Advances in Brief

Delayed-Type Hypersensitivity Reactions to Tumor-associated Antigens in Colon Carcinoma Patients Immunized with an Autologous Tumor Cell/Bacillus Calmette-Guérin Vaccine

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Abstract

Five different colon tumor-associated antigens (CTAA) were tested for their ability to induce an immune response in vivo and in vitro in ten colon carcinoma patients immunized with an irradiated autologous tumor cell/Bacillus Calmette-Guérin vaccine (active specific immunization) after resection of the primary tumor. The CTAA were defined by two different human monoclonal antibodies (MCA 1688 and MCA 28A32) derived by immortalization of peripheral blood B-lymphocytes from an active specific immunization patient. Delayed-type cutaneous hypersensitivity responses against a mixture of CTAA 28A32-50K and -32K were positive in seven of ten patients tested. In vitro T-cell responses upon stimulation with CTAA 28A32-32K were found to be positive in seven of ten patients and correlated with delayed-type cutaneous hypersensitivity responses to the antigen mixture. These data suggest that CTAA 28A32-32K might contain an important tumor-related T-cell epitope. Moreover, this method is suitable to define potential future candidates for antitumor vaccine development.

Introduction

Development of anticancer immunotherapy has attracted much attention in the last few decades (1). In colon carcinoma, the relatively high recurrence rate after radical surgical resection of the primary tumor in patients with no evidence of metastatic disease at the time of surgery has prompted a search for adjuvant treatment, preferably with minor side effects. One approach comprises ASI (2). This modality of therapy, developed in the L10 guinea pig model by Hanna and Peters (3), was translated into a clinical Phase I/II trial in colon carcinoma patients in the early 1980s (4). It was demonstrated that patients immunized with irradiated autologous tumor cells after surgical resection of the primary tumor developed a cutaneous DCH reaction to their tumor cells without responding to autologous mucosa cells (5). In another study, the DCH correlated well with in vitro lymphocyte-proliferative responses to mitomycin C-treated autologous tumor cells (6).

Promising results with regard to improved time to recurrence obtained with this adjuvant therapy (4-7) have led to a comprehensive multicenter randomized Phase III trial in the Netherlands. In this trial, colon carcinoma patients, Astler-Coller-Dukes’ Stages B2, B3, and C, are treated with radical surgical resection of the tumor alone or surgery followed by ASI with the irradiated autologous tumor cell/Bacillus Calmette-Guérin vaccine. Vaccinated patients receive four vaccines composed of 10⁷ irradiated autologous tumor cells; the first two vaccines are admixed with BCG as an immunostimulant. This therapy can be administered on an outpatient basis and is well tolerated with only minor side effects (8).

However, a major drawback of this treatment modality is the limited availability of autologous tumor cells and the laborious efforts to prepare single cell suspensions for vaccination. These problems could be circumvented if a generic antigen vaccine could be developed. Therefore, investigations were aimed at defining relevant CTAA (9) with potential for formulation into a generic vaccine. Here, we describe in vivo and in vitro reactivity of 10 immunized patients to five CTAA preparations defined by the human monoclonal antibodies MCA 1688 and MCA 28A32, reactive with epitopes shared by many colon tumors (9). We demonstrate that DCH testing and in vitro lymphocyte-proliferative responses are valuable tools in defining potential candidates for future vaccine development.

Materials and Methods

Patients. After approval of the ethical committee of our hospital and with written consent, 10 colon carcinoma patients, randomized into the adjuvant treatment arm of the colon ASI trial, were enrolled in the DCH protocol. As adjuvant therapy, all patients received four vaccines starting 1 mo after radical surgical resection of the primary tumor. The first three vaccines were administered at weekly intervals. The fourth vaccine was given 6 mo after surgery. The first two vaccines consisted of irradiated autologous tumor cells and 10⁷ colony-forming units of BCG (Tice strain; Organon Teknika Corp., Chicago, IL) as immunostimulant. The last two vaccines consisted of irradiated tumor cells only. DCH testing with CTAA was done at the time of the third and the fourth vaccinations and 1 mo thereafter.

Preparation of Autologous Tumor Cell Vaccines. Colon tumor cell vaccines were prepared as described previously (3-6). Briefly, viable tumor tissue was obtained at the time of surgery, minced with a scalpel, and dissociated in HBSS (Whittaker Bioproducts, Inc., Walkersville, MD) with 0.14% collagenase type I and 0.1% DNase type I (Sigma Chemical Co., St. Louis, MO). The cells were cryopreserved in aliquots of 15 to 20 × 10⁶/ml and stored in liquid nitrogen until use. After rapid thawing in HBSS/DNase, cells were washed in HBSS and irradiated (20,000 rads). Viability of the tumor cells after thawing was always >70% as judged by trypan blue exclusion. Before vaccination, the tumor cells were pelleted and resuspended in 0.1 ml of HBSS. A vaccine contained 10 to 12 × 10⁷ irradiated autologous tumor cells and was injected intradermally.

