CRM197 and cancer: effects of intratumoral administration

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Background: Cross-reacting material 197 is a nontoxic mutant of diphtheria toxin that cross-reacts immunologically with the native counterpart. The molecule was recently tested as a systemic anticancer agent with promising results but its exact mechanism of action has remained unclear. Objective: The main goal of this study was to investigate this mechanism through the possible histologic changes induced by direct inoculation of the molecule into a tumor lesion. A secondary objective was to evaluate toxic, hematologic and antitumor effects of the procedure. Methods: Cross-reacting material 197 was administered by injection into accessible lesions of 12 patients with advanced cancer refractory to standard therapies. Two different doses were used according to the maximal diameter of the selected lesions. Lesions up to 2.5 cm were injected with 0.017 mg/day contained in 0.33 ml of a dilution buffer. Lesions up to 5.0 cm were injected with 0.051 mg/day contained in 1.0 ml of dilution buffer. The treatment schedule consisted of six injections administered on alternate days. A biopsy was taken from each selected lesion before the start of treatment and again 15 days later. Results: Toxicities and effects of cross-reacting material 197 on the injected lesion varied according to the patients’ degree of reactivity to diphtheria toxin. Patients with poor reactivity experienced negligible toxicity and no change or minimal change in the number of circulating neutrophils, in the post-treatment histology of their lesions and in the final lesion size. Patients with strong reactivity experienced mild-to-moderate flu-like syndrome, a prominent increase in the number of circulating neutrophils and a dense neutrophilic infiltration of the injected lesion leading to inflammation and, sometimes, to suppuration. Shrinkage of the index lesion ranging from 60 to 100% was observed in three of the patients. Conclusion: Neutrophils seemed to play a crucial role in achieving these results. One patient was available for post-treatment biopsy only at day 30 from the start of treatment. Examination of these specimens evidenced a striking granulomatous reaction surrounding a core of dead and viable tumor cells.

The aim of immunotherapy in cancer is the stimulation of a host antitumor reaction, either humoral or cellular. This goal can be pursued actively, by means of biological response modifiers (BRMs) to generate an immune response to malignant cells. Since the 1960s, several BRMs have been proposed, such as bacille Calmette–Guérin (BCG), interferons (IFNs), tumor necrosis factor (TNF), monoclonal antibodies (mAbs) and interleukins (ILs). Even diphtheria toxin (DT) might be included in this group of products. In fact, this powerful inhibitor of protein synthesis was tested as an anticancer agent many years ago with some success [1–4]. However, while the product had been tested in the hope that its toxicity exerted an antitumor effect, unexpectedly, the following observations suggested that its immunological properties also had some antiproliferative activity. First, mice surviving an intraperitoneal transplantation of tumor cells after a DT treatment rejected a new transplantation of the same cells [3]. Second, a woman treated with DT for an inoperable gastric cancer had striking tumor regression and a post-treatment dense infiltration of her adenocarcinoma by mononuclear cells and giant cells of the foreign body type [2]. Third, the majority of responders to DT treatment demonstrated the maximal reactivity of humoral immunity and delayed-type hypersensitivity (DTH) [4]. Currently, cross-reacting material 197 (CRM197), a mutant of DT that cross-reacts immunologically with the toxin [5], may represent an ideal BRM. It is completely nontoxic, shares the strong immunogenicity of the native molecule and has a unique ability to link heparin-binding epidermal growth factor (HB-EGF), the specific cell membrane receptor for...
DT. This receptor is expressed in a wide range of cell types, however it is often overexpressed in cancer [6]. Therefore, it is possible that the administration of CRM197 to cancer patients results in facilitated binding of the mutant toxin to cancer cells. It is also conceivable that the linkage of such a foreign and immunogenic protein with cancer cells renders them heterogeneous and capable of eliciting an immune response.

Based on the immunologic phenomena observed previously after a cancer treatment with DT [2–4] and on the above speculations, in the last few years this group administered CRM197 subcutaneously to advanced cancer patients with promising results [7,8]. After injection of the molecule, most of the patients enrolled in these trials displayed a transient neutrophilia and some also experienced neutrophilic infiltration and partial necrosis of tumor lesions. Recently, these findings were confirmed with subcutaneous administration of higher doses of CRM197 to another group of advanced cancer patients [9].

In this study, 12 advanced cancer patients were treated with direct intralesional inoculation of CRM197. The first aim of the trial was to investigate the mechanism of action of the product through the possible histologic changes induced by its close contact with malignant cells. A secondary objective was to evaluate toxic, hematologic and antitumor effects of the procedure.

