Dendritic Cells Pulsed with an Anti-Idiotype Antibody Mimicking Carcinoembryonic Antigen (CEA) Can Reverse Immunological Tolerance to CEA and Induce Antitumor Immunity in CEA Transgenic Mice

Asim Saha,1 Sunil K. Chatterjee,1 Kenneth A. Foon,2 F. James Primus,3 Sunil Sreedharan,4 Kartik Mohanty,1 and Malaya Bhattacharya-Chatterjee1

1Department of Internal Medicine and the Barrett Cancer Center, University of Cincinnati, Cincinnati, Ohio; 2University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania; 3Department of Pathology, Vanderbilt University Medical Center, Nashville, Tennessee; and 4Titan Pharmaceuticals Inc., South San Francisco, California

ABSTRACT

In this report, we have studied the immunogenicity of the nominal antigen, carcinoembryonic antigen (CEA), and that of an anti-idiotype antibody, 3H1, which mimics CEA and can be used as a surrogate for CEA. We have demonstrated that immunization of CEA transgenic mice with bone marrow-derived mature dendritic cells (DC) loaded with anti-idiotype 3H1 or CEA could reverse CEA unresponsiveness and result in the induction of CEA-specific immune responses and the rejection of CEA-transfected MC-38 colon carcinoma cells, C15. Immunized mice splenocytes proliferated in an antigen-specific manner by a mechanism dependent on the functions of CD4, MHC II, B7-2, CD40, CD28, and CD25. However, immune splenic lymphocytes isolated from 3H1-DC-vaccinated mice when stimulated in vitro with 3H1 or CEA secreted significantly higher levels of Th1 cytokines than did CEA-DC vaccinated mice. DC vaccination also induced antigen-specific effector CD8+ T cells capable of expressing interleukin-2, IFN-γ, and tumor necrosis factor (TNF)-α and displayed cytotoxic activity against C15 cells in an MHC class I-restricted manner. 3H1-DC vaccination resulted in augmented CTL responses and the elevated expression of CD69, CD25, and CD28 on CD8+ CTLs. The immune responses developed in 3H1-DC-immunized mice resulted in rejection of C15 tumor cells in nearly 100% of experimental mice, whereas only 40% of experimental mice immunized with CEA-DC were protected from C15 tumor growth. These findings suggest that under the experimental conditions used, 3H1-DC vaccination was better than CEA-DC vaccination in breaking immune tolerance to CEA and inducing protective antitumor immune responses in this murine model transgenic for human CEA.

INTRODUCTION

Human carcinoembryonic antigen (CEA) is a 180-kDa cell surface and secreted glycoprotein expressed in trace amounts on some normal epithelial tissues but overexpressed in a high percentage of adenocarcinomas, particularly those of the colon, pancreas, breast, and lung (1). CEA present in the serum of patients with CEA-positive tumors has been used to monitor responses to therapy and disease progression. CEA is recognized as self-antigen (Ag) by the immune system, and thus individuals, including cancer patients, are immunologically tolerant to this tumor-associated Ag. However, recent reports have provided evidence that CEA may be immunogenic in humans under defined conditions (2–10). Thus, tolerance to CEA is not absolute and may be overcome, but the immune responses generated have been generally weak because tumors continue to grow and metastasize in these patients. Therefore, active immunization strategies must use potent mechanisms to enhance the anti-CEA immune response to therapeutic levels. A number of different approaches including an anti-idiotype (Id) vaccine approach have been investigated in both human (2–10) and animal studies (11) in an attempt to develop an effective CEA vaccine. Mice expressing human CEA as a transgene have provided a potential preclinical model to assess the induction of anti-CEA immune responses (12, 13). CEA transgenic (CEA.Tg) mice are tolerant to immunization with this self-Ag. Recent studies, however, have documented that when CEA was administered in different immunogenic forms to CEA.Tg mice, tolerance to this self-tumor-associated Ag was overcome as evidenced by the development of CEA-specific MHC-restricted CTL responses, T-cell proliferation, as well as CD4+ T-cell and anti-CEA antibody responses (14–21). The induction of anti-CEA host immune responses also correlated with tumor rejection in vaccinated CEA.Tg mice (14–21).

