Phase I Clinical Trial of Mixed Bacterial Vaccine (Coley's Toxins) in Patients with NY-ESO-1 Expressing Cancers: Immunological Effects and Clinical Activity

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Abstract

**Purpose:** Mixed bacterial vaccine (MBV, Coley's toxins) is a historical, vaguely defined preparation of heat-inactivated *Streptococcus pyogenes* and *Serratia marcescens* used as nonspecific immunotherapy in the treatment of cancer. The mechanism of action is suspected to have an immunologic basis, yet it is poorly defined up to now. We developed a new, biochemically well defined and current good manufacturing practice–compliant MBV preparation, which has been investigated in patients with NY-ESO-1 expressing cancers.

**Experimental Design:** Patients received MBV subcutaneously at a starting dose of 250 EU (endotoxin units) twice a week. The MBV dose was escalated in each patient until a body temperature of 38°C to 39.5°C was induced or up to the maximum dose of 547,000 EU. Changes in serum cytokine levels were determined and immune responses to NY-ESO-1 were evaluated. Tumor response was assessed according to RECIST.

**Results:** Twelve patients were enrolled and 11 of them developed fever after the administration of MBV. Ten of 12 patients showed a consistent increase in serum IL-6 levels with the highest levels coinciding with the highest body temperature. A subgroup of patients showed increasing levels of TNF-α, IFN-γ, and IL1-β. A patient with metastatic bladder cancer showed a partial tumor response strongly correlated with MBV-induced fever and highly elevated levels of several cytokines.

**Conclusions:** MBV at fever-inducing dose levels can lead to a massive induction of immunoregulatory cytokines that may be involved in inducing tumor regressions. We propose to further explore the role of MBV as a potent immune modulator at higher dose levels and in conjunction with antigen-specific cancer vaccines. *Clin Cancer Res;* 1–11. ©2012 AACR.

Introduction

Nonspecific immunotherapies with bacterial products have been used in the treatment of cancer for more than a century and have shown beneficial effects in multiple clinical settings. The prototype composition for this approach is Coley’s toxins, a mixture of heat-killed *Streptococcus pyogenes* and *Serratia marcescens* named after William B. Coley, who developed the mixture in the late 19th century as a treatment of cancer. This bacterial mixture was used in a large number of patients by Coley and colleagues (1–6). Coley observed that a strong fever reaction was the key indicative aspect of a successful intervention leading to tumor regression. However, a systematic investigation of the mechanisms involved in mediating tumor regression was not possible at the time. In a review of 186 cases of soft tissue sarcomas treated with Coley’s toxins, Helen Coley Nauts concluded that the toxins were most effective when given every 24 or 48 hours initially followed by less frequent further injections, when they caused fever of 38.9°C to 40°C, and if the toxins were injected into or in close vicinity to the tumor. In addition, beneficial effects were observed when the toxins were given more than an extended period of 6 to 12 months (7, 8). In the 1970s, Coley’s toxins were investigated again. The mixture of heat-killed *Streptococcus pyogenes* and *Serratia marcescens* used in these more recent clinical trials was called mixed bacterial vaccine (MBV) that was administered against different types of cancer with...
There is increasing evidence that nonspecific activation of the innate immune system may critically support the initiation of a functional specific immune response against cancer. On the basis of anecdotal reports on the therapeutic effects of Coley’s toxins, we developed a new, well defined and current good manufacturing practice–compliant bacterial preparation (mixed bacterial vaccine, MBV) that led to the production of immunoregulatory cytokines at fever-inducing dose levels in the treatment of 12 patients. The study also showed that MBV-induced fever and elevated levels of various cytokines may have mediated the tumor regression in a metastatic bladder cancer patient. One patient showed a partial tumor response; 6 patients showed prolonged overall survival. The novelty of our study is that the immunostimulatory effects of MBV were investigated systematically on the basis of a defined endotoxin quantity of the preparation. The immunomodulatory effects render MBV as a potent adjuvant that needs to be evaluated in combination with different strategies of cancer vaccines including prophylactic therapy for the establishment of a protective immune response.

