Hemorrhagic Tumor Necrosis During a Pilot Trial of Tumor Necrosis Factor-α and Anti-GD3 Ganglioside Monoclonal Antibody in Patients With Metastatic Melanoma

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Hemorrhagic tumor necrosis is an inflammatory event that leads to selective destruction of malignant tissues, with both potentially toxic and beneficial consequences. A pilot clinical trial was undertaken combining tumor necrosis factor-α (TNF-α) with the monoclonal antibody R24 (MoAb R24) against GD3 ganglioside in patients with metastatic melanoma. Patients received MoAb R24 to recruit leukocytes to the tumor followed by low doses of recombinant TNF-α to activate leukocytes. Eight patients were treated and seven patients had mild toxicity. One patient with extensive metastatic melanoma developed tumor lysis syndrome within hours after treatment with almost complete necrosis of bulky tumors in multiple visceral sites. To our knowledge, this is the first documented case of hemorrhagic tumor necrosis in a patient with metastatic cancer in multiple visceral sites.

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MATERIALS AND METHODS

Patient selection. Eligibility criteria included the following: histologically confirmed stage III or IV metastatic melanoma according to the American Joint Committee For Cancer Staging and End-Results Reporting™; Karnofsky performance status ≥70; serum creatinine level less than 1.7 mg/dL (normal, <1.1 mg/dL); serum total bilirubin level less than 1.5 mg/dL (normal, <1.0 mg/dL);

granulocyte count greater than 3,000/µL; platelet count greater than 160,000/µL; age greater than 18 years; written informed consent; life expectancy of at least 3 months; no history of concurrent malignancy other than melanoma, cutaneous basal cell carcinoma, cutaneous squamous cell carcinoma, or carcinoma in situ of the cervix; and measurable or evaluable metastatic disease. Patients were excluded if they had intracranial metastases, previously received either rTNF-α or a MoAb, significant heart disease (New York Heart Association class III or IV), or a serious infection requiring antibiotics, or if they were pregnant or lactating. Anticancer therapy, aspirin, and nonsteroidal anti-inflammatory drugs were withheld for a minimum of 3 weeks before therapy.

Preparation and administration of R24 and recombinant human TNF. MoAb R24 was produced by Cell Tech, Inc and was provided by the National Cancer Institute (Bethesda, MD) as a 2.5 mg/mL solution in 30 mmol/L phosphoric acid, 150 mmol/L sodium chloride buffer, and 2% wt/vol human serum albumin. MoAb R24 was mixed with 100 mL of normal saline and 25 mL of 25% human serum albumin and was administered intravenously through a 0.86-µL filter over 2 to 3 hours. Before the initiation of therapy, 100 µg MoAb R24 diluted in 1 mL of normal saline was administered intravenously as a test dose. Patients received 10 mg/m² of MoAb R24 on days 1 and 3. From the initial phase I study, 10 mg/m² was associated with mild toxicity and histologic evidence of a perivascular inflammatory infiltrate at tumor sites. Thirteen patients were treated with rTNF-α at one of two dose levels. At dose level I received 50 µg/m² 6 hours after the start of MoAb R24. Patients at dose level II received 150 µg/m² 6 hours after the start of MoAb R24. Recombinant TNF-α was mixed with 100 mL of normal saline and administered intravenously 1 hour after the start of MoAb R24. Patients at dose level II received 150 µg/m² 6 hours after the start of MoAb R24. Recombinant TNF-α was mixed with 100 mL of normal saline and administered intravenously 1 hour after the start of MoAb R24.

Response assessment and toxicity. Before treatment, each patient was evaluated with a complete history and a physical examination, a complete blood count, a bleeding time, a prothrombin time, a partial thromboplastin time, a fibrinogen level, serum chemistries, a serum complement (CH50) level, a urinalysis, a chest roentgenogram, a cranial computed tomography (CT) scan, and an electrocardiogram. Response was monitored monthly by physical examination, the above blood work, and appropriate radiologic studies. A partial response was defined as at least a 50% reduction in the sum of the products of the longest perpendicular diameters of indicator lesions for at least 1 month without development of new lesions. Progressive disease was defined as greater than 50% increase in the sum of the products of the longest perpendicular diameters of indicator lesions or the appearance of new lesions. Toxicity was graded by the World Health Organization drug toxicity scale.