We demonstrated recently that bone marrow-derived dendritic cells pulsed with anti-Id 3H1 (22), which mimics a specific epitope of CEA, when injected into naive C57BL/6 mice induced both humoral and cellular anti-3H1 as well as anti-CEA immunity (23). The objective of the present study was to assess the ability of bone marrow-derived dendritic cells pulsed with either 3H1 or CEA to induce antitumor immunity in CEA.Tg mice. Results obtained in several animal models have shown that dendritic cells (DC) pulsed with tumor Ags, proteins, peptides, DNA, or tumor lysates are capable of inducing Ag-specific CTL responses, resulting in protection from tumor challenge and regression of established tumors (reviewed in Refs. 24 and 25).

MATERIALS AND METHODS

Mice and Cell Lines. CEA transgenic mice [C57BL/6J-Tg(CEAGe)18FJP], male and female, were obtained from Dr. F. James Primus and also from Sierra BioSource Inc. (Morgan Hill, CA). Expression of CEA in normal tissues of these CEA.Tg mice is restricted to the large intestine and, to a lesser extent, the stomach, similar to the pattern of expression in humans (12). The mice were treated in accordance with Institutional Animal Care and Use Committee guidelines. Age- and sex-matched mice were used for all experiments.

The murine chemically induced colon carcinoma cells, MC-38 (C57BL/6, H-9), and the human CEA-transfected MC-38 clone C15 (C15–4.3) have been described previously (12). YAC-1, a murine NK-sensitive cell line was obtained from American Type Culture Collection (Manassas, VA).

Anti-Id Vaccine and Purified CEA. Generation, purification, and characterization of anti-Id monoclonal antibody (mAb) 3H1, designated as CeaVac have been described previously (22). 3H1 (Ab2) was generated against an anti-CEA mAb, 8019 (Ab1), which reacts with a specific epitope on CEA that is highly restricted to tumor cells and not found on normal tissues. Isotype matched control anti-Id mAb, 1A7 (26), which mimics a melanoma-associated Ag, ganglioside GD2, was also used in this study. Purified CEA was obtained commercially from Fitzgerald (Concord, MA). CEA was isolated from human liver metastasis of colon tumors and purity amounted to >98% by SDS-
PAGE. Murine anti-CEA mAb, 8019 reacted strongly with this purified preparation of CEA.

Generation of Bone Marrow-Derived DC. DC were generated as described previously (23), and these cells were >85% positive for the expression of MHC I, MHC II, CD11c, CD40, CD54, CD80, and CD86 as determined by flow cytometry (data not shown). The data were comparable with the results described previously (23).

Vaccination with Protein-Pulsed DC. On day 8 of culture, DC were incubated with 3H1, 1A7, or CEA overnight at 37°C, in the presence of 50–100 μg/ml of Ag. After loading, DC were extensively washed in PBS, and 2–3 × 10^5 DC/0.1 ml of PBS were injected s.c. in the lower right flank of syngeneic mice. One group of mice received immunizations with unpulsed DC for comparison. Each mouse received three immunizations every other week. Sera were collected from each group of mice after immunizations and were stored at −20°C.

Serum Antibody Responses. Sera from vaccinated mice were tested for CEA-specific antibodies by ELISA as described previously (11). Assays were performed in triplicate for each sample.

Sera from vaccinated mice were tested for Id and epitope analysis of anti-anti-Id antibody (Ab3). Experiments were performed as described previously (11, 23). Assays were performed in triplicate and sera obtained from mice immunized with 1A7-pulsed DC were used as a control in these experiments.

The lytic ability of Ab3 was tested in the presence of effector cells by standard antibody-dependent cellular cytolysis as described previously (11). Briefly, spleens were removed from three to four immunized mice, pooled, and a single cell suspension was prepared by mechanical dissociation. These cells were used as effector cells. MC-38 and C15 tumor cells were labeled with ^31Cr and used as targets. Assays were performed in triplicate wells. Sera from mice immunized with 1A7-pulsed DC or unpulsed DC were used as controls.

In Vitro T-cell Proliferation. Splenocytes were harvested from three mice per group 2 weeks after the final immunization, and T-cell proliferation was measured by [3H]thymidine incorporation (11). Assays were performed in triplicate wells. In select experiments, CD4+ and CD8+ T cells were purified from immunized mice splenocytes by using magnetic beads, according to the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA). The purity of T cells was >90% as determined by flow cytometry (data not shown). These purified cells were used in T-cell proliferation assays. T-cell proliferation was also assessed in the presence of antibodies (10 μg/ml) against the following Ags: CD4, CD8, MHC I, MHC II, CD25, CD28, CD40, and CD86. All antibodies used in blocking experiments were obtained from BD PharMingen (San Diego, CA).

