculated using the mean NALP5 antibody index + 3 SD of 10 healthy controls. Any serum sample with NALP5 antibody index above the upper limit of normal was designated as NALP5 antibody positive.

Measurement of cytokine antibodies

Antibodies against cytokines were detected in ELISAs (14, 15). IL-22, IL-17A, and IL-17F (R & D Systems, Minneapolis, MN) and interferon (IFN)-ω, IFN-α2A, and IFN-λ1 (IL-29; Sigma-Aldrich) were prepared according to the manufacturers' instructions. For ELISAs, the required cytokine was diluted in PBS to 0.1 μg/ml and 100 μl samples used to coat the wells of a Corning polystyrene 96-well microtiter plate (Bibby Sterilin Ltd., Bargoed, UK). The plates were then incubated overnight at 4°C. Excess peptide was removed by decanting, and the wells were blocked with blocking buffer (PBS containing 0.1% Tween-20 and 3% bovine serum albumin) for 30 minutes at 37°C. Plates were washed four times with washing buffer (PBS containing 0.1% Tween-20). Aliquots (100 μl) of serum at a 1:100 dilution in blocking buffer were added to the wells. PBS was applied as a control. The plates were incubated at room temperature for 1 hour and then washed four times with washing buffer. Aliquots (100 μl) of antihuman IgG alkaline phosphatase-conjugate (Sigma-Aldrich), diluted to 1:2000 in blocking buffer, were added to the wells for 1 hour at room temperature. After washing five times with washing buffer, 100 μl alkaline phosphatase substrate Sigma Fast *p*-Nitrophenyl Phosphate (Sigma-Aldrich) was applied to each well and plates incubated at room temperature to allow color development. A LabSystems Integrated EIA Management System (Life Sciences International, Basingstoke, UK) was used to read absorption of the wells at 405 nm. All sera were tested in duplicate and the average OD_{405} value taken. The binding reactivity of each patient and control sera to each cytokine were expressed as an antibody index calculated as the following: mean OD_{405} of tested serum/mean OD_{405} of 10 healthy control sera. Each serum was tested in two experiments and the mean antibody index calculated. The upper limit of normal for each ELISA was calculated using the mean antibody index + 3 SD of 10 healthy control sera. Patient sera with an antibody index greater than the upper limit of normal were regarded as positive for cytokine antibodies.

Results

Detection of CaSR autoantibodies in the patient's serum

Initially, a CaSR immunoprecipitation assay, as detailed in "Materials and Methods," was used to analyze the patient's serum for CaSR autoantibodies. The upper limit of normal for the CaSR immunoprecipitation assay was estimated as a CaSR antibody index of 2.81, calculated from a population of 10 healthy control sera (Fig. 2). The serum sample from the patient tested positive for CaSR autoantibodies with a CaSR antibody index of 40.1 (Fig. 2). The positive control anti-CaSR antiserum had a CaSR antibody index of 20.5 (Fig. 2).

Subsequently, the response to Ca^{2+} of the CaSR expressed in HEK293 cells was assessed by the measurement of intracellular IP1 accumulation, as described in "Materials and Methods." To determine the effects of IgG from the patient and healthy controls (n = 10) on the response of the CaSR to Ca^{2+}, HEK293-CaSR cells were
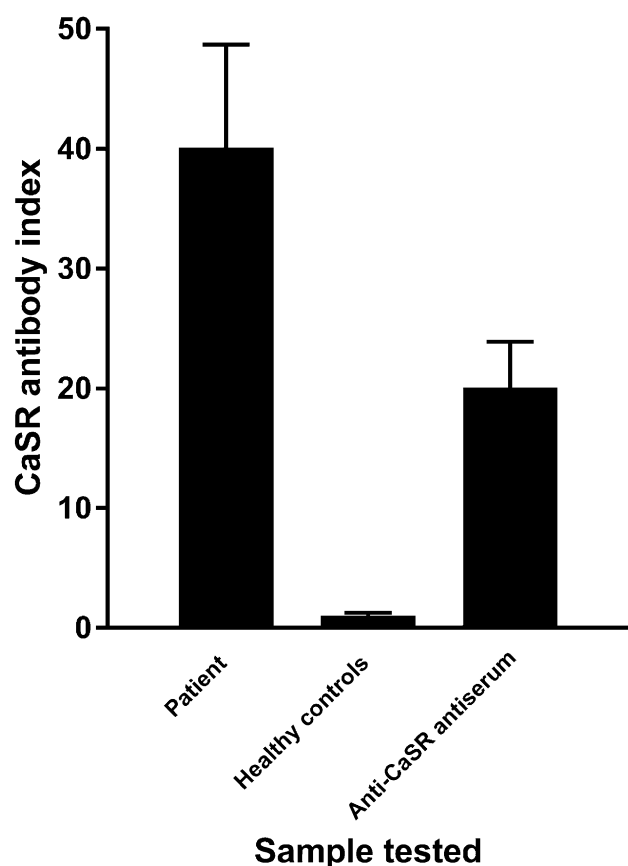
Figure 2. Detection of patient CaSR autoantibodies. Serum from the patient, healthy controls (n = 10), and positive control anti-CaSR antiserum was tested in CaSR immunoprecipitation assays at a 1:100 dilution and assigned a CaSR antibody index, as detailed in "Materials and Methods." The upper limit of normal for the CaSR immunoprecipitation assay was calculated using the mean CaSR antibody index + 3 SD of 10 control sera, which gave a CaSR antibody index value of 2.81. A CaSR antibody index above the upper-normal limit was designated as positive for anti-CaSR antibody reactivity. The CaSR antibody index of the patient's sample (40.1) and the positive control anti-CaSR antiserum (20.5) are shown and are positive.