PMN studies. Peripheral blood from the patients evaluable for response was obtained to determine PMN reactivity in vitro and to assess cytotoxicity against melanoma cells. Peripheral blood from a normal healthy volunteer drawn concurrently with blood from each patient served as the control specimens. Peripheral blood was drawn from the patients and the volunteer into a heparinized tube (1) immediately before starting the MoAb R24 infusion, (2) immediately before starting the TNF-α infusion, and (3) at the end of the rTNF-α infusion. Blood was promptly layered over neutrophil isolation medium (NIM; Los Alamos Diagnostics, Los Alamos, NM) and spun at 400g at 25°C for 30 minutes. The PMN layer was removed and washed twice in Krebs-Ringer phosphate buffer with 5.5 mmol/L glucose (KRP). This procedure yielded greater than 97% PMNs. For H₂O₂ assays, 96-well microculture plates (Falcon Primaria; Becton Dickinson and Co, Lincoln Park, NJ) were coated overnight with fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) in complete medium, consisting of RPMI 1640 (Sigma Chemical Company, St Louis, MO) with 7.5% heat-inactivated FBS, 1% nonessential amino acids (GIBCO Laboratories, Grand Island, NY), 1% L-glutamine (JRH Biosciences, Lenexa, KS), and 1% penicillin-streptomycin (JRH Biosciences). After the PMNs were isolated, plates were washed twice with warm KRPG and the PMNs were added to wells at 2 x 10⁶ PMNs/well. Each of the following were added separately to different wells: (1) 10 ng/mL phorbol myristate acetate (PMA; Sigma), (2) 5.6 µmol/L formyl-methionyl-leucyl-phenylalanine (FMLP; Sigma), and (3) rTNF-α at 0.1, 0.3, or 100 ng/mL. The H₂O₂ produced by the neutrophils in response to these agents was measured using a scopoletin fluorescence assay. In this assay, H₂O₂ oxidizes up to 35 µmol/L scopoletin (Sigma) to a nonfluorescent state in a reaction catalyzed by 1 purpuragulin unit/mL horseradish peroxidase (Sigma). The presence of 1 mmol/L sodium azide (Fisher Scientific Co, Fair Lawn, NJ) in the reaction mixture prevents the action of catalase and myeloperoxidase on H₂O₂. Fluorescence was measured using an automated plate reader (MicroFluor MR6000; Dynatech Laboratories, Alexandria, VA), as previously described.

The human melanoma cell line SK-MEL-29 was provided by Dr Harvey Babich (Rockefeller University, New York, NY). Cells were maintained as adherent cultures in 25-cm² tissue culture flasks (Corning Glass Works, Corning, NY) in complete medium. The melanoma cells were grown in a humidified atmosphere of 5% CO₂ in air at 37°C and were detached with trypsin (Flow Laboratories, McLean, VA) as needed. An enzyme-linked immunosorbent assay (ELISA) was used for cytotoxicity. Melanoma cells at 3 x 10⁴ cells/mL were seeded in 0.1 mL/well complete medium (containing phenol-free RPMI; Sigma) in 96-well microculture plates and allowed to adhere overnight. The medium was changed and the PMNs isolated from the patients and healthy control donor (as described above) were then added to the wells. The ratios of PMNs (effector cells) to plated melanoma cells (target cells) were 10:1 and 50:1. The PMN and melanoma cells were coincubated for 24 hours, at which time glutaraldehyde (Fisher Scientific; 1% vol/vol final concentration) in phosphate-buffered saline (PBS; JRH Biosciences) was added to the wells. (Previous work has shown that ELISA cytotoxicity results were maximal and no different after coincubation periods between 4 hours and 48 hours [unpublished data] ). After 10 minutes at 25°C, the plates were washed three times with PBS and then incubated with 10% FBS in PBS for 1 hour at 37°C to block nonspecific binding of antibody. After washing, the plates were incubated for 1 hour at 37°C with MoAb R24 at 2 µg/mL in FBS-PBS. After a second washing, the plates were incubated for 1 hour at 37°C with β-galactosidase–conjugated goat anti–mouse IgG3 (Fisher-Biotech, Pittsburgh, PA) diluted 1:400 in FBS-PBS. Finally, the bound β-galactosidase was measured by reaction for 30 minutes in the dark with buffered 1.0 mg/mL n-octyl-β-D-galactopyranoside (Sigma). Optical density (OD) was read as absorbance at 405 nm using an automated plate reader (Dynatech MR5000).

