Advances in Brief

Immunization with Haptenized, Autologous Tumor Cells Induces Inflammation of Human Melanoma Metastases

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Abstract

Twenty-four patients with metastatic melanoma were treated with a novel form of active immunotherapy, autologous tumor cell vaccine conjugated to the hapten, dinitrophenyl. This approach is based on the idea, well established in animal systems, that presentation of tumor antigens in the context of a strongly immunogenic hapten augments the development of immunity to those antigens. After being sensitized to dinitrophenyl, patients were given injections of dinitrophenyl-vaccine every 28 days following pretreatment with low dose cyclophosphamide. The vaccine induced a striking inflammatory response in superficial metastases in 14 of 24 patients, consisting of erythema, swelling, warmth, and tenderness over tumor masses. Immunohistochemistry and flow cytometric analysis of biopsy specimens showed marked infiltration with lymphocytes, the majority of which were CD8+, HLA-DR+ T-cells. These observations suggest that a T-cell-mediated immune response against melanoma-associated antigens was facilitated by the “helper” effect of the anti-hapten response.

Introduction

There is accumulating evidence that human cancer cells have tumor-associated antigens, i.e., determinants expressed exclusively by tumor cells or to a much greater extent than by their nonmalignant counterparts (1, 2). However, immunizing cancer-bearing patients against such antigens has proved to be difficult (3, 4). One major obstacle may be preexisting immunological tolerance to those antigens, a concept for which there is considerable support in animal systems (5, 6). The cytotoxic drug cyclophosphamide has been shown to heighten the immune response and to break tolerance to a variety of exogenous antigens, as well as to self-antigens, when given prior to immunization (7–10). In clinical trials, we have shown that the administration of cyclophosphamide prior to injection of an autologous tumor vaccine can induce DTH1 to autologous tumor cells and, sometimes, regression of metastatic tumors (3, 11).

Mitchison (12) proposed that immunization with tumor cells conjugated to “helper determinants,” such as DNP or TNP, would augment cell-mediated immunity to tumor-associated antigens. This prediction has been validated in two experimental systems (13, 14). Here we report the first clinical trial of a hapten-conjugated vaccine in patients with melanoma and show that the treatment induces inflammation and T-lymphocyte infiltration of metastatic masses.

Materials and Methods

We treated 24 patients with metastatic, surgically incurable melanoma, all of whom had at least one superficial metastasis in skin or lymph nodes that was directly observable and easily biopsied. Informed consent was obtained. The patients were sensitized to DNP by topical application of 1% dinitrofluorobenzene in acetone-corn oil on two consecutive days. Cyclophosphamide, 300 mg/m² i.v., was administered 3 days before the sensitization. Two weeks later patients were again given cyclophosphamide, followed 3 days later by injection of DNP-conjugated melanoma vaccine; cyclophosphamide plus DNP-vaccine were repeated every 28 days. The vaccine consisted of 10–25 x 10⁶ cryopreserved, autologous, irradiated (2500 R), DNP-conjugated melanoma cells prepared as described previously (3) and mixed with Bacillus Calmette-Guérin. DNP conjugation was performed by the method of Miller and Claman (15). Patients were tested for cell-mediated immunity to hapten-modified cells by skin testing with DNP-conjugated MNC.

Immunohistochemical analysis of tumor biopsies was performed using an avidin-biotin peroxidase technique, as described previously (16). Flow cytometric analysis of these tissues was performed on enzymatically dissociated, cryopreserved cell suspensions. The cells were thawed and stained with monoclonal antibodies on ice for 30 min. Then they were washed and analyzed with a Coulter EPICS C flow cytometer (Coulter Electronics, Hialeah, FL). Lymphocytes were identified by antibody HB12 (American Type Culture Collection) to the T-200 common leukocyte antigen and were analyzed by setting appropriate bit map gates on a plot of forward versus 90-degree light scatter. The panel of monoclonal antibodies used for immunohistochemistry and flow cytometry consisted of Leu-1 (CD5), Leu-2 (CD8), Leu-3 (CD4), Leu-11 (CD16), NKH-1 (CD56), HLA-DR (L243), Leu-M1 (CD15), tac (interleukin 2 receptor, CD25), and ICAM-1 (CD54).