Patients & methods
Between January 2000 and June 2003, 12 outpatients with histologically confirmed, primary or recurrent cancer refractory to standard therapies were enrolled in this study. Ratio of males to females was 7:5, with a median age of 67 years and median Karnofsky performance score (KPS) of 60. All patients required bidimensionally measurable lesions accessible to local injections. Other eligibility criteria were: ambulatory performance status with a KPS ≥ 50; adequate organ function with hemoglobin greater than 9.0 g/l, leukocytes ≥ 3,000/µl, platelets ≥ 100,000/µl, bilirubin less than 2.0 mg/dl and creatinine less than 2.0 mg/dl; no surgery or antitumor therapies for the previous 4 weeks at least; witnessed and fully informed consent. The study was approved by our ethical committee.

Prior to enrollment, all patients underwent a physical evaluation with documentation of performance status, electrocardiogram, routine biochemistry and urinalysis. Physical evaluation was repeated on alternate days during the treatment. Biochemistry and urinalysis were repeated on days 7 and 15 from the start of treatment. A complete blood count was performed prior to the first injection of CRM197 and 24 and 48 hours later. Thereafter, this test was repeated weekly for 4 weeks.

Humans may present titers of serum antitoxin against DT ranging from ≤ 0.01 IU/ml (susceptibility to diphtheria) to 1.0 IU/ml (full protection) [10]. To monitor the pretreatment titer of this antitoxin in our patients we used an enzyme-linked immunosorbent assay (ELISA) test method (Genzyme Virotech GmbH). As many individuals may also acquire DTH to DT as a result of subclinical infections [11], we tested all our patients by injecting intradermally into the forearm 0.003 µg of CRM197 contained in 0.1 ml of phosphate buffer of pH 7.2. A DTH condition was scored when an erythematous reaction of at least 1.0 cm in diameter appeared within 24 hours.
Intratumoral administration of CRM197 – RESEARCH ARTICLE

CRM197 (Lot CRM003, 20,000 Lf and 53.8 mg/ml) was provided by Rino Rappuoli of Chiron Vaccines, Italy. The preparation was diluted to a concentration of 0.051 mg/ml in sterilized dilution buffer ([DB] 10 mM sodium phosphate buffer, pH 7.2) containing 10% sucrose as stabilizer. The final product, tested for sterility and general safety on the depilated skin of young New Zealand rabbits, was aliquoted in pyrogen free vials and stored at -20°C.

Treatment was performed by injecting CRM197 into and around a single index lesion. The lesion selected for injection was required to have the largest diameter, ranging from 1.0 to 5.0 cm, and was designed to undergo a biopsy 1 week before the start of treatment and again at day 15 from the first injection. Treatment consisted of six injections with a 25-gauge needle administered on alternate days. Two different doses were used according to the diameter of the lesions. Patients with lesions sizing up to 2.5 cm received 0.017 mg/day contained in 0.33 ml of DB. Patients with lesions up to 5.0 cm received 0.051 mg/day contained in 1.0 ml of DB. The treatment schedule was adopted in view of the long half-life of CRM197, which in our latest clinical trial with systemic administration of CRM197 was found to be approximately 18 hours. The present doses were equivalent to 0.01 and 0.03, respectively, of the smallest dose administered in that trial.

Specimens of pre- and post-treatment biopsies were compared after staining with hematoxylin and eosin. Given the preliminary nature of this study, immunohistologic tests aiming at characterizing the cells observed in the specimens were not performed.

Results

Toxicities

Local toxicity consisting of mild pain in the injected lesions and sometimes in peripheral oedema, was experienced by four patients lacking any reactivity to DT and three with humoral immunity. At 6–12 hours from the injection, all patients with humoral immunity and DTH experienced erythematous itching and indurated skin reaction involving the injected lesions and their periphery. The more the treatment progressed, the greater the reaction became. Local erythema was accompanied by moderate fatigue, hypotension, arthralgia, myalgia and fever (from 37.5° to 38.8°C). This flu-like syndrome faded slowly with diaphoresis. No patient was required to discontinue treatment or have therapy delayed because of unacceptable toxicity.

