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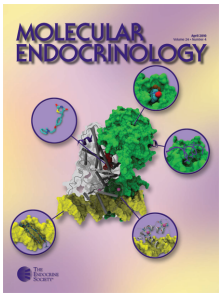

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Hormonal and Biochemical Normalization and Tumor Shrinkage Induced by Anti-Parathyroid Hormone Immunotherapy in a Patient with Metastatic Parathyroid Carcinoma

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Parathyroid carcinoma is a rare cause of primary hyperparathyroidism, and the efficacy of medical therapy and chemo- and radiotherapy is poor in recurrent or metastatic disease. We report the first case of PTH immunization in which tumor shrinkage accompanied hormonal, biochemical, and clinical improvements in a patient with metastatic parathyroid carcinoma.

A 50-yr-old woman with refractory parathyroid carcinoma and pulmonary metastases was immunized eight times between February 2001 and December 2003 with bovine and modified human PTH fragments and intact human PTH, mixed with Freund's adjuvant. Total and ionized calcium and PTH levels were assayed weekly for 6 months and regularly thereafter. Thoracic computed tomography scans were performed regularly.

Antibodies to all PTH fragments were detected after two immunizations. Baseline PTH and total calcium were 213.0 ng/liter and 13.96 mg/dl, respectively, and remained elevated during the first three immunizations. From the fourth immunization onward, PTH and calcium decreased, and the patient's clinical condition improved markedly. PTH and calcium levels have remained controlled for more than 24 months, and the sizes (surface area) of pulmonary metastases decreased from baseline by 39–71%.

This is the first evidence that PTH immunization not only can improve clinical, hormonal, and biochemical measures in parathyroid carcinoma but also has an antitumor effect. (*J Clin Endocrinol Metab* 89: 3413–3420, 2004)

PARATHYROID CARCINOMA IS a rare cause of primary hyperparathyroidism, historically accounting for less than 1% of cases, although more recent studies report a rate of approximately 5% (1). Parathyroid carcinoma is usually symptomatic at presentation, often markedly so, and patients commonly have both active bone and renal disease at diagnosis (1, 2). Important advances have been made in understanding the molecular pathology of parathyroid carcinoma. Recently it has been shown that both familial (3) and sporadic (4, 5) forms of parathyroid carcinoma are associated with various mutations in the HRPT2 gene on chromosome 1q25–1q32 suggesting that HRPT2 acts as a tumor suppressor gene (6).

Treatment of parathyroid carcinoma is surgical in the first instance, with the initial aim of achieving a cure by complete tumor resection (2). Recurrence is common (2, 7) and usually occurs locally in the neck. Parathyroid carcinoma is slow growing and metastasizes late, with the most frequent sites of deposits being the lung (40%), cervical lymph nodes (30%), and liver (10%) (1). Treatment of recurrent disease relies on surgical resection of local and distant deposits to palliate hypercalcemia because the primary cause of mortality is hormonally driven biochemical disturbance, rather than direct tumor invasion. This approach is combined with fluid support and loop diuretics to increase renal calcium excretion. Few other treatment options exist for patients for whom surgery is not possible because parathyroid carcinomas are not radiosensitive and respond poorly to cytotoxic chemotherapy (1, 2). Bisphosphonates, which reduce PTH-induced osteoclast activity, have beneficial but transitory effects on calcium levels; however, they have no impact on tumor progression. Other options include calcitonin, octreotide, gallium, and mithramycin, which are occasionally transiently effective, although the latter two are of limited use because they are nephrotoxic (1). Collins *et al.* (8) successfully used a calcimimetic compound, cinacalcet, to decrease PTH secretion (via increased parathyroid cell sensitivity) and improved symptoms in an elderly patient with refractory parathyroid carcinoma, and recent preliminary reports in six patients with parathyroid carcinoma demonstrated amelioration of hypercalcemia after cinacalcet treatment (9, 10).

Therapeutic immune targeting of cancer cells is a topic of growing interest as the mechanisms of antigen presentation, autoimmunity, and immune tolerance become more clearly defined. A variety of methods have been used to induce and augment targeting of antigens expressed by cancer cells. Previously, Bradwell and Harvey (11) reported the success-

Abbreviations: CT, Computed tomography; IRMA, immunoradiometric assay.

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ful treatment of a patient with refractory parathyroid carcinoma by immunization with fragments of the PTH molecule to break natural tolerance and induce an antibody reaction against PTH. This method proved successful in reducing hypercalcemia and concomitantly improved the patient's physical status, but there was no antitumor effect. In the current study, we report for the first time the long-term hormonal, biochemical, and antitumor effects of anti-PTH immunotherapy in a patient with refractory metastatic parathyroid cancer.