Results and Discussion

DTH to DNP-MNC was not present before treatment but was induced in 22 of 24 patients by dinitrofluorobenzene sensitization [diameter of induration at 48 h, 18.6 ± 4.6 (SE) mm]. Patients did not exhibit DTH to unconjugated MNC or MNC conjugated to the non-cross-reactive hapten, TNP.

Following DNP-vaccine therapy, 14 of 24 patients developed clinically evident inflammatory responses in superficial (nodal, s.c., or i.d.) metastatic masses. These responses consisted of marked erythema, warmth, and tenderness of the tumors and the overlying skin (Fig. 1). In one patient, this progressed to frank necrosis with purulent drainage.

The time from the beginning of vaccine treatment to an observable inflammatory response was 2–4 months (i.e., 2–4 vaccine injections). Responding metastatic lesions varied in size from 5 mm diameter (dermal metastases) to 10–12 cm (lymph node masses). The number of inflamed tumors on a single patient ranged from 1 to >100 (Table 1). In 5 of 11 patients
who had multiple superficial metastases, the inflammatory response involved all of the observable lesions; the remaining 6 patients had inflammation in 25–75% of their visible tumors.

In 10 of these 14 patients, inflamed tumors were biopsied and analyzed by immunohistochemistry. A typical result is shown in Fig. 2. Melanoma cells were infiltrated with T-lymphocytes, the majority of which were CD8+ [suppressor-cytotoxic phenotype] and HLA-DR+ [indicative of activation (17)]. In contrast, biopsies of s.c. melanoma metastases taken before vaccine treatment showed only rare scattered T-cells. Zones of tumor immediately adjacent to T-cell infiltration demonstrated abundant HLA-DR+ dendritic cells that proved to be Leu-M1+ macrophages. Furthermore, melanoma cells in areas of dense lymphocytic infiltration expressed the adhesion molecule ICAM-1 more intensely than tumor cells in areas where lymphocytes were sparse (Fig. 2E). ICAM-1 is known to be expressed by melanoma cells, and its expression is up-regulated by γ-interferon (18, 19). Thus, it is possible that this observation reflected γ-interferon production by T-cells at the tumor site.

Of the 24 patients studied, 10 did not exhibit clinically evident inflammation at the tumor sites, and 7 of these underwent biopsy. Tumor specimens from all 7 patients showed lymphocytic infiltration of tumor masses similar to that described above. Again, matched specimens obtained before therapy did not show lymphocytic infiltrates. Thus, in 21 of 24 patients treatment with a DNP-haptenized vaccine induced grossly evident inflammation and/or histologically documented lymphocytic infiltration or both.

We performed flow cytometric analysis of cell suspensions made by enzymatic dissociation of excised tumors to enumerate tumor-infiltrating lymphocytes and to determine their phenotype. In 9 inflamed tumors, 40.2 ± 2.7% (SE) of the total viable cells were T-lymphocytes as indicated by expression of CD3; in contrast, analysis of a panel of 14 s.c. melanoma metastases from untreated patients showed T-lymphocytes to be sparse (10.2 ± 2.2%). As shown in Fig. 3, CD8+ T-cells were much more numerous than CD4+ T-cells. Moreover, a high percentage of T-cells expressed HLA-DR, indicating that they were activated (17); however, fewer than 5% expressed the p55 interleukin 2 receptor (tac, CD25; data not shown).

In 5 patients, the development of clinically evident tumor-inflammatory responses was followed by regression of the tumor mass, while in 2 patients inflamed tumors remained unchanged in size for 3–6 months. In the 17 remaining patients there was progressive tumor growth with the persistence of inflammation.

The grossly evident inflammatory responses described in this communication do not occur spontaneously in metastatic melanomas. Furthermore, they are not induced by cyclophosphamide alone or by other cytotoxic drugs (10) and have not been reported by other investigators using human tumor vaccines (20–23). In our previously reported study of 40 melanoma patients treated with cyclophosphamide plus an autologous nonhaptenized vaccine (11), we observed tumor inflammation in s.c. metastases of a single patient. Thus, it appears that immunization with DNP-haptenized tumor cells was the critical element in the induction of this reaction.