RESULTS

Patient characteristics. The patient characteristics are shown in Table 1. Eight patients with metastatic melanoma were enrolled. All patients had visceral involvement. The sites of primary disease varied, with five cutaneous, one oc-
Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)/Sex</th>
<th>KPS</th>
<th>Prior Therapy</th>
<th>Sites of Disease</th>
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<td>1</td>
<td>31/F</td>
<td>90</td>
<td>Chemotherapy</td>
<td>Nodes, liver</td>
</tr>
<tr>
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<td>76/M</td>
<td>70</td>
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<td>Nodes, liver, spleen, pelvic mass, lung</td>
</tr>
<tr>
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<td>Chemotherapy</td>
<td>Skin nodules, lung, pleura, liver</td>
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<td>Pelvic mass</td>
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<td>Nodes, liver, spleen, pelvic mass</td>
</tr>
<tr>
<td>8</td>
<td>52/M</td>
<td>90</td>
<td>Chemotherapy</td>
<td>Nodes, lung</td>
</tr>
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</table>

Abbreviation: KPS, Karnofsky performance status.

Results and toxicity. Eight patients were evaluable for toxicity and seven patients were evaluable for response. Six patients were enrolled at rTNF-α dose level I (50 μg/m²); two patients were enrolled at rTNF-α dose level II (150 μg/m²). The clinical toxicities are shown in Table 2. All patients experienced typical grade 2 or 3 urticarial reactions to MoAb R24, which were controlled with diphenhydramine. One patient developed acute dyspnea that was relieved by a single dose of epinephrine. The one patient who was invaluable for response had a significant decrease (50% of baseline) in blood pressure after receiving half of the planned MoAb R24 dose. The patient with hypotension responded to hydration, but she received no further MoAb R24 or any rTNF-α. Subsequent evaluation showed no cardiac, infectious, or hematologic mechanism for the hypotensive episode, and there were no sequelae. Seven patients received full treatment with both MoAb R24 and rTNF-α. Of the seven patients receiving rTNF-α, six experienced moderate fever and chills, no worse than grade 2. All symptoms resolved with supportive care. Six patients had eventual progression of melanoma after treatment with a median time to progression of 8 weeks. One patient (patient no. 2) developed massive, acute tumor lysis syndrome shortly after finishing treatment. This case is discussed below.

Tumor lysis syndrome. Patient no. 2 was a 76-year-old man with metastatic melanoma in the lungs, liver, spleen, peripheral and retroperitoneal lymph nodes, and subcutaneous tissues. A pituitary adenoma had been resected 2 years earlier and he was receiving replacement doses of steroids (5 mg prednisone per day) until 3 weeks before therapy. His pretreatment evaluation was significant only for elevated lactate dehydrogenase level (693 U/L; normal, <200 U/L), alkaline phosphatase level (960 U/L; normal, <88 U/L), and serum glutamic oxaloacetic transaminase level (143 U/L; normal, <37 U/L), which were thought to be secondary to extensive metastatic melanoma in the liver and other sites. The patient received intravenous MoAb R24 (10 mg/m²) and developed a grade 2 urticarial reaction that resolved with diphenhydramine treatment. Six hours after beginning MoAb R24, rTNF-α (50 μg/m²) was administered and the patient developed characteristic rigors (grade 2) and fever during the infusion. Other than fatigue, the patient's symptoms resolved shortly after finishing treatment. During and after treatment there was no episode of hypotension.