Patients and Methods

A 50-yr-old female patient was referred to the University Hospital Center, Liège, in September 2000, suffering from hypercalcemia [total calcium 14.76 mg/dl (3.69 mmol/liter); normal range 8.6–10.2 mg/dl (2.15–2.55 mmol/liter)] caused by a metastatic parathyroid carcinoma. She was originally diagnosed with primary hyperparathyroidism at another center in 1992 at which time both her total calcium [14.0 mg/dl (3.5 mmol/liter)] and her PTH (736 ng/liter; normal range 10–55 ng/liter) were markedly elevated. A right-sided parathyroid mass was identified during cervical exploration and was excised; histology demonstrated a well-differentiated parathyroid carcinoma with lymph node involvement and local invasion. Surgery was initially successful, with the patient experiencing a normalization of PTH levels and hungry bone syndrome, and she was treated with calcium supplementation and vitamin D. Thereafter the patient was well for approximately 6 yr, after which time she again developed symptomatic hypercalcemia [total calcium 13.32 mg/dl (3.33 mmol/liter)] and was found to have a PTH of 230 ng/liter. Radiological imaging revealed multiple pleuropulmonary metastases of less than 1 cm in diameter, and the diagnosis of metastatic parathyroid carcinoma was confirmed after thoracoscopic wedge resection of a metastasis. Radiographs demonstrated cyst formation and osteopenia, suggestive of osteitis fibrosa cystica (brown tumors). Initial treatment of the patient included iv rehydration and a loop diuretic (furosemide). Over this period the patient also received calcitonin, octreotide, and pamidronate treatment. Although calcium levels decreased initially, normalization did not occur, and in September 2000, the patient's total calcium was 14.76 mg/dl (3.69 mmol/liter), whereas PTH was 223 ng/liter. A thoracic computed tomography (CT) scan at this time revealed tumor progression, compared with the 1998 CT scans, although no new metastases were visualized. Chemotherapy and radiotherapy were discussed, but these were declined by the patient.

The patient's clinical condition continued to deteriorate due to the metabolic effects of hypercalcemia. She experienced extreme fatigue, nausea, and weight loss of 3 kg due to dehydration despite 3 liters/d fluid intake and treatment with loop diuretics (80 mg/d furosemide). Against this background of disease progression and the failure of therapy, it was decided to offer the patient immunization against PTH. After approval of the treatment plan by the ethics committee of the hospital and the provision of informed written consent, the patient underwent the first immunization in February 2001.

Anti-PTH immunization

Immunogenic preparations of PTH were generated as described previously by Bradwell and Harvey (11). Briefly, the peptide sequences of human and bovine $PTH_{1–34}$ (bioactive aminoterminal sequence), $PTH_{33–52}$, and $PTH_{51–84}$ were synthesized. Modification of human PTH fragments involved the insertion of single amino acid substitutions in each peptide fragment. This was achieved during synthesis of the human peptides by adding a variety of amino acids at position 2, allowing a random substitution. The bovine peptides were unmodified. Immunogenicity was increased by synthesizing each of the PTH fragments as multiantigenic peptides (octamers) on lysine webs, connected to a lysine core (Alta Biosciences, University of Birmingham, Birmingham, UK). These immunogens were then mixed with commercially produced whole human PTH molecules (Peninsula Laboratoires, Europe Ltd., St. Helens, Merseyside, UK), dialyzed against sterile saline and combined in a proportion of 40:60 with complete Freund's adjuvant.

The initial immunization (February 12, 2001, d 1) contained 200 μg human and bovine PTH peptide immunogens and 50 μg human intact PTH. Using a 28-gauge needle, the immunization was administered as eight individual 30-μl intradermal injections across the shoulders/upper thorax, upper arms, and upper thighs. Subsequent immunizations were performed using a similar technique in April 2001 and September 2001. Further 100-μl booster immunizations in December 2001, June 2002, and November 2002 contained alum as an adjuvant and were administered as two 50-μl intradermal injections in close proximity to the cervical lymph nodes. The booster immunizations in March 2003 and December 2003 again contained complete Freund's adjuvant and were administered following the same technique and at the same sites as the first three immunizations.

Serum calcium and PTH assays

At baseline and during immunization, serum ionized calcium (Nova, Waltham, MA) and total calcium (Hitachi-Boehringer Mannheim, Indianapolis, IN) were measured on a weekly basis.