That the inflammatory response occurred in one or more metastatic tumors, but not in normal tissue, strongly suggests that the DNP-vaccine induced cell-mediated immunity against melanoma-associated antigens. An alternative explanation is that the immune response in these patients was directed against DNP-modified HLA antigens and that the tumor infiltrates were a nonspecific response to tumor necrosis, perhaps caused by systemic release of lymphokines, such as tumor necrosis factor. However, this scenario seems unlikely, since intense T-cell infiltrates were frequently seen in the absence of necrosis. Moreover, coadministration of other potent antigens that could elicit a release of lymphokines, e.g., Bacillus Calmette-Guérin, does not cause inflammation in distant tumor sites (3).

It appears more likely that a T-cell-mediated immune response against melanoma-associated antigens was facilitated by the "helper" effect of the anti-hapten response. As hypothesized by Mitchison (12) and as demonstrated experimentally (14), T-cell helper activity induced by a hapten is antigen nonspecific: it can provide help for the generation of cytotoxic T-cell response against an unrelated hapten, virus-infected cells, or tumor cells (24). For example, Fujikura et al. (13) showed in mice that tumor cells haptenized with TNP could induce systemic T-cell immunity against the same modified tumor cells. Spleen cells from these mice completely and specifically prevented the growth of the tumor in untreated recipient animals. Flood et al. (14) found that mice immunized with a syngeneic, TNP-haptenized, UV light-induced "regressor" tumor were able to reject another TNP-haptenized tumor that was otherwise nonimmunogenic and ordinarily grew progressively. More-

Table 1 Metastatic tumors with grossly evident inflammatory responses following treatment with DNP-melanoma vaccine

<table>
<thead>
<tr>
<th>Patient</th>
<th>Site</th>
<th>No.</th>
<th>Mean diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dermal</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>s.c.</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>s.c.</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>s.c.</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>Lymph node</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>Dermal</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>s.c.</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>8</td>
<td>Dermal</td>
<td>&gt;100</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>s.c.</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>s.c.</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>11</td>
<td>s.c.</td>
<td>5</td>
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</tr>
<tr>
<td>12</td>
<td>s.c.</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>13</td>
<td>Lymph node</td>
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<td>120</td>
</tr>
<tr>
<td>14</td>
<td>s.c.</td>
<td>2</td>
<td>15</td>
</tr>
</tbody>
</table>

Fig. 1. Clinically evident inflammatory responses in melanoma skin metastases. (A) Before DNP-vaccine treatment, a typical s.c. metastasis is barely visible as a mass distorting the skin surface. (B) Four months after beginning DNP-vaccine treatment, there is an intense erythema and swelling involving the tumor mass and surrounding skin. (C) One month after beginning DNP-vaccine, dermal metastases are surrounded by a faint rim of erythema. (D) After 4 months of DNP-vaccine, the same nodules are identifiable but are now engulfed by inflammation with erythema and purulent drainage.
HAPTENIZED MELANOMA VACCINE

Fig. 2. (A) Immunohistochemistry of s.c. metastatic melanoma before DNP-vaccine therapy; only rare T-cells defined by anti-CD5 monoclonal antibody (arrows) are observed. After immunization at the time of clinical response (B), viable melanoma is diffusely infiltrated with CD5+ T-cells; other sections (not shown) indicated that most of these T-cells expressed HLA-DR, but not the p55 interleukin 2 receptor (tac, CD25). Adjacent tumor (C) shows marked increase in number of HLA-DR+ dendritic cells which proved to be Leu-M1+ monocyte/macrophages. The majority (75%) of T-cells infiltrating melanoma nodules were also CD8+ (D). Sites of infiltration (D, boxed region) demonstrated enhanced ICAM-1 (CD54) expression by tumor cells (E). CD8+ T-cells frequently aggregated about small nests of tumor ("T") cells at these sites (E, inset). Natural killer cells, as defined by expression of CD56 or CD 16, were rare in both pre- and posttreatment specimens (not shown).

Fig. 3. Flow cytometry analysis of tumor-infiltrating lymphocytes (TIL) from patients treated with DNP-vaccine. Columns, means of 9 patients; bars, SE. The phenotypes of peripheral blood lymphocytes (PBL) obtained from the same patients at the same time are shown for comparison.

CD8/CD4 RATIO

HLA-DR

PBL TIL

PBL TIL

acknowledgments

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references


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