Detection of Cytokines. Cell-free supernatants from T-cell cultures were harvested at 48 h for the presence of interleukin-2 (IL-2), IL-4, and IL-10 or at 72 h for IFN-γ and TNF-α. These cytokines were quantified by ELISA kits (R&D Systems, Minneapolis, MN). All samples were tested in triplicate. The results are expressed in pg/ml.

To measure the production of intracellular cytokines, spleens were harvested from mice 2 weeks after the final immunization. These cells (2 × 10^6/ml) were cultured in the presence of 3H1 or CEA (10 μg/ml) for 2–3 days and GolgiPlugs (1 μM/ml; BD PharMingen) was added to the culture for the last 5 h of incubation. Stimulated cells were washed, stained with either phycoerythrin-conjugated anti-CD4 or anti-CD8 antibody (BD PharMingen), washed, then fixed and permeabilized with Cytofix/Cytoperm kit (BD PharMingen), followed by staining with FITC-conjugated anti-IL-2, anti-IFN-γ, or anti-TNF-α antibody (BD PharMingen), or isotype-matched controls. The cells were then washed twice and subsequently analyzed in a flow cytometer. Naïve mice splenocytes cultured in the presence of 3H1 or CEA were used as controls in these experiments, and these cells were presented as a percentage of positive cells.

Cytotoxicity Assay. Cytotoxicity assays were performed according to the standard protocols (15, 18). Lymphocytes were isolated from harvested spleen of three mice per group 2 weeks after the final immunization, and these cells (2 × 10^6/ml) were stimulated by coculture with 3H1, 1A7, or CEA (10–25 μg/ml) along with 20 units/ml recombinant human IL-2 (Sigma, St. Louis, MO). On day 5, these in vitro stimulated cells were collected as CTL effector cells, and the CTL activity was determined by a standard 6 h ^51Cr release cytotoxicity assay using a variety of target cell lines. Assays were performed in triplicate wells at different effector to target cell ratios as indicated. Spontaneous release was <25% of maximum release. Antibody-blocking experiments with anti-CD8 (53–6.7) or anti-CD4 (GK1.5) mAb (5 μg/ml) and with anti-H-2Kb/H-2D^d or anti-I-A^d mAb (5 μg/ml) were performed as described previously (23).

Flow Cytometry of CTL Culture. Mice immunized with 3H1-pulsed DC or CEA-pulsed DC were sacrificed 2 weeks after the final immunization, and splenocytes were harvested and stimulated by culture with 3H1 or CEA along with recombinant human IL-2 as described above. After 5 days of culture cells were washed, stained, and analyzed in a flow cytometer for the expression of CD69, CD25, and CD28 on CD4+ and CD8+ T cells as described previously (23). Results are presented as percentage of positive cells.

Murine Model for Determining the Efficacy of 3H1 Vaccine. The human CEA-transfected murine colon carcinoma cells, C15, constitutively express CEA in culture. When the CEA-transfected cells (1 × 10^6 cells/mouse) or nontransfected parental cells (5 × 10^5 cells/mouse) were injected s.c. into syngeneic C57BL/6 (H-2b) mice transgenic for CEA, tumor developed in 100% of the mice within 10–15 days. Experiments included 10 mice/group.

Tumor Protection. Mice immunized with 3H1-pulsed DC, CEA-pulsed DC, 1A7-pulsed DC, or unpulsed DC were divided into two subgroups for tumor challenge. Tumor challenge was performed s.c. with 1 × 10^6 of CEA-transfected murine colon carcinoma cells, C15 or 5 × 10^6 of nontransfected parental MC-38 cells in the lower left flank 2 weeks after the final immunization. Mice were monitored on a regular basis for tumor growth and survival, and tumor size was recorded as tumor area (in mm^2). Mice were sacrificed when tumors became ulcerated or when tumor size exceeded 250 mm^2, and survival was recorded as the percentage of surviving animals of total animals on a given day. All experiments were repeated two to three times using individual groups of 10–14 mice.

Statistical Analysis. Statistical analysis was performed using SigmaStat software (Jandel, San Rafael, CA). Differences between groups were analyzed by Student’s t test. The data are presented as mean ± SE. A value of P < 0.05 was considered to be significant. Survival data were analyzed using the method of Kaplan and Meier.