Three separate assays were used to measure PTH, two routine assays and one specialized assay. PTH was assessed weekly for 6 months and regularly thereafter, using a two-site N-terminal phase assay for intact PTH [N-tact PTH SP immunoradiometric assay (IRMA) kit, DiaSorin, Stillwater, MN; standard: $PTH_{1–84}$; first antibody: polyclonal anti-$PTH_{39–84}$; second antibody: polyclonal anti-$PTH_{1–34}$-^{125}I; $< 0.1\%$ cross-reactivity; normal range: 10–55 ng/liter]. A second, C-terminal/mid-molecule PTH RIA was also used (C/MM PTH parathyroid hormone 100T RIA kit, The Nichols Institute Diagnostics, San Juan Capistrano, CA; standard: $PTH_{44–68}$; antibody: polyclonal anti-$PTH_{44–68}$; tracer: $PTH_{44–68}$-^{125}I; normal range: 61–315 ng/liter). To verify that a genuine decrease in PTH concentration was occurring, rather than PTH-antibody blockade of the assays, a third assay was performed using an acid dissociation procedure, in which 50 μl of the patient's serum was diluted in 200 μl of glycerine/HCl and adjusted to a pH of 2.5 using HCl. After centrifugation in a Centricon 100 (cut-off at 100,000 rpm), the filtrate was diluted 1:5 in hypoparathyroid (PTH-free) serum, adjusted to pH 7.4 with 0.1 M NaOH and assayed (Roche Elecsys, Vilvoorde, Belgium).

Anti-PTH antibody identification

A semiquantitative method was used to measure anti-PTH antibodies. Individual 0.1-μl volumes of PTH peptide fragments (0.5 mg/ml) were applied to nitrocellulose membranes and incubated with the patient's serum diluted 1:500 in PBS. Sheep antihuman IgGFc antibody, conjugated to peroxidase (1 μg/ml) (The Binding Site, Birmingham, UK) was applied, and binding was visualized after the addition of the chromogenic substrate, 3-amino-9-ethyl-carbazole. The control antibody was an affinity-purified sheep antibody against an unrelated multiantigenic peptide, applied at concentrations of 5, 20, 80, 320, and 1280 mg/liter to the dot-blot under the same conditions as the patient's serum. The titers of the patient's anti-PTH antibodies were determined by comparison with the control antibody.

Lymphocyte cytokine stimulation test

An enzyme linked immunospot assay for interferon-γ was performed to assess cytokine release from peripheral blood T cells after antigen stimulation to measure specific T cell responses (12). Then 2×10^5 fresh peripheral blood mononuclear cells were incubated separately with 4 μg each of unmodified and modified multiantigenic PTH peptides used for immunization (see above), and interferon-γ production was assessed. Appropriate negative and positive controls were used including an irrelevant multiantigenic peptide.

Radiological studies

Serial thoracic spiral CT examinations were performed to assess the size and extent of the pulmonary metastases over the course of immunizations. A CT scan performed on September 26, 2000 (d −139), approximately 5 months before the first immunization, was used as a control. Further thoracic CT scans were performed on d 277, 438, 533,

634, and 877, respectively. All CT scans were performed on the same scanner (PQ 5000, Philips, Eindhoven, The Netherlands) and workstation (Voxel Q Philips) using the same technique (noncontrast, breathhold for 20 sec in full inspiration). Each lesion was manually contoured at the largest diameter and the corresponding surface was electronically measured. The statistical significance of any changes in the lesion surface was assessed using Student's *t* test.

Results

Calcium and PTH levels

At baseline before immunization, the serum PTH and total calcium levels were 213.0 ng/liter and 13.96 mg/dl (3.49 mmol/liter), respectively. The hormonal and biochemical responses to the first three immunizations were modest, with a sustained reduction in PTH and serum calcium being seen for the first time after the third immunization. Figures 1 and 2 demonstrate the marked response to the fourth immunization, with PTH falling from 191.5 ng/liter before immunization to 84.5 ng/liter approximately 1 month thereafter. Over the same time period, the total calcium concentration mirrored the PTH response, falling from 12.12 mg/dl (3.03 mmol/liter) to within the normal range [9.48 mg/dl (2.37 mmol/liter)]. Both PTH and calcium were normal at the time of the fifth immunization. Ionized and total calcium normalized first (d 334), followed by PTH (d 365). PTH remained within the normal range or just above the upper limit of normal thereafter. Both ionized and total calcium remained normal for more than 24 months of follow-up, at which time (February 2004) the patient developed mild hypocalcemia with total and ionized calcium concentrations of 8.32 mg/dl (2.08 mmol/liter) and 3.88 mg/dl (0.97 mmol/liter), respectively; PTH was 48.8 ng/liter.