Hematologic, histologic & clinical response

Patient numbers 7, 8 and 9 lacked any reactivity to DT and experienced no change in the number of circulating neutrophils at day 2 from the start of treatment, in the histology of their index lesions at day 15 and in the lesion size at day 30. One patient (no. 4) lacking any reactivity to DT and three others (numbers 2, 10 and 12) with humoral immunity only had a weak increase in the number of circulating neutrophils at day 2, a moderate neutrophilic infiltration of their biopsy samples at day 15 and a minimal shrinkage or no change in size of their index lesions at day 30. One patient (no. 6) with humoral immunity and a weak DTH to DT had an appreciable increase in circulating neutrophils of 92% at day 2, an evident neutrophilic infiltration of his lesion at day 15 and a 60% shrinkage of the lesion at day 30. These patients (numbers 1, 3 and 5) with humoral immunity and a strong DTH to DT had a remarkable increase in the number of circulating neutrophils of 92% at day 2, an evident neutrophilic infiltration of his lesion at day 15 and a 60% shrinkage of the lesion at day 30. Three patients (numbers 1, 3 and 5) with humoral immunity and a strong DTH to DT had a remarkable increase in the number of circulating neutrophils at day 2 (92, 100 and 84% increase, respectively), biopsy specimens showing a fibrinous background of dead tumor cells, debris of tumor cells and thrombotic vessels widely crowded with neutrophils at day 15 (Figure 1). At that time, the
index lesion of patient number 1 appeared enlarged, covered by a thin layer of pus, friable, easy bleeding and very similar to the pseudomembrane of diphtheria (Figure 2). Two weeks later, suppurative waned the base of this index lesion causing the loss of the nodule and the rupture of the carotid artery along with the patient's death.

Patient number 3, after experiencing a 60% reduction in size of her lesion, died of pneumonia. Patient number 5 had an 80% reduction of her chest wall lesion and is still alive and well 24 months after treatment. Patient number 11 had a humoral immunity and a weak DTH against DT. Her index lesion was a recurrent rhabdomyosarcoma of a thigh that had been previously excised three times and treated with chemotherapy. Before we could take the programmed biopsy of day 15, the patient had been urgently hospitalized for a suspected femoral fracture. Two weeks later, the orthopedists decided to disarticulate the leg. Exhaustive histologic examination of the removed tumor evidenced a strong granulomatous reaction surrounding a core of viable tumor cells bordered by a large rim of dead tumor cells and debris of tumor cells (Figure 3). The granuloma consisted of an exuberant deposition of collagen, dense lymphocytic infiltration, macrophages, eosinophils, plasma cells and scattered giant cells of the foreign body type (Figure 4). Results are summarized in Table 1.

Discussion

Toxicities correlated with the degree of immunological reactivity to DT of the patients. Subjects lacking any reactivity, or having only a humoral, suffered from mild pain at the injection sites. Conversely, subjects with humoral immunity and DTH to DT after the administration of CRM197 complained of a disturbing flu-like syndrome and of pruritus around the injected lesions. On the whole, however, the intratumoral injection of CRM197 was safe and relatively well tolerated. The poor clinical condition of several of our patients might have limited their hematologic, histologic and antitumor response to the injected CRM197. Nevertheless, the results seemed to correlate with the immunologic reactivity to DT of the subjects. In fact, the above parameters were not, or little influenced by CRM197 in patients with poor reactivity to DT, while they were greatly affected in those with high reactivity. In particular, patients with humoral immunity and strong DTH to DT after receiving CRM197 had a striking increase in the number of circulating neutrophils and a prominent neutrophilic infiltration of the injected lesion leading to inflammation and, sometimes, to suppuration. Since CRM197 lacks any toxicity, this finding confirms that its antitumor activity is immunologic in nature and strongly suggests that neutrophils might have a central role in this activity.

Many mechanisms have been proposed to explain the ability of neutrophils to exert an antitumor effect. Complement activation, antibody dependent cellmediated cytotoxicity, and release of cationic proteins and/or oxygen radicals contained in their cytoplasmic granules are some of the proposed mechanisms [12,13]. The hypothesis of hyperoxidation is particularly attractive. In fact, the release of oxidants, such as hydrogen peroxide, hydroxyl radicals and singlet oxygen (the respiratory burst) might have a strong tumor-destructive potential, especially as malignant cells have no or lowered levels of superoxide dismutase, an enzyme that may neutralise the toxic oxygen species [14].