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Translational Relevance

There is increasing evidence that nonspecific activation of the innate immune system may critically support the initiation of a functional specific immune response against cancer. On the basis of anecdotal reports on the therapeutic effects of Coley’s toxins, we developed a new, well defined and current good manufacturing practice–compliant bacterial preparation (mixed bacterial vaccine, MBV) that led to the production of immunoregulatory cytokines at fever-inducing dose levels in the treatment of 12 patients. The study also showed that MBV-induced fever and elevated levels of various cytokines may have mediated the tumor regression in a metastatic bladder cancer patient. One patient showed a partial tumor response; 6 patients showed prolonged overall survival. The novelty of our study is that the immunostimulatory effects of MBV were investigated systematically on the basis of a defined endotoxin quantity of the preparation. The immunomodulatory effects render MBV as a potent adjuvant that needs to be evaluated in combination with different strategies of cancer vaccines including prophylactic therapy for the establishment of a protective immune response.

Investigational agent

MBV was manufactured by Cobra Biomanufacturing Plc, and consists of 250,000 endotoxin units (EU)/mL of heat-killed Gram-positive Streptococcus pyogenes and Gram-negative Serratia marcescens in PBS containing 2% glycerol. Both strains were developed from nonpassaged, freshly isolated clinical virulent bacterial isolates from patients with a sepsis syndrome. The identity of the strains was confirmed by standard microbiology technologies and by 16sRNA sequencing. All manufacturing, formulation, and packaging of the investigational agent were done in accordance with applicable current Good Manufacturing Practices and met the applicability criteria for use in humans.

Patients, Materials, and Methods

Patients and treatment plan

This was a phase 1, open-label, multiple dosing, single-arm study according to study protocol LUD2005-003, which was approved by the Ludwig Institute for Cancer Research (New York, NY) as well as by the medical and ethical committees of Hessen, Germany. Twelve patients with NY-ESO-1 expressing tumors or NY-ESO-1 serum antibodies were enrolled to receive MBV subcutaneously at a starting dose of 250 EU twice a week. In the absence of a dose-limiting toxicity, the MBV dose was escalated in each patient to the MBV dose level that elicits a body temperature varying results (9–13). Although, single factors of the bacterial product were identified as active components, for example, lipopolysaccharide (LPS), a component in the cell wall of Gram-negative bacteria (14), the detailed mechanisms of action have not been identified yet. It is suggested, however, that MBV triggered the release of a broad range of fever-inducing substances, such as interleukins, TNF, and interferons. The recent renewed interest in MBV is prompted by 2 new basic developments. First, humoral and cellular immunity to cancer antigens, among them the so-called cancer testis-antigens, expressed by many solid tumors, can now be measured in greater detail. NY-ESO-1 is one of these antigens. It is highly immunogenic in cancer patients and has the capacity for inducing spontaneous antibody responses in as many as 50% of patients with NY-ESO-1–expressing tumors (15). Spontaneous antibody responses are frequently associated with concurrent NY-ESO-1–specific CD4 and CD8 T cells in cancer patients (16). Immunity to NY-ESO-1 has recently been described as a good prognostic biomarker to predict clinical responders to anti-CTLA4 immunotherapy (17). For this reason, we have selected anti–NY-ESO-1–directed immune responses as a surrogate marker to gauge for tumor antigen-specific immune responses. Second, the discovery of the human Toll-like receptor (TLR) gene family in the 1990s as receptors for pathogen-associated molecular patterns led to a better understanding of downstream cytokines that contribute to immunologic cancer recognition and rejection (18, 19). Unmethylated CpG, bacterial DNA, and other TLR agonists are likely to stimulate the innate immune system to initiate the production of a complex cascade of cytokines (20–22). These include IL-1β, TNF-α, and IL-6, which act as endogenous pyrogens that elevate the body temperature. TNF-α regulates the inflammatory response, induces apoptosis, and disrupts neovascularization of solid tumors. TNF-α also amplifies the innate immune response by inducing IL-12 production that provides a critical link between innate and adaptive immunity by driving type-1 responses through the induction of IFN-γ production by natural killer cells, type-1 helper cells, and cytotoxic T cells. IFN-γ plays potentially several important roles in the immune response to tumors including tumor immunosurveillance and direct antitumor activity. Thus, IL-6, TNF-α, IL-12, and IFN-γ, collectively represent cytokines that are critical at the various stages of the host response to bacterial products. Over the years, numerous versions of Coley’s toxins have been produced under different names (e.g., Vaccineurin, Novopyrexal, MBVax, etc.), but each of these products had a different specific formulation and a standard dosage or standardized route or time frames of administration have never been explored systematically. To address these issues, we have developed a new, well defined, and biochemically well-characterized current good manufacturing practice–compliant preparation of MBV, and have initiated a dose escalation phase I clinical study to determine the fever-inducing dose of MBV and to explore the immunologic and clinical effects following treatment with MBV. We focused on NY-ESO-1–specific immune responses and on serum cytokine induction for TNF-α, IFN-γ, IL-1β, and IL-6. We found that increased levels of cytokines strongly correlated with the increase of MBV–related body temperature and, in 1 patient, with favorable clinical outcome.
of 38°C to 39.5°C or up to the maximum dose level 8 (547,000 EU). If a fever of 39.5°C to 40°C was observed, the patient continued to receive MBV at the previous dose level for 4 additional doses. For patients who had not achieved the desired pyrogenic effect at dose level 8, no additional MBV was administered. The trial design schema is illustrated in Supplementary Fig. S1. On the basis of MBV pyrogenicity in rabbits and the assumption that endotoxins are equipotent in rabbits and in man, the initial MBV injection dose of 250 EU was considered acceptable for use in patients without undue risk of overwhelming endotoxin reactions. Toxicity was evaluated according to the National Cancer Institute CTCAE Scale (Version 3.0). Tumor response was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST).

