Anaphylaxis caused by repetitive doses of a GITR agonist monoclonal antibody in mice

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Key Points

- Repeated doses of agonist antibodies targeting the costimulatory receptors GITR and OX40 result in anaphylaxis in mice.
- Anaphylaxis caused by the GITR agonist antibody DTA-1 is dependent on GITR, IL-4, basophils, and platelet-activating factor.

Immunotherapy for cancer using antibodies to enhance T-cell function has been successful in recent clinical trials. Many molecules that improve activation and effector function of T cells have been investigated as potential new targets for immunomodulatory antibodies, including the tumor necrosis factor receptor superfamily members GITR and OX40. Antibodies engaging GITR or OX40 result in significant tumor protection in preclinical models. In this study, we observed that the GITR agonist antibody DTA-1 causes anaphylaxis in mice upon repeated intraperitoneal dosing. DTA-1–induced anaphylaxis requires GITR, CD4+ T cells, B cells, and interleukin-4. Transfer of serum antibodies from DTA-1–treated mice, which contain high levels of DTA-1–specific immunoglobulin G1 (IgG1), can induce anaphylaxis in naïve mice upon administration of an additional dose of DTA-1, suggesting that anaphylaxis results from anti-DTA-1 antibodies. Depletion of basophils and blockade of platelet-activating factor, the key components of the IgG1 pathway of anaphylaxis, rescues the mice from DTA-1–induced anaphylaxis. These results demonstrate a previously undescribed lethal side effect of repetitive doses of an agonist immunomodulatory antibody as well as insight into the mechanism of toxicity, which may offer a means of preventing adverse effects in future clinical trials using anti-GITR or other agonist antibodies as immunotherapies. (Blood. 2014;123(14):2172-2180)

Introduction

Immune modulation using monoclonal antibodies has a significant impact on the overall survival of patients with cancer, based on the results of clinical trials using antibodies to block CTLA-4 and PD-1.1-4 In an approach that differs from using antibodies to mitigate immune checkpoint, agonist monoclonal antibodies can be used to directly stimulate T-cell function. Antibodies that engage members of the tumor necrosis factor receptor (TNFR) superfamily have shown promising tumor protection in preclinical models.3,7-13 Glucocorticoid-induced TNFR-related (GITR) is a costimulatory receptor in the TNFR superfamily with high homology to the other TNFR superfamily members OX40, 4-1BB, and CD27.14 GITR and OX40 are expressed primarily on activated CD4+ and CD8+ T cells as well as on CD4+Foxp3+ regulatory T cells (Tregs).15,16 Engagement of GITR and OX40 through agonist monoclonal antibodies results in increased T-cell activation, cytokine secretion, proliferation, and survival.17-21 We and others have shown that the GITR agonist antibody DTA-1 and the OX40 agonist antibody OX86 are very effective antitumor therapies in murine tumor models by increasing antitumor CD4+ and CD8+ T-cell effector function as well as destabilizing and causing apoptosis of Tregs in the tumor microenvironment.7,24-32 Additionally, B cells are required for DTA-1–mediated protection from certain tumor models, indicating a humoral component to the antitumor effects of DTA-1.33

Although antibodies targeting costimulatory pathways have shown unquestionable potential in preclinical models, clinical trials using a CD28 superagonist antibody and preclinical experiments using a 4-1BB agonist antibody have had severe adverse immune-mediated side effects.34,35 This indicates that agonist monoclonal antibodies must be treated with great caution, and potential side effects should be investigated comprehensively. In this study, we show that engagement of the TNFR superfamily members GITR and OX40 with repetitive intraperitoneal doses of the agonist antibodies DTA-1 and OX86, respectively, causes anaphylaxis in mice. Anaphylaxis induced by repetitive doses of DTA-1 is caused by serum antibodies and is dependent on CD4+ T cells, B cells, basophils, platelet-activating factor (PAF), and GITR. We suggest a mechanism in which anaphylaxis results from generation of anaphylactic anti-DTA-1 antibodies. Anaphylaxis caused by DTA-1 can be reduced or prevented by an antibody that neutralizes interleukin-4 (IL-4), a PAF antagonist, or a basophil-depleting...
antibody. These results suggest that anaphylactic antidrug antibody generation may be of particular concern when using agonist antibodies targeting GITR and OX40.