Eight hours after finishing rTNF-α, the patient developed acute dyspnea. Laboratory evaluation showed marked lactic acidosis (peak serum lactic acid level of 36 mmol/L; normal, <1.3 mmol/L) with evidence of disseminated intravascular coagulation indicated by a peak fibrin split product level of 320 μg/mL (normal, <10 μg/mL) and a nadir fibrinogen level of 280 μg/mL of a baseline of 384 μg/mL (normal, >150 μg/mL), a markedly elevated serum uric acid level of 11.5 mg/dL (normal, <8.5 mg/dL and serum phosphorus level of 8.5 mg/dL (normal, <4.2 mg/dL), and severe acute renal insufficiency (Fig 1). These findings suggested a tumor or tissue lysis syndrome. Pulmonary artery catheterization showed high output cardiac failure and a vasodilated state consistent with acidosis or sepsis. The patient’s renal status was most consistent with acute tubular necrosis. Microscopic evaluation of the urine showed hematuria and many coarse, granular casts. The serum creatine phosphokinase level was normal and the urine myoglobin level was minimally elevated (5.7 μg/mL; normal, <2.0 μg/mL). The patient was intubated, given ventilatory support, and started on intravenous bicarbonate, stress doses of steroids, allopurinol, diuretics, and fresh frozen plasma. All blood cultures were negative, except for a culture from the tip of the pulmonary artery catheter that grew coagulase-negative staphylococcus. A chest roentgenogram showed bilateral patchy infiltrates. Sputum cultures after intubation grew Klebsiella pneumoniae and yeast. A CT scan showed a slight increase in the size of the lesions in both the liver and spleen compared with a baseline CT scan performed 10 days before starting treatment. With supportive care, the patient’s condition improved and he was extubated 10 days after MoAb R24 and rTNF-α treatment. However, after extubation, and 12 days after treatment, his condition worsened again and aggressive support measures were discontinued. The patient died from worsening respiratory compromise 13 days after treatment.

An autopsy was performed to clarify the cause of death and to determine if tissue lysis was restricted to tumor sites. On gross examination, pigmented nodules and mass lesions...
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Fig 1. Laboratory data for patient no. 2. (A) Serum lactate dehydrogenase (LDH) in units per liter; (B) serum phosphate and uric acid in milligrams per deciliter; (C) white blood count (WBC) per microliter and platelets (PLT) per microliter; and (D) prothrombin time (PT) in seconds and partial thromboplastin time (PPT) in seconds. Day 0 is the day of treatment with rTNF-α and MoAb R24.

were seen in both lungs, lymph nodes (perihilar, paraaortic, and retroperitoneal areas), heart, gallbladder, colon, liver, pancreas, spleen, adrenals, and kidneys. By histologic examination, pigmented lesions in all of these organs (and, in addition, microscopic deposits in the bone marrow) showed massive necrosis, with most pigmented lesions being completely necrotic and devoid of recognizable malignant cells (Fig 2A). Nodules of necrotic debris were encircled by melamin-containing macrophages (Fig 2B), indicating that a necrotizing process had occurred more than 1 week before autopsy and was not merely an agonal event. A few nodules showed microscopic tumor nests persisting at the periphery of areas of necrosis. Pigment was found within necrotic nodules. The pigment stained black with the Fontana-Mason stain and was negative for hemosiderin, indicating that it was melanin. Renal tubules were loaded with pigmented casts and exhibited necrosis of lining epithelium. In the liver, lung, spleen, kidney, and other organs, areas of parenchyma unaffected by tumor showed no evidence of necrosis. Blood vessels within and adjacent to necrotic areas showed evidence of fibrinoid necrosis (Fig 2C), whereas vessels within the normal tissue parenchyma were unaffected. Sections of the lung showed acute bronchopneumonia and special stains disclosed septic hyphae. Lung cultures grew Aspergillus fumigatus. The cause of death was deemed to be aspergillus pneumonia, complicated by renal insufficiency. The autopsy confirmed that the patient had developed massive tumor lysis induced by treatment with rTNF-α and MoAb R24.