Body temperature

Body temperature measurement was conducted before each MBV vaccination and every hour for 6 hours after each MBV injection using a tympanic thermometer. Patients were instructed to further monitor body temperature using a tympanic thermometer hourly on the day of vaccination up to 6 hours as an outpatient. Results were documented in a body temperature log.

NY-ESO-1 serum antibody

NY-ESO-1-specific antibodies were measured in the serum by ELISA analysis at baseline and every 4 weeks during MBV treatment as described in ref. (23).

Analysis of NY-ESO-1–specific T cells

For the analysis of CD8⁺ and CD4⁺ T cells in enzyme-linked immunospot (ELISPOT), purified CD8⁺ and CD4⁺ T cells were presensitized with NY-ESO-1 peptide–pulsed irradiated autologous peripheral blood mononuclear cells (PBMC) depleted of CD4 and CD8 T cells. Presensitized CD4⁺ and CD8⁺ effector cells were tested on days 10 to 14 by IFN-γ ELISPOT against NY-ESO-1 peptide–pulsed autologous antigen-presenting cells (Epstein-Barr virus–transformed B cells, T cell antigen-presenting cell or dendritic cells) as described in ref. 23. A response was considered positive if the number of spots in the peptide-exposed well was 2-fold or more higher than the number of spots in the unstimulated well, and if there was a minimum of 10 (after subtraction of background spots) peptide-specific spots/25,000 T cells or fewer cells if T-cell clones were used.

Analysis of cytokines

Serum samples were obtained 6 hours after each MBV injection, processed immediately and stored at −80°C for subsequent cytokine measurement by ELISA assays using multiplex technology from Meso Scale Discovery as described by the manufacturer.

Anti-MBV antibodies

MBV specific antibodies were measured in the serum by standard ELISA analysis using 1,250 EU per well MBV lysate as antigen coating.

Results

Patients

Twelve patients with NY-ESO-1–expressing tumors were entered into the clinical trial at Krankenhaus Nordwest, Frankfurt, Germany: 6 patients with melanoma; 2 with sarcoma; 2 with prostate cancer; 1 with head and neck cancer and 1 patient with bladder cancer. Two patients had resected disease at study entry. The patients presented with tumor stages III and IV. Seven patients (#2, 3, 4, 5, 7, 9, and 12) had received NY-ESO-1–specific immunotherapy before entering this study. All patients were clinically followed until progression or death (Table 1).