Methods

Mice and tumor cell lines
All mouse procedures were performed in accordance with Institutional Animal Care and Use Committee protocol guidelines at Memorial Sloan-Kettering Cancer Center (MSKCC) under an approved protocol. Veterinary care was given to any animals requiring medical attention. C57BL/6 and Kitw/Kitw−/− mice were obtained from the Jackson Laboratory. Major histocompatibility complex (MHC) class I−deficient (strain B2MN12) and MHC class II−deficient (strain ABBN12) were obtained from Taconic. GITR−/− and littermate controls (Sv129 × C57BL/6 background)26 were a gift from Dr. P. P. Pandalit (MSKCC, New York, NY) and were backcrossed >10 generations onto C57BL/6 background by using a speed congenic system.27 Mice with the µMT mutation were purchased from the Jackson Laboratory and backcrossed >10 generations onto C57BL/6 background and bred at MSKCC. The B16-F10 mouse melanoma line was originally obtained from L. Fidler (MD Anderson Cancer Center, Houston, TX). In therapeutic tumor protection experiments, mice were challenged with 0.75 to 1.0 × 106 B16-F10 cells injected intradermally into the flank (50 microliters per injection) and monitored every 2 to 3 days for 80 days.

Monoclonal antibodies and drug treatments
DTA-1 (anti-GITR), OX86 (anti-OX40), FKG45.5 (anti-CD40), GK1.5 (anti-CD4), 11B11 (anti-IL-4), and TA99 (anti-Tyrp-1) were produced and purified by the Monoclonal Antibody Core Facility at MSKCC. LOB12.3 (anti-4−1BB), 1A8 (anti-Ly6G), LTF-2 (rat immunoglobulin G2b [IgG2b] isotype control), and HRPN (rat IgG1 isotype control) were purchased from Bio X Cell. Murinized DTA-1 (mDTA-1) was obtained from Merck Research Biosciences. Plates were read with a SpectraMax 340PC (Molecular Devices) using plates coated with either DTA-1 or LTF-2 in PBS and biotinylated rat antimouse IgG1 (BD Biosciences) as a secondary antibody. The SAv-HRP enzyme reagent and the OptEIA detection reagent were purchased from BD Biosciences. Plates were read with a SpectraMax 340PC (Molecular Devices) at 650 nm.

Statistical analysis
Statistical differences between experimental groups were determined by using the unpaired 2-tailed Student t test and GraphPad Prism software.

Results

Repetitive intraperitoneal doses of DTA-1 cause anaphylaxis in a GITR-dependent manner
We and others have previously shown that DTA-1 is a very potent immunotherapy when used as a single agent.24−27,38,39 A single dose of DTA-1 protects up to 60% of mice bearing established tumors.25 To improve on the antitumor effects of DTA-1, we administered multiple doses of DTA-1 in combination with a tumor antigen−specific antibody (TA99) to mice bearing established tumors. Although the combination therapy of TA99 and DTA-1 provided considerable protection from tumor challenge, we observed that about 20% of the mice succumbed to a treatment-related toxicity (supplemental Figure 1). Upon monitoring mice immediately following DTA-1 injections, we observed that most of the mice became ill-appearing and lethargic by 20 minutes after the final dose of DTA-1. Additionally, we observed erythema of the footpads and piloerection in all affected animals, as well as very low blood volume when the affected mice were euthanized. These effects were observed only after the final dose and were not observed after the first or second doses of DTA-1. In addition, the adverse effects of DTA-1 were independent of tumor challenge and TA99 treatment. The symptoms and time course were suggestive of anaphylaxis,40−42 which we confirmed by examining body temperature after each dose of DTA-1. Mice experienced a rapid and severe drop in body temperature following the third intraperitoneal dose of DTA-1 (Figure 1A and supplemental Figure 2A). mDTA-1 caused the same anaphylactic symptoms as DTA-1, demonstrating that the foreign nature of DTA-1 is not the cause of anaphylaxis (Figure 1B and supplemental Figure 2B). mDTA-1 caused more severe anaphylaxis symptoms when administered at lower (75 µg) doses by intravenous injection into the tail vein (supplemental Figure 3), but neither DTA-1 nor mDTA-1 caused anaphylaxis when administered subcutaneously. Three doses of isotype control antibody (rat IgG2b) did not cause symptoms of anaphylaxis (Figure 1C).