Biochemical Characterization of CTAA. CTAA were identified by two human monoclonal IgM antibodies, MCA 1688 and MCA 28A32, derived from B-cells of a patient undergoing ASI (10). MCA 1688 recognizes a tumor-associated antigen designated as CTAA 1688. This antigen comprises a complex of polypeptides with molecular weights of 38,000 to 43,000. Protein sequence analyses have indicated that these polypeptides represent altered forms of cytokeratins. This antigen is not glycosylated and is found in the cytoplasm of tumor cells (9). MCA 28A32 recognizes four antigens, based on...
sodium dodecyl sulfate-polyacrylamide gel electrophoresis, identified as follows: CTAA 28A32-50K, CTAA 28A32-46K, CTAA 28A32-36K, and CTAA 28A32-32K with molecular weights of 50,000, 46,000, 36,000, and 32,000, respectively. The antigens were purified to near homogeneity and were found to be reactive with MCA 28A32 in both native form (using an enzyme immunoassay) and denatured form (using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analyses). In addition, using both isoelectric focusing and comparative peptide mapping, these proteins were not related to each other nor were they derived from a larger precursor molecule. Only CTAA 28A32-36K was glycosylated. The remaining antigens were non-glycosylated, cytoplasmic proteins.3

**DCH Testing.** At the time of the third and fourth vaccination and 1 mo thereafter, 0.1 mg of purified antigen in 0.1 ml of phosphate-buffered saline was injected intradermally on the back. In order to minimize the number of injections, CTAA 28A32-50K and CTAA 28A32-32K were admixed. As a control, 0.1 mg of human serum albumin was administered. Reactions were read 24 and 48 h after administration of the antigens. To reduce interobserver variation, skin tests were always performed and evaluated by the same investigators (H. G. and J. B. V.). The results are expressed as the mean of two perpendicular diameters of erythema. A positive response is defined as ≥5 mm erythema, irrespective of induration.

**In Vitro Proliferative Responses of Peripheral Blood Lymphocytes to CTAA 1688 and CTAA 28A32.** PBL were isolated from heparinized blood of the patients drawn at the time of DCH testing by Ficoll-Hypaque density gradient centrifugation and cryopreserved. In vitro tests with the PBL of a patient were all done at the same time after completion of the DCH protocol.

Fifty thousand PBL were cultured in round-bottomed microtiter plates in 100 μl of RPMI 1640 supplemented with 10% fetal bovine serum, γ-interferon (60 units/ml), and gentamicin sulfate (50 μg/ml). γ-Interferon has been shown to augment in vitro proliferative responses to soluble antigens in this assay. As stimulants, 100 μl of soluble antigens were added at a concentration of 10 μg/ml. All incubations were performed in a humidified atmosphere of 5% CO2 in air. Proliferation was determined after 7 days of culture by measuring the incorporation of [3H]thymidine (1 uCi/well) (NEN, Boston, MA) added 18 h before harvest of the cultures. All cultures were performed in quadruplicate with standard deviations below 7%. Stimulation indexes were calculated as follows:

\[
\text{Stimulation index} = \frac{\text{mean cpm of cells in the presence of test antigen}}{\text{mean cpm of cells in the presence of medium}}
\]

Stimulation indexes above 3.0 were considered positive at the 99% confidence interval based on calculation of interassay coefficients of variance for PBL tested with control antigens.

**Immunohistochemical Staining of Primary Tumors with 1688 and 28A32.** Paraffin sections of the primary tumors of the patients enrolled in the DCH protocol were tested for reactivity with the human IgM MCA 1688 and 28A32 and a rabbit polyconal antiserum to CTAA 28A32-32K. Paraffin slides were deparaffinized, hydrated through xylene and graded alcohol washes, and air dried. Blocking solution (50 to 100 μl of lysine-BSA per slide) was added and incubated for 30 min at room temperature. Per slide, 200 μl of 2-fold serial dilutions of 1688 (1.25 μg/ml to 0.078 μg/ml) and 28A32 (20 μg/ml to 1.25 μg/ml) were added. Rabbit anti-CTAA 28A32-32K was used at a 1:500 dilution. Antibodies were applied and incubated for 2 h at room temperature. After incubation, slides were washed 3 times with PBS and incubated for 1 h at room temperature with a goat anti-human IgM peroxidase conjugate (KPL, Gaithersburg, MD) (1:120 dilution in PBS/1% BSA) or goat anti-rabbit immunoglobulin peroxidase conjugate. Normal human IgM or normal rabbit serum was used as control. Sections were then washed again 3 times in PBS, and the peroxidase reaction was performed by incubating the sections for 15 min at room temperature with 3,3'-diaminobenzidine-tetra-HCL (Sigma, St. Louis, MO) in PBS containing 0.01% H2O2. Sections were then washed under running tap water and counterstained with hematocoealin for 30 s, washed again with running tap water, dehydrated, and mounted.