All but one of our patients had been biopsied at day 15 from the initiation of treatment.
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Table 1. Patient characteristics, doses of CRM197 and effects of intratumoral injection.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex/Age</th>
<th>Tumor sites</th>
<th>Reactivity to DT</th>
<th>CRM197 (mg/day)</th>
<th>Circulating neutrophil (at day 2)</th>
<th>Lesion histology (at day 15)</th>
<th>Lesion reduction (at day 30)</th>
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<tbody>
<tr>
<td>1</td>
<td>M/64</td>
<td>Mouth</td>
<td>+</td>
<td>0.04</td>
<td>++</td>
<td>+ 0.017</td>
<td>+ 92%</td>
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<td>2</td>
<td>M/69</td>
<td>Mouth</td>
<td>-</td>
<td>0.05</td>
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<tr>
<td>4</td>
<td>M/48</td>
<td>Mouth</td>
<td>-</td>
<td>0.05</td>
<td>-0.051</td>
<td>+ 28%</td>
<td>Weak Inflammation - 25%</td>
</tr>
<tr>
<td>5</td>
<td>F/89</td>
<td>Breast</td>
<td>++</td>
<td>0.04</td>
<td>++</td>
<td>+ 0.017</td>
<td>+ 84%</td>
</tr>
<tr>
<td>6</td>
<td>M/65</td>
<td>Colon</td>
<td>+</td>
<td>0.03</td>
<td>+0.051</td>
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<td>7</td>
<td>F/73</td>
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<td>11</td>
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<td>Sarcoma</td>
<td>+</td>
<td>0.03</td>
<td>+0.051</td>
<td>+ 65%</td>
<td>Granuloma - 60%</td>
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<td>12</td>
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Abd: Abdominal; DT: Diphtheria toxin; DTH: Delayed-type hypersensitivity; F: Female; M: Male.

From the start of treatment; Inflammation: dense neutrophilic infiltration, abundant exudate and fibrin; Mild inflammation: moderate neutrophilic infiltration and slight exudate.

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to study the acute reaction to the injection of CRM197. The excepted patient (number 11) had been urgently hospitalized before we took a biopsy and had had a leg disarticulation at day 30. The histologic specimens obtained from this case evidenced a striking granulomatous reaction surrounding a core of dead and viable tumor cells. The granulomatous reaction represents the progression of an inflammatory response from an acute stage to a chronic stage. Usually, such progression depends on the persistence of foreign material in the involved site and may lead to serious damage of the whole area. However, CRM197 had been injected for the last time at day 11 and its persistence in the index lesion presumably had not been longer than 48–72 h. Therefore, a different foreign material might have contributed to convert the inflammatory acute stage into a chronic stage. Thus, it cannot be excluded that even cancer cells, after their link to CRM197, became heterologous, behaved as foreign material and co-operated in triggering the conversion of inflammation to the chronic stage.

Conclusion
The intratumoral injection of CRM197 was safe and well tolerated. Toxicities and effects of the mutant toxin on the injected lesion were directly correlated with the degree of reactivity to DT of the patients. Neutrophils appear to have a central role in the acute inflammation and antitumor effect elicited by CRM197. A late effect of the mutant toxin consisted of a chronic inflammation surrounding the tumor core.

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Highlights

- Cross-reacting material 197 is a nontoxic mutant of diphtheria toxin that cross-reacts immunologically with the native toxin.
- Moreover, it binds to the same cell membrane receptor of the toxin.
- The receptor for diphtheria toxin and CRM197 is heparin-binding epidermal growth factor (HB-EGF). This molecule is often overexpressed in cancer cells.
- In previous dose-finding clinical trials, CRM197 demonstrated some degree of antitumor activity.
- The present trial was based on the intratumoral injection of CRM197. The primary goal was to study from a histologic viewpoint the immune reaction to the injected mutant toxin.
- The treatment plan consisted of six intratumoral injections administered on alternate days.
- The intratumoral administration of CRM197 was safe and well tolerated.
- The effect of CRM197 correlated with the patients’ degree of reactivity to diphtheria.
- Patients with a strong reactivity to diphtheria toxin had an increase in the number of circulating neutrophils and a striking neutrophilic infiltration of the injected lesion, leading to inflammation and, sometimes, to suppuration and shrinkage of the lesion.
- Neutrophils appear to play a crucial role in the antitumor activity of CRM197.
- Histologic specimens obtained from a patient at day 30 after the start of treatment showed a striking granulomatous reaction surrounding a core of dead and viable tumor cells. It probably represented the chronic stage of the inflammation following the injection of CRM197.

Bibliography