Figure 3. Effect of patient IgG on CaSR activity. Intracellular IP1 accumulation in HEK293-CaSR cells was measured in response to Ca^{2+} stimulation (final concentrations of 0 to 5 mM) after cells were preincubated with the patient's IgG sample or healthy control IgG at a 1:100 dilution. Cells without preincubation with IgG were also included. The accumulation of intracellular IP1 was measured using an IP-One ELISA Kit, an immunoassay based on competition between free IP1 and IP1-HRP conjugate for binding to anti-IP1 monoclonal antibody. The results for IP1 accumulation were expressed as the following: percentage inhibition of IP1-HRP binding = (1 − IP1-HRP binding in stimulated cells/IP1-HRP binding in unstimulated cells) × 100. Increasing IP1 accumulation in the HEK293-CaSR cells is reflected by an increase in the percentage inhibition of IP1-HRP binding. The results show IP1 accumulation (mean ± SD of six experiments) in Ca^{2+}-stimulated HEK293-CaSR cells that were preincubated with either IgG from the patient or IgG from a single control or that were not preincubated with IgG. Only the patient's IgG sample significantly increased the levels of IP1 accumulation when compared with Ca^{2+} stimulation (at 0.5, 1.5, and 3 mM) of HEK293-CaSR cells not preincubated with IgG. *$P < 0.001$ (one-way ANOVA).

incubated with IgG samples at a 1:100 dilution before no stimulation or stimulation with a range of Ca^{2+} concentrations (0.5, 1.5, 3.0, and 5.0 mM). Each experiment included HEK293-CaSR cells stimulated with Ca^{2+} alone. Immunoprecipitation accumulation was measured using the IP-One ELISA Kit, as detailed in "Materials and Methods," and the percentage inhibition of IP1-HRP binding was calculated.

Of the 10 control IgG samples analyzed, none had any effect on the levels of IP1 accumulation when compared with stimulation by Ca^{2+} alone (Fig. 3). In contrast, the IgG sample from the patient significantly increased the levels of IP1 accumulation at Ca^{2+} concentrations of 0.5, 1.5, and 3.0 mM when compared with Ca^{2+} stimulation alone ($P < 0.001$, one-way ANOVA; Fig. 3). The IgG from the patient had no detectable effect on IP1 accumulation, however, when no Ca^{2+} was added to the
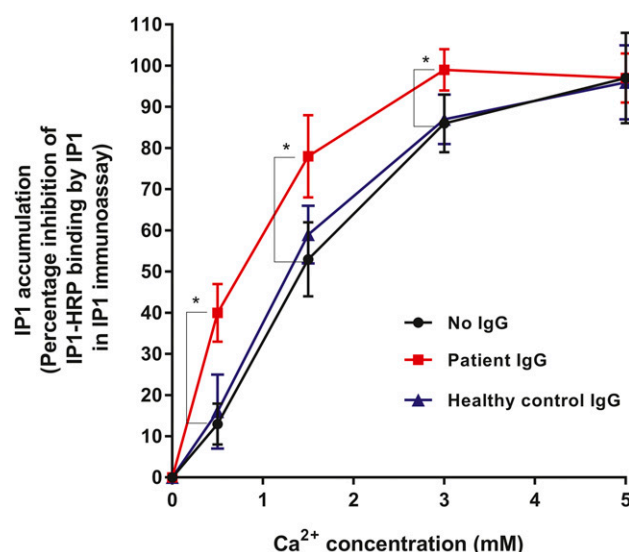
buffer, indicating that some degree of receptor activation by its natural ligand Ca^{2+} was needed to see the functional effect of the IgG. The results indicated the presence of CaSR-stimulating activity in the patient IgG sample.