We found that GITR−/− mice were protected from DTA-1−induced anaphylaxis (Figure 1D). GITR−/− mice showed symptoms of severe
anaphylaxis in an anaphylaxis model caused by ovalbumin, indicating that protection of GITR−/− mice from DTA-1–induced anaphylaxis is not a result that can be generalized to all anaphylaxis models (Figure 1E). These results show that GITR expression is critical for DTA-1–induced anaphylaxis.

Anaphylaxis is caused by repetitive dosing of antibodies targeting GITR and OX40

To determine whether anaphylaxis caused by DTA-1 was a general side effect of repetitive dosing of antibodies targeting the TNFR superfamily of costimulatory receptors, we treated mice with agonist antibodies to OX40 (clone OX86), 4-1BB, and CD40. We observed that mice treated with repetitive intraperitoneal doses of OX86, but not anti-4-1BB or anti-CD40, underwent anaphylaxis (Figure 2 and supplemental Figure 4). Anaphylaxis caused by OX86 was comparable in incidence and severity to anaphylaxis caused by both antibodies is similar. We used DTA-1 to study the mechanism of anaphylaxis in the subsequent experiments.

CD4+ T cells, B cells, and IL-4 are required for DTA-1–induced anaphylaxis

Because GITR is expressed most highly on T cells, we were particularly interested in whether T cells are involved in anaphylaxis caused by repetitive dosing of DTA-1. We observed that both RAG−/− and TCRβ−/− mice were protected from DTA-1–induced anaphylaxis (Figure 3A-B). We found that MHC class I–deficient mice were not protected from DTA-1–induced anaphylaxis, indicating that CD8+ T cells do not play a major role in anaphylaxis caused by DTA-1 (Figure 3C). Conversely, mice that were depleted of CD4+ T cells (supplemental Figure 5) as well as MHC class II–deficient mice (Figure 3D) were protected from DTA-1–induced anaphylaxis, suggesting a role for CD4+ T cells in anaphylaxis caused by...
DTA-1. Although GITR is expressed at much lower levels on B cells than on CD4⁺ T cells (supplemental Figure 6), we also examined whether B cells were required for DTA-1–induced anaphylaxis, because GITR agonist antibodies have recently been shown to have potent humoral adjuvant effects.33,43 We found that μMT mice, which lack mature B cells, were protected from anaphylaxis caused by DTA-1 (Figure 3E). Because CD4⁺ T cells typically do not secrete vasoactive mediators such as histamine that are important for the pathology of anaphylaxis, the role of CD4⁺ T cells in DTA-1–induced anaphylaxis is most likely to provide helper signals to B cells to facilitate an anaphylactic antibody response. One such signal may be IL-4, a critical cytokine for anaphylaxis as well as other Th2-type allergic responses.44,45 We observed that neutralizing IL-4 considerably reduced anaphylaxis severity (Figure 3F).

Anaphylactic activity can be transferred via serum antibodies

The findings that CD4⁺ T cells and B cells were required for DTA-1–induced anaphylaxis suggested that an antibody response is the cause of the observed symptoms. Indeed, the difference between anaphylaxis and other types of shock such as sepsis is the involvement of antibodies, which trigger release of inflammatory mediators from innate immune cells through Fc receptors.46 To further elucidate the mechanism underlying anaphylaxis induced by repetitive dosing of DTA-1, we attempted to transfer the anaphylactic activity from one cohort of mice to another through either cells or serum. Mice were treated with 2 doses of DTA-1 administered 4 days apart. Six days after the second dose of DTA-1, the mice were euthanized, and cells from lymphoid organs or pooled sera were transferred into naive animals following the schema in Figure 4A. No toxicity was observed immediately following transfer of cells or serum. Three hours after the transfer, all mice were treated with a single dose of DTA-1. The mice that had received the serum fraction displayed symptoms of anaphylaxis upon DTA-1 administration, whereas the mice that had received cells did not (Figure 4B–C). Additionally, control mice that had received sera from IgG-treated mice did not show signs of anaphylaxis upon an additional dose of DTA-1 (Figure 4D).