RESULTS

Humoral Response to CEA in CEA.Tg Mice. In our initial studies, we determined whether DC vaccination could induce humoral immune responses in CEA.Tg mice. In these experiments, mice were immunized with 3H1-pulsed DC, and serum samples were analyzed for the presence of anti-anti-Id antibodies (Ab3) by RIA. Ab3 induced in mice was detectable after the first immunization, however the antibody titers peaked after the third immunization (data not shown). Next we tested whether Ab3 induced in mice also contained anti-CEA antibody. We also analyzed whether CEA-pulsed DC vaccinated mice could induce a humoral response to this self-Ag. CEA.Tg mice immunized with 3H1-pulsed DC developed significant anti-CEA IgG antibody titers after three immunizations (Fig. 1A). CEA-DC vaccinations also induced anti-CEA antibody responses in these mice as determined by ELISA. Sera from control 1A7-pulsed DC immunized mice did not induce detectable anti-CEA antibody. Of interest, the anti-CEA antibody titers developed in 3H1-DC immunized mice were significantly higher compared with those in CEA-DC immunized mice (P < 0.001) after the third immunization.

The Id of Ab3 developed in mice immunized with 3H1-pulsed DC was analyzed by the inhibition of binding of Ab1 (8019) to Ab2 (3H1) by mice sera. The binding of Ab1 to Ab2 was inhibited by sera from 3H1-DC immunized mice, and the inhibition was significant (percent inhibition, 35.2 ± 5.1) at 20-fold serum dilution. The binding inhibition of ^125I-labeled 8019 to CEA was also analyzed in the presence of immunized mice sera. Sera from 3H1-DC immunized mice inhibited this binding, and the inhibition was significant (26.2 ± 4.2%) at 20-fold dilution. Sera from CEA-DC-immunized mice demonstrated low levels of inhibition in these assays (data not shown) suggesting that the antibodies generated in these mice were probably directed against different epitopes of CEA or that the titer and affinity of the antibodies were low.
whether T cells from CEA.Tg mice can be primed to induce an tumor cell lysis was Ag specific.

Dendritic cells.

B

Sera were obtained from groups of mice after final immunization and were diluted 1:3 and

A

Results in

Fig. 1. Humoral immune responses developed in CEA transgenic mice. A, induction of anti-CEA IgG serum titers in immunized mice. Mice were immunized three times with 3H1-pulsed DC, CEA-pulsed DC, or 1A7-pulsed DC at 2-week intervals and were bled after each immunization. Sera were diluted 1:20 with PBS and tested for the presence of anti-CEA antibody by ELISA. Data represent mean ± SE of six mice per group. DC, dendritic cells. B, antibody-dependent cellular cytotoxicity by sera from immunized mice. Sera were obtained from groups of mice after final immunization and were diluted 1:3 and 1:5 with PBS. Specific cell lysis was determined by 6-h 51Cr release cytotoxicity assay. Results in A and B are representative of three independent experiments. CEA, carcinoembryonic antigen.

Cytotoxic Activity by Immune Sera. In our subsequent studies we focused on whether the immunization of CEA.Tg mice could also result in the generation of CEA-specific antibody-dependent cellular immune responses. Antibody-dependent cellular cytotoxicity was performed at different serum dilutions to determine whether the anti-CEA antibody generated by DC vaccination is cytolytic for CEA-expressing colon carcinoma cells, C15. As shown in Fig. 1B, significant antibody-dependent cellular cytotoxicity was observed in mice immunized with 3H1-pulsed DC. Antibody-dependent cellular cytotoxicity with sera from mice immunized with CEA-pulsed DC were lower than those measured for the corresponding 3H1-DC-immunized littermates, although this difference was not significant at the serum dilutions tested (P > 0.06). MC-38 cells lacking CEA expression as targets resulted in a background lysis, suggesting that tumor cell lysis was Ag specific.

Ag-Specific T-Cell Proliferation in CEA.Tg Mice. To determine whether T cells from CEA.Tg mice can be primed to induce an anti-CEA response, splenocytes were isolated from different groups of immunized mice and were stimulated in vitro in the presence of different Ags. For a positive control, 1.0 μg concanavalin A was used in these experiments. The results demonstrated that spleen cells from mice immunized with 1A7-pulsed DC or unpulsed DC, failed to proliferate in the presence of CEA Ag (Fig. 2A). By contrast, significant proliferation was obtained in the presence of 3H1 or CEA with spleen cells isolated from mice immunized with 3H1-pulsed DC. CEA-pulsed DC immunized mice splenocytes also proliferated in the presence of CEA and 3H1, although the proliferation in the presence of 3H1 was significantly lower compared with that of CEA (P < 0.001). T-cell proliferation in the presence of CEA was relatively higher in 3H1-DC-immunized mice compared with CEA-DC-immunized littermates (P < 0.009).