Staining intensity was scored at concentrations of antibodies determined in earlier studies as cut-off criteria for the selection of patients with sufficient antigen expression to permit good in vivo localization of the antibody in an imaging study (11). This was 0.625 μg/ml for 1688 and 5 μg/ml for 28A32. The rabbit anti-CTAA 28A32-32K dilution was selected, based on the best concentration for differential reactivity between tumor and normal tissue. The rabbit antiserum was raised by immunizing rabbits to purified CTAA 28A32 emulsified in complete Freund’s adjuvant. This antiserum did not cross-react with any of the other CTAA 28A32 antigens. Reactivity was scored as follows:

- + , negative to very weak focal (<5%) staining;
- 1+, weak staining;
- 2+, weak to moderate staining;
- 3+, moderate staining;
- 4+, strong staining.

**Results**

**DCH Reactions to CTAA 1688, CTAA 28A32-50K and -32K, CTAA 28A32-46K, and CTAA 28A32-36K.** Seven of the 10 patients responded to the CTAA 28A32-50K and -32K mixture with an erythematous skin reaction. Induration was never observed. Table 1 shows the maximum DCH responses to CTAA 28A32-50K and -32K recorded during the DCH protocol. As to the development of the reactivity during the vaccination protocol, only one patient reacted to CTAA 28A32-50K and -32K at the time of the first DCH test (at the time of the third vaccination). Most patients had a delayed erythematous reaction at the second test (at the time of the fourth vaccination).

One of 10 responded to CTAA 28A32-46K and 5 of 10 responded to 28A32-50K and -32K. At the time of the third DCH test, 2 of the latter patients’ reactivity to CTAA 28A32-50K and -32K was found to persist, whereas one patient showed a delayed erythematous reaction for the first time (Fig. 1). In most patients, DCH reactivity was positive after both 24 and 48 h. In all cases, the erythematous reaction at 48 h, was equal to, or more extensive than, the reaction read at 24 h (data not shown).

None of the patients ever reacted to CTAA 1688 or CTAA 28A32-36K. Only one patient showed an erythematous reaction to CTAA 28A32-46K.

All patients had erythema and induration at the vaccine site during the third and fourth vaccinations with irradiated autologous tumor cells (Table 1).

**In Vitro Lymphoproliferative Responses.** Three of the PBL samples, collected before vaccination, showed a proliferative response to tuberculin PPD, used as a positive control for BCG immunization. Eventually, PBL of all patients became responsive to PPD in vitro, albeit with different kinetics (data not shown). This indicates that all patients were able to mount a T-cell response upon immunization.

At the time of the first DCH test, no in vitro lymphoproliferative responses were obtained to any CTAA. Responses to CTAA 1688 and CTAA 28A32-50K remained negative at all time points tested. One

<table>
<thead>
<tr>
<th>Table 1</th>
<th>DCH responses and PBL proliferative stimulation indexes to CTAA 28A32-50K and -32K in ten patients</th>
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<tbody>
<tr>
<td>Patient</td>
<td>Response</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
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<tr>
<td>2</td>
<td>18</td>
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<td>3</td>
<td>9</td>
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<td>9</td>
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</table>

* Numbers represent the mean of two perpendicular diameters (mm).

**Notes:**
- DCH response to a mixture of CTAA 28A32-50K and -32K. Numbers represent the mean of two perpendicular diameters of erythema (mm).
- Erythema <5 mm was considered negative (−).
- Stimulation indexes <3.0 were considered negative (−).
- Numbers represent the mean of two perpendicular diameters (mm).

patient responded to CTAA 28A32-46K, and another showed a positive reaction to CTAA 28A32-36K in vitro in the course of the DCH protocol.

The majority of patients developed positive in vitro reactivity to CTAA 28A32-32K (7 of 10) (Table 1), although the kinetics were different among individuals.