Toxicity

Treatment with MBV was considered safe. In all patients, MBV–related local and systemic reactions were mild to moderate. Three patients had no reactions at all. Five patients experienced local reactions, such as reddening, swelling, pain, or pruritus at the injection site. Four patients had systemic and local reactions, which were likely to be related to pyrogenicity and associated inflammation. Any side effects resolved within 1 to 5 days. No dose limiting toxicity and no treatment-related serious adverse events occurred. Inflammatory local reactions were seen predominantly after injection of dose level 6 and higher (Table 1).

Pyrogenicity of MBV

The pyrogenic dose levels varied among the patients between dose level 2 and dose level 8. Eleven of 12 patients developed fever ≥38°C, 8 of them at dose level 5 or higher. There were 3 patients in which fever was observed at dose level 8, 2 patients at dose level 7, 2 patients at dose level 6, and 1 patient at dose level 5. Three patients developed fever at dose level 3 and lower. Of those, patient #2 possibly suffered from a latent infection that might have caused fever unrelated to MBV. Patient #9 did not experience any fever, although the dose was escalated up to level 8. Fever was symptomatically managed with the antipyretic drug paracetamol when measured 38°C or higher. Therefore fever of 39°C or higher was seen just once in a single patient. Although, the treatment was continued at the pyrogenic dose level for 4 times, 5 patients (#1, 4, 6, 7, and 12) did not respond again with fever to the subsequent MBV administrations. Two patients (#10 and 11) had 1 more and 3 patients (#2, 3, and 5) had 2 more fever reactions at the pyrogenic dose level. One patient (#8) responded with fever after every subsequent MBV administration (Fig. 1).

In vitro immunostimulatory activity of MBV

To evaluate the immunostimulatory activity of our vaccine preparation in vitro, human PBMC were stimulated with various concentration of MBV and cytokine levels in supernatant including TNF-α, IFN-γ, IL-6, IL-12, IL-10 were measured by ELISA. As shown in Supplementary Fig. S2, MBV induced all 5 cytokines in a dose-dependent manner at a level comparable with or higher than that induced by phorbol 12-myristate 13-acetate (PMA) or LPS. We further tested activation of human monocye–derived dendritic
cells (mo-DC) by MBV. After stimulation with MBV, mo-DC upregulated surface MHCs (HLA-DR and HLA-ABC), costimulatory molecules (CD80 and CD86), and the DC-maturation marker CD83 (Supplementary Fig. S3A and S3B). In addition, MBV stimulation of mo-DC induced cytokine production including immunopotentiating IL-12, inflammatory TNF-α, and immunosuppressive IL-10 (Supplementary Fig. S3C).

In vivo cytokine activation by MBV treatment and correlation with body temperature

In addition to TNF-α, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, and IL-12, TNF-α levels rose in 5 patients (#1, 3, 4, 9, and 11) and dropped again in 3 of these patients (#3, 4, and 11). IFN-γ levels rose in 4 patients to a moderate level (#1, 2, 4, 10), and in 1 patient to a high level (#11). Levels for IL-1β were generally low but significantly rose in 2 patients (#9 and #11). Levels for IL-2 rose in 3 patients (#1, 4, and 11). Levels for IL-5 were low in all but 1 patient (#7) and did not increase during MBV treatment. Levels of IL-6 increased in 11 of 12 patients (#1, 2, 3, 4, 5, 6, 8, 9, 10, 11, and 12) and dropped again in 8 patients. Two of these patients (#2 and #4) showed baseline cytokine levels of IL-6 significantly higher as compared with normal endogenous levels. Levels for IL-8 and IL-10 rose in 2 patients (#4 and #11). IL-12 levels rose in 1 patient (#11). IL-13 levels decreased in 1 patient (#7), whereas all other patients did not respond with changes of IL-13 levels. Levels of granulocyte macrophage colony-stimulating factor, IL-4, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, and IL-12. TNF-α levels rose in 5 patients (#1, 3, 4, 9, and 11) and dropped again in 3 of these patients (#3, 4, and 11). IFN-γ levels rose in 4 patients to a moderate level (#1, 2, 4, 10), and in 1 patient to a high level (#11). Levels for IL-1β were generally low but significantly rose in 2 patients (#9 and #11). Levels for IL-2 rose in 3 patients (#1, 4, and 11). Levels for IL-5 were low in all but 1 patient (#7) and did not increase during MBV treatment. Levels of IL-6 increased in 11 of 12 patients (#1, 2, 3, 4, 5, 6, 8, 9, 10, 11, and 12) and dropped again in 8 patients. Two of these patients (#2 and #4) showed baseline cytokine levels of IL-6 significantly higher as compared with normal endogenous levels. Levels for IL-8 and IL-10 rose in 2 patients (#4 and #11). IL-12 levels rose in 1 patient (#11). IL-13 levels decreased in 1 patient (#7), whereas all other patients did not respond with changes of IL-13 levels. Levels of granulocyte macrophage colony-stimulating factor, IL-4,