Given the potential for false-low levels of PTH being reported due to masking of the immunoassay by circulating anti-PTH antibodies, two alternative assays were used. As shown in Fig. 2, A and B, RIA measurements confirmed the decrease in PTH levels demonstrated by the IRMA assay. No excess free PTH was noted after acid dialysis, indicating that no circulating PTH was being shielded by antibody binding.

Other measures of calcium metabolism were also assessed. Serum 1, 25-dihydroxyvitamin D concentration was 65.5 ng/ml (normal range: 3–48 ng/ml) at baseline when the corresponding PTH was 213 pg/ml. When PTH fell to 82.4, the serum 1, 25-dihydroxyvitamin D normalized at 13.1 ng/ml. Subsequently the serum 1, 25-dihydroxyvitamin D remained in the range 20 to 55 ng/ml over the series of immunizations. Decreased total alkaline phosphatase was also noted. Total alkaline phosphatase was 753 IU/liter (normal range 34–117 IU/liter) when the concomitant PTH was 209.5 ng/liter and total calcium was 3.05 mmol/liter. As PTH and total calcium fell to 2.59 mmol/liter and 55.2 ng/ml, respectively, alkaline phosphatase normalized to 70 IU/liter, and remained normal thereafter.

Anti-PTH antibody identification

Semiquantitative dot-blot analyses demonstrated that antibodies against the human $PTH_{33–52}$ and $PTH_{51–84}$, and the bovine $PTH_{51–84}$ fragments were present 3 wk after the first immunization. From 2 wk after the second immunization

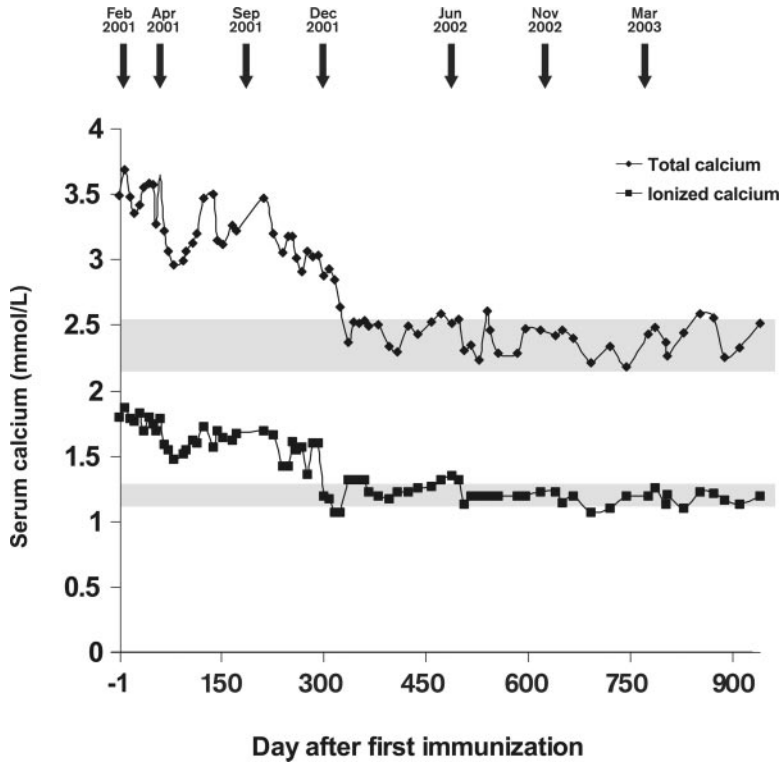

FIG. 1. Evolution of ionized and total calcium levels in a patient with metastatic parathyroid carcinoma after anti-PTH immunization. The *arrows* indicate the date of each immunization, and the normal ranges for total and ionized calcium are outlined in *gray*. Normal range for ionized calcium: 1.13–1.3 mmol/liter; total calcium: 2.15–2.5 mmol/liter. To convert micromoles per liter to nanograms per liter, divide by 0.25.