Immunohistochemistry of Primary Tumors with 1688, 28A32, and Rabbit Anti-CTAA 28A32-32K. The relative antigenic content of vaccines was evaluated by assessing immunoreactivity of antibodies to paraffin-embedded sections of the primary tumor to determine whether this influenced the development of DCH responses to the CTAA (Table 2). The 1688 antibody was weak to strongly positive on 8 of 10 tumors. Therefore, the lack of DCH reactivity to CTAA 1688 is probably due to the absence of an antigenic stimulus. The monoclonal antibody 28A32, which recognizes four different molecular species, had positive reactivity for all tumors. Immunohistochemical analysis with this antibody, however, cannot discriminate between expression of the different CTAA 28A32. Therefore, we developed polyclonal rabbit antisera to each antigen. Although these antisera recognized the purified antigens by Western blot and enzyme-linked immunosorbent assay analysis, only the antisera to CTAA 28A32-32K reacted with paraffin-embedded tumor tissues with good tumor specificity. The three patients that did not develop a DCH response to the CTAA 28A32-50K and -32K mixture had no detectable to weak (2+) reaction to tumor with this antisera. The majority of the responders had high levels of staining (3+ to 4+). The lack of response may therefore be due to incomplete sensitization to this antigen.

Table 2 Comparison of antigen expression by immunohistochemical analysis with DCH response

<table>
<thead>
<tr>
<th>Patient</th>
<th>CTAA 1688 MCA 1688</th>
<th>CTAA 28A32-50K MCA 28A32</th>
<th>Rabbit anti-CTAA 28A32-32K</th>
</tr>
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<tbody>
<tr>
<td>1</td>
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<td>4</td>
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<td>4</td>
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* Mean diameter of erythema (mm). No response (-).

Discussion

In this study, we have investigated immune responsiveness of 10 colon carcinoma patients vaccinated with an autologous tumor cell/BCG vaccine toward five different CTAA identified by MCA 1688 and 28A32. Immunohistochemically, the majority of the primary tumors of all patients stained with both MCA or rabbit antisera to CTAA 28A32-32K, albeit with different intensities. This indicates that all CTAA tested contained putative epitopes against which an immune response could be mounted or augmented in the course of the vaccination procedure.

None of the patients showed either DCH reactivity or in vitro T-cell responses to CTAA 1688. Therefore, it seems that CTAA 1688 carries a B-cell epitope but no strong T-cell epitope(s). The same holds true for CTAA 28A32-46K and CTAA 28A32-36K, although each antigen induced an in vitro proliferative response or a DCH reaction in one individual.

In contrast, in the course of the vaccination procedure most patients had a positive skin test to the CTAA 28A32-50K and -32K mixture and showed in vitro T-cell reactivity to CTAA 28A32-32K. Although no induration was observed, we argue that the erythematous reaction can be considered as a T-cell-mediated event. This is based on the following observations. (a) The erythematous reaction was always maximal at 48 h, in contrast to an Arthus-type reaction. (b) There was some correlation between in vitro T-cell proliferation and the DCH response. Since the response in vitro was directed solely against CTAA 28A32-32K, it seems likely that the DCH is mediated by the presence of this antigen in the mixture. Presently, studies are under way to address this point. (c) It has been found that an erythematous reaction is a very important and often underestimated aspect of T-cell-mediated DCH reactivity (12).

Both DCH and in vitro T-cell responses to the antigens were relatively low. This might be due to the fact that the presentation and/or concentration of the antigens was not optimal. Possibly, induction of lymphokine production, necessary to elicit both a DCH reaction and T-cell proliferation in vitro, was suboptimal with the preparations used.

Moreover, it is unknown whether and to what extent these CTAA bind to MHC molecules and are presented in an MHC-dependent fashion. In general, there is some controversy about the role of the MHC in the antitumor response in vivo and in vitro. In vivo, decreased MHC Class I expression on tumor cells was observed to be associated with increased tumorigenicity and frequently correlated with a poor prognosis (13). Ransom et al. (14) reported a correlation between MHC Class II molecule expression on the autologous colon tumor vaccine cells and vaccine efficacy. From in vitro studies in several human tumors, both MHC-restricted (15, 16) and MHC-nonrestricted (17) but sometimes tissue-specific (18) cytotoxic T-cell responses have been described. Presently, the relative contribution of the several
mechanisms in the in vivo human immune response against tumors still has to be established.

Bystryn et al. (19) described a correlation between strong DCH reactivity to the vaccine used for adjuvant treatment in melanoma patients and clinical outcome. However, this could not be confirmed by Mitchell et al. (20). In the latter study there was a correlation between in vitro T-cell cytotoxicity to melanoma-associated antigens and clinical response to immunization. Jessup et al. (6) showed that DCH responses to autologous tumor cells in vaccinated colon carcinoma patients correlated well with in vitro T-cell proliferation to the tumor cells. Therefore, at the present time, it seems that both in vivo and in vitro studies are needed in the search for tumor-associated antigens as possible candidates for vaccines. In this study, we have shown that CTAA with potential T-cell-stimulatory capacity could successfully be identified by monoclonal antibodies derived from B-cells of immunized patients. This can be a very important tool in future vaccine development.

References

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