![Figure 1](https://example.com-figure1.jpg)

**Table 1.** Patient characteristics, highest MBV dose level and summary of vaccine-related toxicity and NY-ESO-1 specific humoral and cellular immune responses

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Disease</th>
<th>Stage</th>
<th>Escalated dose level of MBV</th>
<th>Toxicity (grade)</th>
<th>NY-ESO-1 tumor status</th>
<th>Antibody&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CD4</th>
<th>CD8</th>
<th>CD8</th>
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<th>CD8</th>
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<td>L (0), S (0)</td>
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<td>+</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>PD</td>
</tr>
<tr>
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<td>IV</td>
<td>3</td>
<td>L (0), S (0)</td>
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<td>++</td>
<td>++</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>PD</td>
</tr>
<tr>
<td>3</td>
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<td>III</td>
<td>7</td>
<td>L (2), S (0)</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>5</td>
<td>Prostate cancer</td>
<td>IV</td>
<td>8</td>
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<td>+</td>
<td>+</td>
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<td>6</td>
<td>Prostate cancer</td>
<td>IV</td>
<td>3</td>
<td>L (2), S (0)</td>
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<td>++</td>
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<td>L (1), S (2)</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>L (2), S (0)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>PD</td>
</tr>
</tbody>
</table>

Abbreviations: L, local injection reaction including reddening, swelling, pain, pruritus; S, systemic toxicity including Flu-like syndrome, sweating, weakness, CRP elevation.

<sup>a</sup>Antibody reciprocal serum titer: +, <25,000; ++, 50–100,000; ++++, >100,000.

<sup>b</sup>Relapse 2 months after study termination.
and IFN-α were undetectable in all patients throughout the study. In summary, all but 1 patient (#8) responded to MBV with increasing levels of at least 2 cytokines. For IL-6, maximum increase and duration of the peak showed a direct correlation to the fever-inducing MBV dose level except for patient #9 who showed an increase of IL-6 at maximum MBV dose level 8 but did not develop fever of 38°C. Cytokine results of all patients are presented in Fig. 2. The most impressive changes in cytokine levels were observed in patient #11 who developed high serum levels of TNF-α, IFN-γ, IL-2, IL-6, IL-8, and IL-10 simultaneously after the injection of MBV at the pyrogenic dose level 8. Maximum cytokine values were 10- to 100-fold higher as compared with baseline values or individual and median reference ranges. Serum levels further increased with the subsequent administration and then rapidly decreased to baseline values during the following MBV administrations. Levels of cytokines IL-1β, IL-5, and IL-12 clearly became detectable with the increase of body temperature, but values did not reach very high levels. These results indicate that MBV treatment at a fever-inducing dose level can lead to a massive induction of immunoregulatory cytokines.