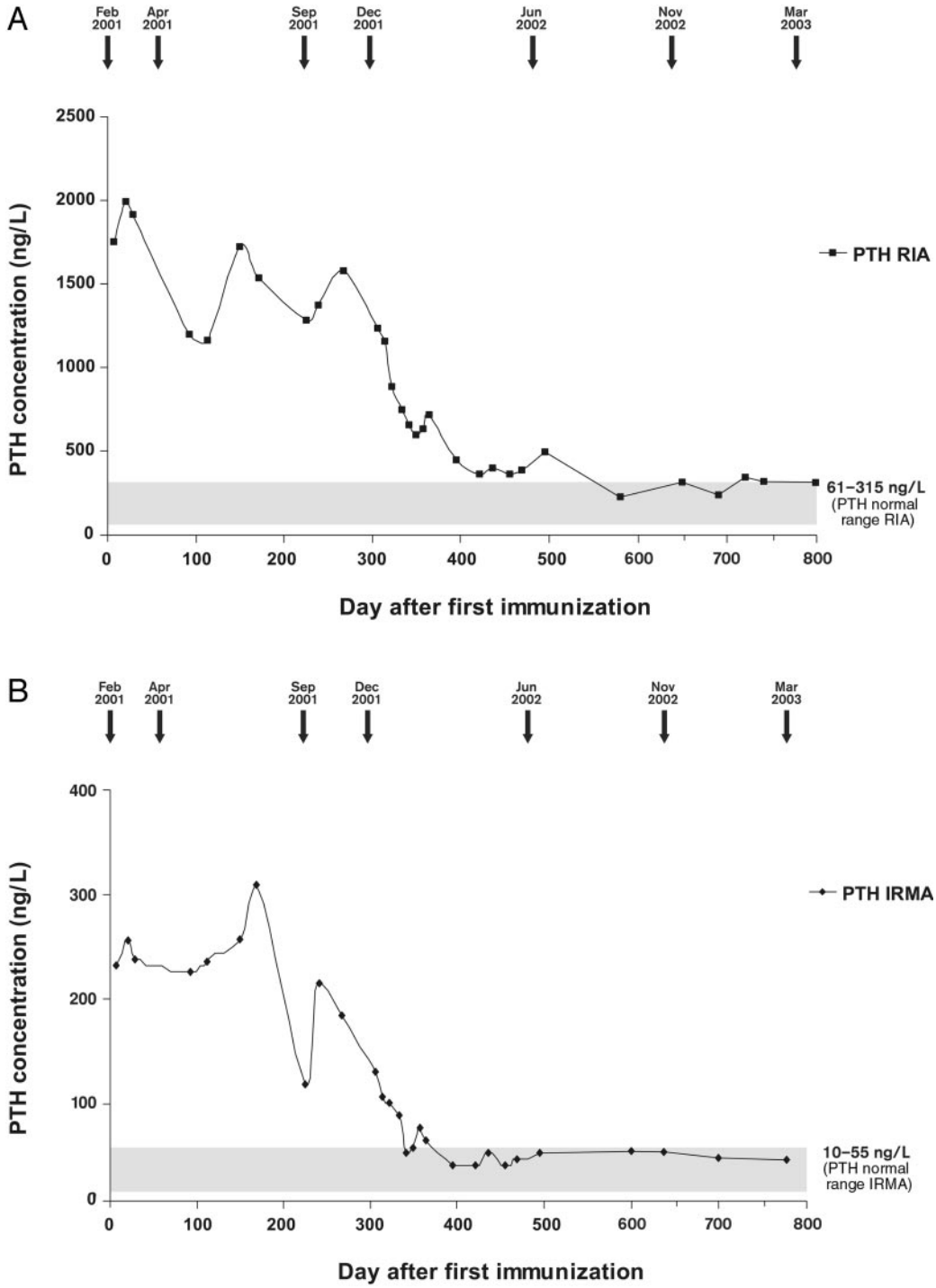

FIG. 2. PTH concentrations during the treatment period, measured using a C-terminal/midmolecule PTH RIA (normal range: 61–315 ng/liter, A) and a two-site N-terminal phase assay for intact PTH, IRMA (normal range: 10–55 ng/liter, B).

onward, antibodies against all human and bovine immunogens were identified.

Lymphocyte cytokine stimulation

The enzyme linked immunospot assay showed an antigen-specific response against the three fragments of unmodified and modified PTH with a slightly greater response against the C-terminal peptide. Incubation of the peptides with the patient's cells gave a median response of 4 cells/10,000 (range 1–7). This increased to a median response of 7 cells/10,000 (range 1–12) when the peptides were incubated with the patient's serum. Responses using an unrelated control peptide were 0–1 cells/10,000.

Radiological response

The mean axial surface of the pulmonary metastases increased slightly from the baseline CT scan on d −139 to the scan performed on d 277 after the first immunization. The last CT scan performed at d 877 revealed a total decrease of 39.2–71.4% in the size of the pulmonary metastases, which was statistically significant ($P < 0.05$) (Figs. 3 and 4). No additional metastatic pulmonary lesions developed during treatment. Radiological studies of the skeletal lesions showed no progression over the course of treatment and follow-up.