Although the sera transferred from DTA-1–treated mice most likely contained DTA-1, we do not believe that the transferred DTA-1 was responsible for anaphylaxis, because naive mice that were treated with 2 doses of DTA-1 (3 hours apart) did not undergo anaphylaxis (supplemental Figure 7). Therefore, DTA-1 induces production of a factor present in the serum that can elicit anaphylaxis in mice upon an additional dose of DTA-1. Serum from DTA-1–treated wild-type mice could also induce anaphylaxis upon an additional dose of DTA-1 in GITR⁻/⁻ mice (Figure 4E), indicating that although GITR is required for anaphylaxis caused by repeated doses of DTA-1 (Figure 1D), GITR triggering is not required for the actual symptoms of anaphylaxis observed upon the final dose.

To determine which component in the serum of DTA-1–treated mice is responsible for rendering naive mice susceptible to DTA-1–induced anaphylaxis, we fractionated sera from DTA-1–treated mice over a protein A/G column into a fraction containing only serum antibodies and a fraction from which antibodies had been depleted (termed “flowthrough” fraction). We observed that transfer of the antibody fraction, but not the flowthrough fraction, from DTA-1–treated mice into naive mice resulted in anaphylaxis when mice were treated with a single dose of DTA-1 (3 hours later) (Figure 4F–G). As mentioned above, although DTA-1 is likely to be present in the antibody fraction we purified, we do not believe that the DTA-1 transferred in the antibody fraction is responsible for transferring the susceptibility to anaphylaxis. Rather, taking into account the humoral adjuvant properties of DTA-1, we believe that the mice are making an anaphylactic antibody response to DTA-1 itself, and that the final intraperitoneal bolus injection of DTA-1 forms immune complexes with anti–DTA-1 antibodies and triggers release of inflammatory mediators that cause vasodilation, leading to the observed drop in body temperature and other anaphylactic symptoms in the affected mice.

Repetitive dosing of DTA-1 induces anaphylaxis through the IgG1-basophil-PAF pathway

It has become apparent that 2 pathways of anaphylaxis exist in mice: the best characterized is caused by antigen crosslinking of IgE on
mast cells followed by release of histamine,47–50 while the other pathway is mediated by IgG1 and basophil or neutrophil release of PAF.51–56 We observed that mast cell–deficient Kit−/−Kitw−/− mice were not protected from anaphylaxis caused by DTA-1 (Figure 5A). This led us to investigate the IgG1- and PAF-mediated pathway of anaphylaxis. Treatment with the PAF inhibitor CV6209 30 minutes before the final dose of DTA-1 resulted in decreased anaphylaxis severity, whereas the histamine inhibitor triprolidine had no effect on anaphylaxis caused by DTA-1 (Figure 5B), suggesting that PAF-secreting cells mediate anaphylaxis in our model. Depletion of basophils, but not neutrophils, 24 hours before the final dose of DTA-1 resulted in protection from anaphylaxis (Figure 5C), supporting the hypothesis stated above that DTA-1–induced anaphylaxis results from an anti-DTA-1 anaphylactic antibody response.

**Inhibiting anaphylaxis does not reduce the efficacy of DTA-1 as a tumor therapy**

We next investigated whether anaphylaxis caused by repetitive dosing of DTA-1 could be prevented while preserving the antitumor activity of DTA-1. Mice were challenged with B16 melanoma and treated with the combination therapy of DTA-1 and the tumor antigen–specific antibody TA99 (supplemental Figure 1). As in Figure 5, PAF was inhibited 30 minutes prior to the final dose of DTA-1. Blocking PAF protected tumor-bearing mice from anaphylaxis, as observed in non–tumor-bearing mice (Figures 5B and 6A). Additionally, protection from anaphylaxis did not adversely affect the tumor protection resulting from TA99 plus DTA-1 therapy (Figure 6B). These results suggest that DTA-1–induced anaphylaxis can be realistically mitigated in therapeutic settings.