PMN studies. PMN responses to fMLP, PMA, and rTNF-α were studied using blood samples taken from the patients immediately before starting treatment, immediately before the rTNF-α dose, and at the end of rTNF-α infusion. The PMNs of patient no. 2, who developed tumor lysis syndrome, exhibited a diminished respiratory burst to all stimuli for both baseline and treatment samples (Fig 3 a through c). Responses were compared with those of PMNs from the healthy volunteer and the other patients. Control PMNs showed a typical respiratory burst pattern with release of H2O2 when stimulated with PMA, fMLP, or rTNF-α (Fig 3 g through i). The respiratory burst of PMN exposed to fMLP and rTNF-α was typically 30 to 60 minutes later than the response to PMA, and the response to rTNF-α was attenuated at lower rTNF-α concentrations as previously described. For five of the six patients studied, the PMN in vitro responses were similar (data not shown). Representative results from patient no. 4 exhibited a normal burst pattern at baseline and after MoAb R24 administration (pre-rTNF-α), but a sharply attenuated response in vitro for the PMNs drawn after rTNF-α administration (Fig 3 d through f). Similar results were observed in patients treated at both dose levels I and II of rTNF-α (data not shown).

Unlike the PMNs from the other patients in the study or from the normal subject, the PMNs isolated from patient no. 2 floated on, rather than migrated through, the medium used for density gradient separation. Hypodensity such as this is also seen in the PMNs of burn patients and is believed to result from partial degranulation, suggesting a stimulated state in vivo.

Results of cytotoxicity assays are shown in Fig 4. Optical density signal reflects viable melanoma cells, measured by MoAb R24 binding to target SK-MEL-29 melanoma cells.
A cytotoxic effect of PMNs from the healthy control was observed at effector to target cell (PMN:melanoma SK-MEL-29) ratios of 10:1 and 50:1 (Fig 4 g through i). A similar pattern of cytotoxicity emerged for all treated patients (data not shown), as illustrated by patient no. 4 (Fig 4 d through f). The PMNs from patient no. 2 showed intact cytotoxic capacity (Fig 4 a through c) despite their deficient generation of H$_2$O$_2$ (Fig 3 a through c).

DISCUSSION

Hemorrhagic tumor necrosis, typically associated with severe infections, is an example of selective tissue destruction mediated by inflammatory events, and formed a basis for the eventual identification of TNF. The inflammatory destruction of tumors by induction of TNF-α in animal models led to the isolation of TNF-α and the production of a recombinant pharmacologic molecule that could be used to induce inflammation in cancers. Recent trials of rTNF-α in patients with advanced cancer have shown its severe toxicity and indicated that further strategies will have to be developed if rTNF-α is to be used for human cancer therapy. One possibility is that other agents may synergize with rTNF-α to amplify inflammatory events at tumor sites. For instance, it has been suggested that the alkylating agent melphalan and interferon-γ augment the antitumor effects of high-dose rTNF-α when administered together by regional limb perfusion.

Many cellular and soluble components can participate in inflammatory responses that lead to tissue necrosis. We have been particularly interested in a role for PMNs and complement. One model for inflammatory tissue destruction is the local Shwartzman reaction, induced by a subcuteaneous injection of endotoxin followed 24 hours later by an intravenous challenge of endotoxin.$^{24}$ This sequence leads to necrosis of the prepared skin. The Shwartzman reaction does not occur in neutropenic animals, and histologic and experimental correlates suggest that PMN aggregation and adhesion to endothelium are crucial for tissue damage.$^{14,25}$ A Shwartzman-like reaction can be triggered by substituting intravenous zymosan, which activates complement, for the endotoxin challenge, and necrosis can be prevented by anti-C3 antisera or complement depletion by cobra venom factor.$^{25-27}$ Indeed, generation of the complement component C5a plays a central role in hemorrhagic necrosis induced by TNF-α.$^{28}$ PMN aggregation mediated by complement activation has been proposed to produce tis-

![Fig 2. Histology of hemorrhagic tumor necrosis of liver in patient no. 2. (A) Low power photomicrograph (× 100) of a representative metastatic nodule shows intact hepatic parenchyma at the upper left and a mass of necrotic debris at the lower right that is bounded by a cuff of melanophages. (B) Higher magnification (× 400) of (A). (C) Fibrinoid necrosis of a blood vessel found adjacent to one of the necrotic tumor nodules in this patient's liver. All photomicrographs are hematoxylin-eosin-stained sections.](image-url)
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Fig 3. PMN H2O2 release. H2O2 production from the PMNs of two patients and a normal donor (control) after in vitro stimulation with PMA, fMLP, or rTNF-α. PMNs were obtained at baseline, after in vivo treatment with MoAb R24 alone, and after in vivo treatment with rTNF-α. (a through c) Patient no. 2, who developed tumor lysis syndrome; (d through f) patient no. 4, a representative patient (who did not develop tumor lysis syndrome); and (g through i) a healthy control, whose PMNs were isolated concurrently with those of patient no. 2.