Humoral and cellular immunity to NY-ESO-1

NY-ESO-1 antibodies. Nine out of 12 patients (#1, 3, 5, 6, 7, 9, 10, 11, and 12) were baseline seropositive and remained seropositive during the course of MBV treatment. Changes in antibody titer were observed in 2 patients; patient #7 increased from baseline titer 1:12,800 to 1:100,000 at the end of study, and patient #10 decreased from baseline titer 1:100,000 to 1:25,000 at the end of study. One out of 3 baseline antibody-negative patients (#8) seroconverted late at the end of the study to a low titer of 1:400. NY-ESO-1 antibody results are shown in Table 1 and Fig. 3A. In patients #5, 7, 9, 10, and 11, follow-up sera were obtained 24, 9, 21, 5, and 12 months after the end of study, respectively. We observed an increase of NY-ESO-1 antibody titer in patient #9 from 1:1,600 to 1:200,000 21 months after MBV treatment. NY-ESO-1 antibody results are shown in Table 1 and Fig. 3A. In patients #5, 7, 9, 10, and 11, follow-up sera were obtained 24, 9, 21, 5, and 12 months after the end of study, respectively. We observed an increase of NY-ESO-1 antibody titer in patient #9 from 1:1,600 to 1:200,000 21 months after MBV treatment. One out of 3 baseline antibody-negative patients (#8) seroconverted late at the end of the study to a low titer of 1:400.

NY-ESO-1 epitopes recognized by CD4 and CD8 T cells were clustered in the known immunodominant NY-ESO-1 regions p81-110, p119-143, and p151-170. Following MBV treatment, changes in NY-ESO-1–specific epitope recognition were observed in 2 patients. Patient #5 developed a de novo CD8 T-cell response against p119-143 and patient #3 developed a de novo CD4 T-cell response against p151-170. Patients who had no baseline NY-ESO-1–specific T-cell reactivity remained negative during the study. A summary of CD4 and CD8 responses pre- and post-MBV treatment is presented in Table 1. Immunodominant NY-ESO-1 epitopes recognized by CD4+ and CD8+ T cells are shown in Fig. 3B. ELISPOT data of the entire patient cohort and all time points including background spots are presented in the Supplementary Table S1. These results indicate that treatment with MBV did not seem to impact on NY-ESO-1–specific immune responses during the course of MBV treatment.

MBV–specific antibodies

Because the pyrogenic effect of MBV at a certain dose level and the accompanying elevated cytokine levels were only transient, and additional administrations at the same dose level did not further influence the body temperature, we analyzed patients sera for antibody responses against MBV. Anti-MBV serum IgG antibodies developed in nearly all patients to varying levels. Strong MBV–specific antibody responses were found in patients #1, 5, 10, and 11. These patients received MBV at the maximum dose level of 547,000 EU (Fig. 4). MBV antibodies decreased to baseline values after completion of MBV treatment.

Tumor responses

Two patients (#9 and #10) had no evidence of disease at study entry. Patient #9 relapsed 2 months after study termination. Patient #10 entered the study in a status of resected lymph node metastases associated to an unknown primary melanoma and has remained free of disease until today. Ten of 12 patients had measurable disease at study entry and 8 of them progressed under MBV treatment. Six patients (#5, 7, 9, 10, 11, and 12) have more than average survival times and 4 of these patients (#5, 10, 11, and 12) are still alive with survival times of 547,000 EU (Fig. 4). MBV antibodies decreased to baseline values after completion of MBV treatment.

Discussion

In addition to modern antigen-specific antibody- and vaccine-based immunologic cancer therapies, “nonspecific” immunotherapies with bacterial products have also shown antitumor effects (24–26). Over the past 2 decades, immunologic research has broadened our understanding of how these bacterial products may stimulate the immune system,
Figure 2. Serum cytokine levels for TNF-α, IFN-γ, IL-1β, IL-2, IL-5, IL-6, IL-8, IL-10, IL-12, and IL-13 measured at baseline (b), 6 hours after each MBV administration and 4 weeks after the last MBV administration (post = p) in correlation with the increase of body temperature. Cytokine levels are shown on the left y-axis (blue bars) in pg/mL. Body temperatures are shown on the right y-axis (red lines) in °C. Related MBV dose levels are indicated on the x-axis. Cytokine levels under the detection limit are recorded as 0. Reference range in pg/mL: TNF-α: 1 to 8, IFN-γ: 0 to 30, IL-1β: 0.2 to 7, IL-2: 0.7 to 90, IL-5: 0.1 to 2, IL-6: 0.8 to 10, IL-8: 3 to 300, IL-10: 0.7 to 20, IL-12: 1 to 80, and IL-13: 1 to 45.
and how endogenous mediators, induced by nonspecific immune stimulation influence the immune defense (22, 27, 28). In previous studies with MBV, also known as Coley’s toxins, fever induction has been considered to be the key indicative aspect of successful treatment (29). However, there has been no systematic investigation of the mechanism by which MBV–induced fever would contribute to mediate tumor regression.