**Discussion**

Preclinical models have demonstrated that stimulation of the GITR and OX40 pathways using DTA-1 or OX86 can result in striking immune modulation, leading to tumor protection and induction of autoimmunity. In this report, we show that repetitive doses of the GITR agonist antibody DTA-1 cause anaphylaxis in mice, which is mediated by IgG1, basophils, and PAF. We observed similar effects using the OX40 agonist antibody OX86, suggesting that antibodies engaging members of the TNFR superfamily may be particularly prone to anaphylaxis upon repeated dosing. Interestingly, GITR was critically required for anaphylaxis caused by repetitive dosing of DTA-1, but not for anaphylaxis caused by a single dose of DTA-1 after transfer of serum antibodies from previously dosed mice. This strongly suggests that costimulation of T cells through GITR drives anaphylactic sensitization. The critical mechanistic role for GITR in DTA-1–mediated anaphylaxis is strongly supported by the observation that GITR−/− mice were protected from DTA-1–induced, but not ovalbumin-induced, anaphylaxis. DTA-1–induced anaphylaxis was mediated by serum antibodies generated after DTA-1 injection, and because no additional foreign antigens were injected into the mice, the data suggest that anti-DTA-1 antibodies are responsible for the rapid symptoms observed after the final dose of DTA-1.
Therefore, it appears that engaging GITR using DTA-1 enhances the immunogenicity of DTA-1 itself, leading to anaphylaxis upon repetitive dosing.

The observation that the OX40 agonist antibody OX86 also caused anaphylaxis whereas the 4-1BB agonist antibody did not cause any anaphylactic symptoms supports further examination of the similarities between OX40 and GITR in signaling and expression. Interestingly, Tregs express very high levels of GITR and OX40, but not 4-1BB; this expression pattern may explain the more potent antibody responses caused by agonizing GITR and OX40, especially in light of recent findings that Tregs are involved in control of the germinal center response and that depletion of Tregs can enhance anaphylaxis.57-63

An important question is whether anaphylaxis caused by repetitive intraperitoneal dosing of DTA-1 and OX86 in mice is translatable to humans treated with humanized versions of these antibodies. Our preclinical observations will heighten awareness that anaphylaxis may be of particular concern when using antibodies targeting GITR and OX40. Indeed, anaphylaxis caused by antidrug antibodies has been observed in patients retreated with OKT3 (mouse anti-CD3) and basiliximab (chimeric anti-CD25).64-66 Careful immune monitoring of sera from these patients may have detected high levels of antidrug IgE antibodies and could have precluded these patients from receiving another dose of antibody. Although the foreign and chimeric natures of OKT3 and basiliximab, respectively, were considered the primary reasons for anaphylaxis upon retreatment, it is interesting that both OKT3 and basiliximab target and modulate the function of T lymphocytes. Together with our data showing anaphylaxis upon retreatment with DTA-1 and OX86 in mice, this suggests that antibodies targeting T cells may be particularly susceptible to anaphylactic sensitization. Our results indicate that caution should be exercised when administering multiple doses of anti-GITR or anti-OX40 to patients, especially if high levels of antidrug IgE or IgG1 antibodies are observed after the first dose. Of additional concern when translating anti-GITR to the clinic is our observation that

Figure 5. Anaphylaxis caused by DTA-1 is mediated by PAF, basophils, and IgG1 antibodies. (A) C57BL/6J or Kitw/Kitw−/− mice were treated with 1 mg DTA-1 on days 0, 4, and 11. Rectal temperatures of individual mice were monitored for 1 hour following the final injection, represented by individual lines in the graphs. Data are representative of 2 independent experiments (n = 5 mice per group). (B) C57BL/6J mice were treated with 1 mg DTA-1 on days 0, 4, and 11. Thirty minutes prior to the final DTA-1 injection, mice were injected intraperitoneally with either 125 μg of the histamine inhibitor triprolidine or 125 μg of the PAF inhibitor CV6209. Rectal temperatures of individual mice were monitored for 1 hour following the final dose of DTA-1, represented by individual lines in the graphs. Data are representative of 3 independent experiments (n = 5 mice per group). (C) C57BL/6J mice were treated with 1 mg DTA-1 on days 0, 4, and 11. One day before the final dose of DTA-1, mice were either injected intravenously with 25 μg of anti-CD200R3 (clone Ba103) or intraperitoneally with 0.5 mg of anti-Ly6G (clone 1A8). Rectal temperatures of individual mice were monitored for 1 hour following the final dose of DTA-1, represented by individual lines in the graphs. Data are representative of 3 independent experiments (n = 5 mice per group). (D) Sera from DTA-1–treated mice were collected as in Figure 4A. The pooled sera were heat inactivated at 56°C for 3 hours. Heat-inactivated sera was then transferred by intravenous injection into the tail vein of recipient mice. Three hours later, a single dose of DTA-1 was administered, and rectal temperatures were monitored, represented by individual lines in the graphs. Data are representative of 2 independent experiments (n = 5 mice per group). (E) C57BL/6J mice were treated with DTA-1 or isotype control (rat IgG2b, clone LTF-2) on days 0 and 4. On day 11, sera were collected from individual mice and assayed by enzyme-linked immunosorbent assay for IgG1 antibodies recognizing DTA-1 or LTF-2. Data are representative of 3 independent experiments.
anaphylaxis was also observed in mice treated with mDTA-1. Also of note is that mDTA-1 and OX86 are different isotypes from rat DTA-1; therefore, the observations that mDTA-1 and OX86 cause anaphylaxis invalidates concerns that anaphylaxis caused by DTA-1 is an isotype-specific artifact. The finding that repetitive dosing of mDTA-1 causes anaphylaxis when dosed by intravenous bolus, but not when dosed subcutaneously, indicates that intravenous doses in the clinic should be delivered slowly. Regardless of the method of administration, however, administering a drug when high levels of antidrug antibodies are present will result in reduced bioavailability of the drug even if anaphylaxis does not occur, because the drug will be cleared from the bloodstream by antidrug antibodies. Given the high costs of monoclonal antibodies as therapies, administering an antibody to patients with high levels of antidrug antibodies should be avoided from an economic as well as a safety standpoint.