Detection of NALP5 and cytokine autoantibodies in the patient's serum

The patient's serum sample was evaluated for NALP5 and cytokine antibodies using RLBAs and ELISAs, respectively, as detailed in "Materials and Methods." The upper limits of normal for antibody assays against IL-22, IL-17F, IL-17A, IFN-ω, IFN-α2A, IFN-λ1, and NALP5 were antibody indices of 1.29, 1.25, 1.33, 1.89, 2.03, 1.70, and 1.81, respectively. The patient was negative for autoantibodies against NALP5 and all cytokines tested; antibody indices were 1.01, 0.98, 0.91, 0.75, 1.02, 1.11, and 1.07 for assays measuring autoantibodies against IL-22, IL-17F, IL-17A, IFN-ω, IFN-α2A, IFN-λ1, and NALP5, respectively.

Discussion

The biochemical investigations carried out here strongly suggest hypoparathyroidism as the cause of the patient's hypocalcemia. As there was no history of neck radiation, surgery, or infiltration of the parathyroids on imaging, an autoimmune etiology of hypoparathyroidism seemed most likely. Furthermore, persistently low PTH, despite normalization of serum magnesium, excluded the possibility of hypomagnesemia as the cause of hypocalcemia and hypoparathyroidism.

Although the parathyroid glands are not the target of most autoimmune diseases, autoimmunity is an important cause of hypoparathyroidism, either as an isolated endocrinopathy or as a component of APS1 (16, 17). Autoimmune hypoparathyroidism can be caused by permanent hypoparathyroidism, owing to irreversible, immune-mediated damage to the parathyroid glands or functional hypoparathyroidism as a result of antibody-induced activation of signaling pathway(s) regulating parathyroid function (18). In APS1, common autoantibody targets include parathyroid-expressed NALP5 and cytokines. However, the testing carried out here revealed that the patient was not positive for autoantibodies against NALP5, a marker for parathyroid autoimmunity, or a panel of IFNs and ILs that are diagnostic for APS1 (13–15). As such, the patient was not diagnosed with multiple endocrine autoimmune disease to account for her low parathyroid function. Autoantibodies directed against the parathyroid-expressed CaSR have been recognized to be present in the serum of patients with autoimmune hypoparathyroidism (16), including autoantibodies that can activate the receptor, thereby causing reduced PTH secretion (10, 11).

Antibody tests of the patient's serum confirmed our diagnosis of hypoparathyroidism caused by the development of CaSR-activating autoantibodies. Autoantibody activation of the CaSR was indicated by increased IP1 levels in a cell line expressing the CaSR, following treatment with the patient's IgG—the phosphoinositide pathway being a key intracellular mediator of CaSR activation. Thus, by shifting the calcium-PTH curve to the left and decreasing the set-point of the CaSR, PTH is not released at lower serum calcium concentrations, resulting in hypocalcemia. The temporal relationship between the initiation of nivolumab infusions suggests that this drug is likely responsible for the development of the CaSR-activating autoantibodies, although we cannot rule out with certainty that the development of anti-CaSR antibodies and hypoparathyroidism was coincidental. Although the patient's hypoparathyroidism appears persistent, despite discontinuation of nivolumab, it is possible that improvement could take place with longer follow-up. This report illustrates a case of autoimmune hypoparathyroidism induced by ICI blockade as the underlying cause.

The reasons for differential autoimmune involvement of the endocrine glands between anti-PD-1 and anti-CTLA-4 therapy are not entirely clear (2). Higher risk of hypophysitis with anti-CTLA-4 therapy, such as ipilimumab compared with anti-PD-1 pembrolizumab, may be a result of the expression of CTLA-4 in the normal pituitary gland (2). In contrast to the high risk of hypophysitis with anti-CTLA-4 therapy, autoimmune thyroiditis appears to be more common in anti-PD-1 therapy. It has been speculated that in addition to effects on T cell immunity, anti-PD-1 therapy may augment humoral immunity, thus boosting antithyroid antibody formation (2). Careful studies of ICIs *in vitro* and in animal models have also shown that their complex role in T cell immunity and blockade may involve the inhibition of regulatory T cells, which could be a factor in the induction of autoimmunity (19).

Given the high risk of autoimmune diseases, including endocrinopathy, during treatment with ICIs, the risk of toxicity should always be balanced against the benefit that may derive from the ICI blockade. As ICIs are now used to treat many cancers, clinicians should be aware of the potential risk for hypocalcemia associated with their use.

Acknowledgments

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