Clinical status

After the fourth immunization, the patient's clinical condition improved markedly. A dramatic improvement in the severity of asthenia, nausea, and muscle weakness was noted. Over the next 3 months, her appetite returned to normal and she gained 10 kg. The patient's fluid intake was 2 liters daily and furosemide was stopped. The patient became more dynamic and her clinical status normalized.

Adverse events

After each immunization the patient developed 5-mm firm nodules at the injections sites accompanied by local lymphadenopathy. Superficial ulceration of nodules occurred but healed spontaneously to leave small scars. Approximately 10 h after the second immunization, the patient developed a 24-h episode of fever and chills. This was considered retrospectively to be due to an immune reaction arising from PTH/antibody complexes. Apart from anti-PTH antibodies, no evidence was found of autoantibodies being raised against other self-antigens (smooth muscle, skin, liver, and

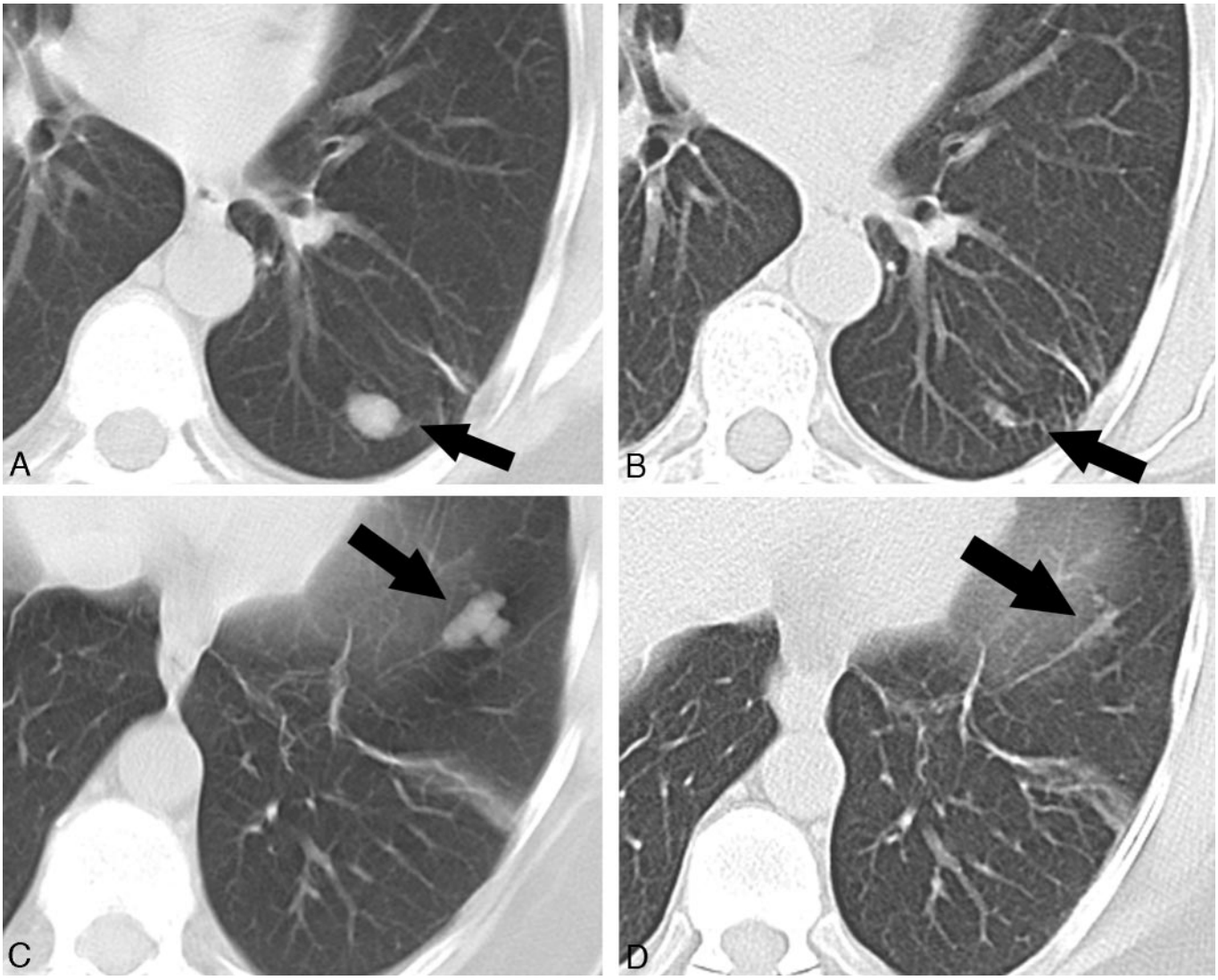

FIG. 3. Axial CT scans showing shrinkage of pulmonary metastases. Shrinkage of the pulmonary metastasis in the left VI segment (*arrow*) is shown in scans performed on d 277 (A) and d 877 (B), while a pulmonary metastasis in the left VIII lung segment (*arrow*) also shrank in size between the d 277 (C) and d 877 (D) CT scans. The same magnification was used for each image. CT technology at the time of the initial scan did not permit volume assessment due to anisotropism in the z-axis. Therefore, surface measurements underestimate the actual volume reduction.