Therefore, a primary objective of our study was to identify the dose of standardized MBV that increased the body temperature from 38°C to 39.5°C. Eleven of 12 patients developed fever of ≥38°C at a given MBV dose level. The pyrogenic dose level varied among patients. Considering the fact that the pyrogenic dose level distribution in most of the patients (7 of 11) was within a 3-level span (3, 2, and 2 patients at dose levels 8, 7, and 6, respectively), a starting dose of MBV level 6, that is, 60,000 EU, may be determined for future trials using this approach. Because of the intervention with antipyrogenic drugs, fever was well managed and generally not increase more than 39°C. However, antipyretic therapy in this immunologic approach may be critical, as these preparations include, for example, inhibitors of the prostaglandin synthesis and glucocorticosteroids that might interfere with the efficacy of the treatment approach at the immunologic level (30, 31). Also, the short pulse of relatively low fever in the patients may have had an adverse impact on cytokine levels and clinical outcome. Repeated induction of fever over several days or at frequent intervals may be required to optimize the production and maintenance of effective cytokine levels.

![Figure 3. NY-ESO-1 specific antibody (A) and T-cell response (B) in all patients. NY-ESO-1 antibody titers did not change during MBV treatment, except in patient #7 whose titer increased from prestudy and #8 who seroconverted late to a reciprocal titer of 1:400, MAGE-A3 was used as an internal assay control. No antibody reactivity against MAGE-A3 protein was observed. NY-ESO-1 specific CD4 and CD8 T-cell responses generally remained unchanged during the study as shown by the recognition of NY-ESO-1 overlapping 20mer peptides at baseline (○) and after MBV treatment (●).](image-url)
As a second objective, the study evaluated the effects of MBV on changes in serum cytokine levels. As expected, we observed a wide inter- and intrapatient variability of cytokine levels about their baseline detectability and to their changes during MBV treatment. Notably, the evaluation of cytokine levels in cancer patients is complicated because of concomitant conditions and of disease-related situations. Also, a typical pretreatment cytokine profile has not yet been identified in cancer patients, and data about the behavior of cytokines during different treatment approaches are very few (32). In our study, the most remarkable effect of MBV on changes in cytokine levels was observed for IL-6. Eleven of 12 patients showed a consistent increase in serum levels of IL-6 with the highest levels coinciding with the MBV dose that elevated body temperature. These results clearly showed the efficacy of MBV to induce fever and cytokines in a dose-dependant manner. In a proportion of patients, significant increases in serum levels of TNF-α (5/12), IFN-γ (4/12), IL-1β (2/12), and IL-2 (5/12). Highest levels of these cytokines mostly appeared unrelated to the body temperature. Some patients showed baseline serum cytokine levels significantly higher as compared with normal endogenous levels. In particular, patient #1 had very high baseline levels of IL-5, IL-10, IL-12, and IL-13, and patient #11 had elevated levels of IL-13. During the course of MBV treatment, significant changes of baseline elevated cytokine levels were observed in patient #7, who responded with a consistent decrease of cytokines IL-5, IL-10, and IL-13. The impact of high baseline serum levels of the anti-inflammatory cytokines IL-10 and IL-13 was significant, because this patient was the only one who did not respond to MBV with the production of IL-6, although fever was developed. These findings suggest that the pattern of cytokines at baseline and during MBV treatment may be related to significant biologic differences most likely associated with the tumor disease and the ongoing immune responses in individual patients. In conclusion, the most remarkable cytokine response during MBV treatment was the increase of IL-6 in 11 of 12 patients. IL-6 is a multipotent cytokine engaged in numerous biologic activities (22), and it is also a potent mediator of fever and the acute-phase immune response. Along with TNF-α, these inflammatory signals are essential for the recruitment of tumor-infiltrating lymphocytes, as well as natural killer cells.