Additionally, several recent advances have been made in antibody engineering technology that enables the creation of less immunogenic antibodies. Algorithms have been developed to predict epitopes within a given protein sequence that will bind to MHC molecules, as well as to determine the structural consequences of mutations that will abrogate the immunogenic peptides. Epitopes specific for Treg cells (Tregitopes) have also been determined in conserved domains of Fc and F(ab) portions of antibodies in mice and humans. Activation of regulatory T cells through engagement with Tregitope-MHC complexes results in expansion and activation of regulatory T cells, along with enhancement of the immunosuppressive Treg functions such as IL-10 and transforming growth factor-β secretion. Deimmunization strategies and engineering to incorporate Treg epitopes may be of particular importance when designing antibodies targeting GITR and OX40 for clinical use.

In conclusion, we found that repetitive intraperitoneal bolus doses of DTA-1 cause anaphylaxis in a manner that requires GITR, CD4+ T cells, B cells, basophils, PAF, IL-4, and serum antibodies. These data are important because patients with cancer are currently being treated with anti-GITR and anti-OX40 in clinical trials, and anticipating immune-based side effects will result in safer trials and possibly expedite FDA approval for these and other immunotherapies. Understanding anaphylaxis caused by antidrug antibodies is particularly significant because it is a phenomenon that has been observed in patients re-treated with other antibodies. It is tempting to speculate that antibodies targeting T cells, especially Tregs, are more susceptible to anaphylactic sensitization than antibodies targeting tumor or stromal cells. It will be important to dose slowly and include quantitative monitoring of antidrug IgE and IgG1 antibodies in clinical trials using GITR agonist antibodies. Moreover, the availability of anti-PAF drugs in the clinic for use in cardiac rehabilitation suggests that inclusion of these drugs in future clinical trials to mitigate adverse immune-mediated events is a possibility.

Figure 6. Inhibition of PAF protects mice from anaphylaxis while maintaining tumor protection provided by DTA-1 as part of a combination therapy. C57BL/6J mice were challenged intradermally with 1 × 10⁶ B16 melanoma cells. Groups of mice were either left untreated (naive) or were treated with 200 μg TA99 on days 5 and 9 after tumor challenge, 1 mg DTA-1 on days 5, 9, and 16 after tumor challenge, or a combination of both TA99 and DTA-1 at the aforementioned doses and schedules. One group treated with TA99 plus DTA-1 received 125 μg CV6209 by intraperitoneal injection in PBS 30 minutes before the day 16 dose of DTA-1. (A) Rectal temperatures were monitored following the day 16 dose of DTA-1. (B) Tumor growth was measured every 2 to 3 days, represented by individual lines in the graphs. Mice were euthanized when tumor diameter reached 1 cm. Data are representative of 3 independent experiments (n = 15 mice per group). *P < .05, unpaired 2-tailed Student t test.
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Authorship


Conflict-of-interest disclosure: A.M.B., D.G., and L.G.P. are employees of Merck Research Laboratories. The remaining authors declare no competing financial interests.

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