Tissue damage in pathologies as diverse as acute myocardial infarction and acute respiratory distress syndrome. Complement-fixing MoAb can induce localization of neutrophils at tumor sites. These considerations raised the possibility of harnessing the cytotoxic potential of PMNs to destroy tumor through direct damage of tumor or through destruction of tumor vasculature. PMNs circulate in numbers that are considerably greater than other potential effector cells, including T lymphocytes, monocytes, or natural killer cells. PMNs secrete potent and defined cytotoxic molecules, including reactive oxygen intermediates, proteases, and cationic proteins in response to defined stimuli. Low concentrations of TNF-α trigger PMNs to secrete reactive oxygen intermediates in vitro, but only if PMNs are adherent to proteins of extracellular matrix or to endothelial cells. Thus, low doses of TNF-α, which are achievable in vivo without serious toxicity, may trigger selectively those PMNs that are adherent to tumor endothelium, are in the process of emigrating, or have emigrated from vessels into tumor. Vascular endothelium is especially sensitive to the cytotoxic effects of PMNs, particularly the release of reactive oxygen intermediates. However, we do not know if PMNs contributed to the tumor lysis syndrome reported here.

MoAb R24 binds to the disialoganglioside GD3, which is highly expressed on melanoma cells and expressed at low levels on normal melanocytes and other neuroectoderm-derived cells. Malignant transformation markedly increases the expression of GD3 on melanocytes. MoAb R24 mediates antibody-dependent cellular cytotoxicity and activates human complement. By itself, MoAb R24 can produce antitumor responses; in an initial clinical trial, 4 of 20 patients experienced major responses. One responding patient developed necrosis of extensive subcutaneous melanoma metastases during several days of treatment and a second patient had marked hemorrhagic necrosis in a biopsy of a responding subcutaneous lesion. Complement activation plays a crucial role in the antitumor effects of MoAb R24 against human melanoma xenografts growing in nu/nu mice. Thus, treatment with complement-fixing MoAb alone may be able to induce inflammatory responses in tumors of some patients with metastatic melanoma. During this trial, we observed an extraordinary case of massive tumor lysis in a patient with extensive visceral metastases of melanoma. A similar septic shock-like picture has been recently reported after systemic endotoxin administration. To our knowledge, this is the first report of documented tumor lysis syndrome in a patient with metastatic melanoma and the first report of this syndrome after systemic treatment with TNF-α or MoAb. In fact, tumor lysis syndrome in patients with solid tumors has been rarely observed. Pathologic and histologic evaluation of tumor and tissues from patient no. 2 confirmed that necrosis was restricted to tumor masses. This observation is all the more
remarkable because necrosis occurred in visceral sites, including liver, lung, spleen, kidney, heart, bone marrow, and adrenal glands. Animal models have suggested that TNF-α induces necrosis in subcutaneous tumors, but hemorrhagic tumor necrosis has not been typically evident in visceral sites in experimental models.

Why only one patient had such an intense response to treatment is unclear. In vitro white blood cell studies showed a blunted PMN response of patient no. 2 to all stimuli tested before treatment. The PMNs from the other five patients responded normally, and only their PMNs drawn immediately after TNF-α exhibited a blunted response to TNF-α. These findings suggest that PMNs from patient no. 2 were in a stimulated state before treatment.

An underlying condition unique to patient no. 2 was an apparent functional adrenal insufficiency. The patient had taken replacement steroids for a resected pituitary adenoma, but had discontinued them for 3 weeks before treatment. Autopsy results showed bilateral adrenal atrophy. After adrenalectomy, mice are sensitized to a lethal response to endotoxin. Likewise, pretreatment with cortisone has sensitized rabbits to develop a local Shwartzman reaction after a single intradermal injection of bacterial toxin. These results and our observations in patient no. 2 may provide clues to the variability of antitumor responses in the patients treated by Coley and others, and could provide the basis for strategies to augment hemorrhagic tumor necrosis after administration of αTNF.

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