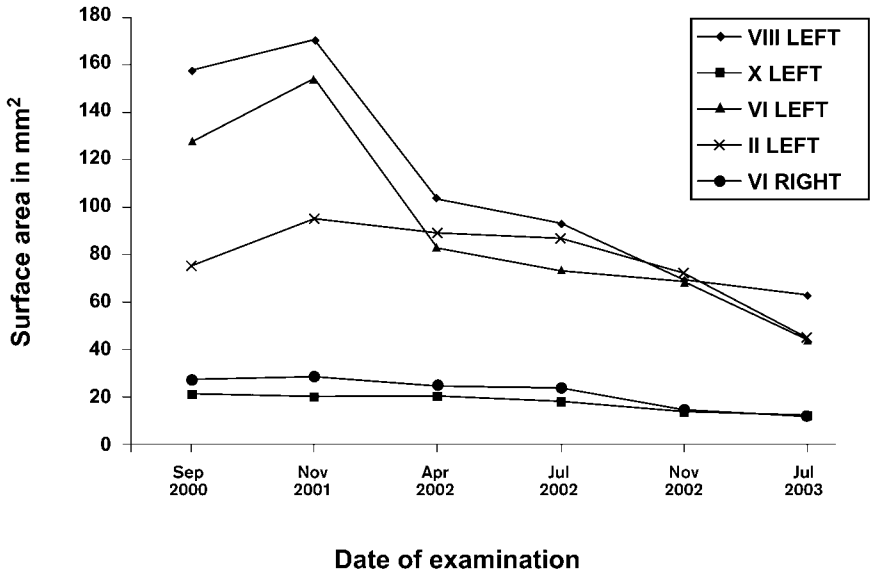

FIG. 4. Evolution of the dimensions (square millimeters) of five pulmonary metastases during the course of PTH immunizations. Data are shown in terms of surface areas.

stomach), and no antinuclear antibodies were identified during the follow-up period.

Discussion

Neuroendocrine tumors are often indolent in character and tend to produce symptoms through hormonal secretion rather than by tumor invasion. In metastatic parathyroid carcinoma, the main problems stem from the uncontrolled effects of excess PTH, and the main causes of morbidity and mortality are hypercalcemia and bone disease. Treatment options for refractory and metastatic disease are limited because parathyroid carcinoma is largely unresponsive to chemo- and radiotherapy. Therefore, novel therapeutic regimens that limit the actions of PTH in parathyroid carcinoma are of interest.

Previous experience has shown that it is possible to reduce hypercalcemia by blocking the effects of high PTH concentrations with neutralizing antibodies. In 1999, Bradwell and Harvey (11) attempted to control severe, intractable hypercalcemia in a patient with terminal parathyroid carcinoma by inducing autoantibody formation to human PTH. This involved breaking normal immune tolerance to PTH by using human and bovine PTH-like immunogenic fragments to stimulate the production of an antibody that would cross-react with human PTH. Whereas this immunologic response was achieved and a reduction in serum calcium occurred, that patient had established severe bone disease and died from complications after the fracture of a femoral bone cyst. One particular feature in that patient had been the rising concentrations of serum PTH because the blocking antibodies formed immune complexes and extended its normal serum half-life. In our patient the high initial PTH and calcium concentrations normalized with repeated immunizations over a 9- to 12-month period. To rule out any interference by induced antibodies with PTH measurements, both a two-site, N-terminal phase assay for intact PTH and a C-terminal/midmolecule assay were used. Although both assays were in agreement, further proof of the reduced PTH concentrations was sought. PTH was separated from potentially binding antibodies by acid dialysis and reanalyzed, but serum PTH concentrations indicated that no shielding of PTH by circulating antibodies had occurred.

In parallel with these observations, axial CT scans of the lung metastases showed dramatic reductions in size. This provided an explanation for the reduced PTH concentrations and was a welcome but unexpected finding, particularly because no tumor regression had been seen in the previous case. There are more than 600 patients recorded with parathyroid carcinoma since 1937 (1, 2), and to our knowledge spontaneous tumor regression has not been reported in the literature. It appears, therefore, that immunotherapy may have not only stimulated antibodies against PTH but also substantially reduced tumor mass because tumor shrinkage is not a natural feature of the disease.