Although, the treatment was continued at the pyrogenic dose level for 4 times, most of the patients had only 1 more fever reaction and did not respond again with fever and cytokine production to subsequent MBV administrations. This could be because of the well-known property of endotoxin to induce tolerance to its own biologic effects (33–36) as well as to the release of anti-inflammatory cytokines, for example, IL-10 in patients #3, 4, 11, and 12, that inhibit TNF-α and IL-1β production in macrophages, and thus contribute to the control of the magnitude of proinflammatory responses. IL-10 also enhances B-cell proliferation and, along with other factors, may support the immune activation against the bacterial product itself leading to a pathogen-specific antibody response. In fact, several patients developed anti-MBV antibodies during treatment with MBV that decreased to baseline levels after MBV treatment was completed (Fig. 4). The MBV-directed antibodies might have had a neutralizing effect on further MBV administrations and may explain, among other aspects, such as endotoxin tolerance, the only transient effect of fever and cytokine induction. To overcome tolerance to the bacterial vaccine, increasing doses of MBV or variations of the bacterial species for MBV may be effective to repeatedly induce fever and cytokines at significant levels. In addition, as endotoxin tolerance has been shown to be reversible, patients who failed to respond to further MBV administrations may have a rest and then start again with less frequent further injections. In fact, preliminary results of patient #11 who received MBV treatment on the basis of a compassionate single-patient protocol after a 3-months break for 16 additional injections at dose level 8, responded again with fever and cytokine production (Supplementary Fig. S4).

Although, the MBV effects on fever and increased synthesis of several cytokines were transient, the short pulse of high systemic cytokine levels may have influenced the induction of an individual tumor response considerably. 
This might have been the case in patient #10 who has been experiencing a disease-free survival of 36, 4+ months, and patient #11 who responded to MBV treatment with a strong increase of a number of cytokines followed by regression of multiple tumor manifestations. The tumor response in this patient may be related to the MBV–induced production of cytokines, such as TNF-α, IFN-γ, IL-6, IL-2, and IL-12 (Fig. 5A and B).

About NY-ESO-1–specific immunity, we did not observe significant changes in humoral and cellular immune responses mediated by MBV treatment. Antibody responses against other cancer antigens, such as MAGE, SSX, CT7, CT10, or CT45-47 were observed in individual patients. Again, all responses were already present at prestudy and no significant changes in titers were observed during or after MBV treatment. However, follow-up blood samples need to be tested, as changes in humoral and cellular immune responses to tumor antigens that might have been initiated during the phase of massive cytokine production may become detectable at a later time after successful translation of the innate response into the adaptive immune response (37). Patient #11 who had preexisting NY-ESO-1–specific antibody and T-cell response showed a partial tumor response after MBV treatment suggesting that MBV possibly broke tolerance and stimulated functional effector cells (38). It is of interest to note that this was the only patient who showed elevated levels of IL-12, a key cytokine augmenting the function of preexisting tumor-specific T cells capable of inducing tumor rejection (39).
In conclusion, this study has shown that MBV treatment at fever-inducing dose levels can lead to a massive induction of immunoregulatory cytokines that may have supported tumor regression mediated by immunologic mechanisms in a patient with advanced bladder cancer. In 6 of 12 patients, a prolonged overall survival was observed without any other treatment correlation. However, because of the small number of patients, the preliminary data on clinical outcome have to be taken with caution and warrant confirmation in a larger phase II clinical study. The individual immune response to MBV seems to be the result of dose, timing, potential additive, or synergistic effects, and genetically determined responsiveness. Our results indicate that depending on individual conditions of cancer patients, the restoration of basic regulatory mechanisms through MBV treatment may be important to introduce an effective antitumor response. In this context, intratumoral MBV administration may "induce a danger signal" and produce a more proinflammatory environment balancing immunosuppressive signals including regulatory T cells. Further studies will have to show whether MBV treatment given at higher doses and over extended periods of time will correlate to antitumor immune responses in larger patient populations. The immunomodulatory effects render MBV as a potent adjuvant that may benefit from the combination with different strategies to treat cancer, including cancer vaccines.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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