We chose a combination of human, modified human and bovine peptides for immunotherapy. Modified human peptides were added to peptides used in the initial immunization protocols but were eventually used alone. The human peptides contained single amino acid substitutions at position 2 of each fragment and were more similar to human molecules than their bovine counterparts. Thus, satisfactory major histocompatability complex presentation of antigens from B cells to T cells was encouraged, while maintaining minimal structural differences to favor cross-recognition of human proteins. These peptides have been used in two other patients with good initial responses and appeared more effective than unmodified bovine and human peptides (13). We used Freund's complete adjuvant because of its high potency for inducing antibodies, but it is equally a very effective agent for inducing cellular immune responses. It is safe to use, although uncomfortable for the patient, and side effects can be minimized with careful intradermal injections.

The progress of immunotherapy was monitored using an antibody dot-blot assay. Immune responses to each of the PTH component fragments were assessed on a weekly basis, and subsequent immunizations were modified to increase the amounts of peptides that had weak antibody responses. By targeting three separate portions of the molecule, anti-

bodies were produced against several parts of the whole human PTH molecule, thus promoting the development of large, circulating PTH-antibody immune complexes, which could be removed by Fc receptor-bearing cells.

Immunotherapy of solid tumors has generally been ineffective for reasons that are not yet wholly clear. The use of whole tumor cell extracts and cell lysates may be inappropriate because of the plethora of antigens in the vaccines. Many of these are not specific to the tumor, whereas those that are tumor specific may be present in low, nonimmunogenic concentrations. Dendritic cells, pulsed with patient's tumor extracts *in vitro*, have been used in parathyroid and other carcinomas but again with poor results (14–17), possibly related to inadequate antigen processing. Furthermore, the use of strong adjuvants to break tolerance has been avoided in many studies because of anxieties about toxicity.

There are a number of potential mechanisms by which immunotherapy could have caused tumor regression in this patient. The induced antibodies would naturally accumulate at the site of the highest concentrations of PTH, adjacent to PTH-producing parathyroid carcinoma cells. Potentially, this may have induced immune activation, leading to tumor cell destruction or the induction of apoptosis. This mechanism was supported by the observation of considerable peripheral blood mononuclear cell responses to the immunized peptides and human PTH. Because the lymphocyte stimulation test for PTH-reacting cells showed a higher level of response to PTH than to a control peptide, this may indicate the recruitment of cytotoxic T cells against the immunogen, which could be responsible for the observed antitumor effect. Studies of tumor responses to immunotherapy in melanoma show similar findings, although patients may have good responses but no evidence of tumor regression and *vice versa* (18). It was recently shown that T cell infiltration of tumors was an important indicator of outcome in ovarian (19) and other tumors (20). Biopsy assessment of the tumor in our patient would have been helpful in ascertaining the antitumor mechanism; however, a lung biopsy to evaluate this possibility was considered clinically inappropriate. Other antitumor mechanisms can be speculated upon. PTH may act as a growth factor for parathyroid carcinoma cells because it activates important signal transduction pathways *in vitro* (21, 22). Hyperparathyroidism and hypercalcemia are associated with a variety of alterations in normal cellular immune function and cytokine production (23–25). Thus, the reduction in PTH may have improved immune targeting of the carcinoma cells by cytotoxic T lymphocytes.

Similar immunotherapy in other patients with parathyroid carcinoma needs to be considered both in terms of the possibility of success and the risk/benefit ratio. Our patient was relatively young and was expected to live several months, which was sufficient time for a good immune response. She had not had chemotherapy, irradiation to the neck, cervical lymph node removal, or thymectomy, and she was not on corticosteroids, all of which were favorable for immunotherapy. Further surgical intervention carried a low probability of success, and chemotherapy was declined. Whereas the risk of immunotherapy cannot be quantified in the limited number of patients treated so far, the satisfactory outcome and benign side effects suggest that it should be considered before patients are terminally ill. It might also be considered before the use of treatments that destroy cervical lymphoid tissues, including repeated cervical surgery.

In conclusion, this is the first case of successful immunization against PTH in a patient with parathyroid carcinoma, in which hormonal and biochemical normalization was accompanied by tumor regression. The patient has been well for more than 24 months; however, it is still too early for long-term predictions because parathyroid carcinomas are very slow growing. It remains to be seen whether tumor regression can be achieved in other patients, but the findings in this case confirm that PTH immunization represents a potentially effective therapy for patients with refractory parathyroid carcinoma.

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