We analyzed the subsets of T-cell populations that become responsive by DC vaccinations. As illustrated in Fig. 2B, purified CD4+ T cells and bulk splenocyte populations obtained from 3H1-DC-immunized mice responded to 3H1 and CEA, whereas CD8+ T-cell populations were associated with a pronounced decrease in proliferation (P < 0.0001). It is likely that purified CD8+ T cells were relatively unresponsive in vitro, as CD4+ T-cell help was not provided. Similar results were obtained when CEA-DC-immunized mice were considered for analysis (Fig. 2C).

Involvement of Costimulatory Molecules in the Proliferation of Immune T Cells. To study the role of costimulatory receptors in the induction of T-cell proliferation to Ag, spleen cells from 3H1-DC-immunized mice and CEA-DC-immunized mice were assayed for proliferation in the presence of antibodies to block specific accessory interactions. Antibodies against CD4 and MHC class II Ags, but not against CD8 and MHC class I Ags, blocked the proliferative response of splenocytes to 3H1 (Fig. 2D) and CEA (Fig. 2E). Antibodies against CD25 and CD28 also inhibited Ag-induced T-cell proliferation (Figs. 2, D and E). T-cell response was also abrogated in the presence of antibodies to CD40 and CD86. However, immune splenocytes exposed to Ag for 4 days before the addition of antibodies failed to exhibit sensitivity to the blocking of accessory interactions (data not shown). These findings indicate that proliferation is irreversible once engagement of costimulatory molecules occurs.

Cytokine Production by Activated T Cells. The release of proinflammatory cytokines from T cells is a well-known indicator of T-cell activation in secondary lymphoid tissues and spleen. Specific subsets of activated T cells produce selective sets of cytokines in response to Ag. To determine whether activated T cells produce type 1 or type 2 associated cytokines, T-cell culture supernatants were collected at different time points and cytokines were analyzed by ELISA. A type 1 response was observed, with a significant enhancement of IL-2, IFN-γ, and TNF-α production in the group of mice immunized with 3H1-pulsed DC (Table 1) irrespective of the stimulant (3H1 or CEA) used. Of interest, if mice received the CEA-DC vaccine, and splenocytes were stimulated in vitro in the presence of CEA, the production of IL-2, IFN-γ, and TNF-α decreased by 40–50% over levels observed in mice vaccinated with 3H1-DC and stimulated in vitro under the same culture conditions. The levels of the type 2-associated cytokines IL-4 and IL-10 obtained from the cultures were much lower compared with that of the type 1 associated cytokines (Table 1). These data support the contention that DC pulsed with 3H1 decisively increased T-cell activation in secondary lymphoid tissues.

We were interested in determining the T-cell subset responsible for production of type 1 cytokines in vitro. Spleen cells obtained from mice immunized with 3H1-pulsed DC or CEA-pulsed DC were stimulated in the presence of CEA, and cytokines released by activated T cells were determined by intracellular staining. The data depicted in
Fig. 2. Proliferation of immunized spleen cells in response to antigen in vitro. T-cell proliferation was determined by [3H]thymidine incorporation. Proliferation in the presence of media alone was deducted from values obtained in the presence of different stimulants. A, splenocytes were obtained from groups of mice immunized with indicated proteins. Analysis of subsets of T cells in splenocytes was performed by flow cytometry (data not shown). How- ever, if mice received the CEA-DC vaccine, the production of IL-2 release was determined by flow cytometry (data not shown). Antibodies (10 μg/ml) against the indicated antigens were added to the stimulation culture. Results in A-E are the mean ± SE of one representative experiment of three performed.

Fig. 3A indicate that 18.8%, 16.0%, and 34.0% of CD4+ T cells were positive for IL-2, IFN-γ, and TNF-α production respectively, whereas 16.0%, 22.6%, and 24.9% of CD8+ T cells were positive for IL-2, IFN-γ, and TNF-α production, respectively, when stimulated spleen cells obtained from 3H1-DC-immunized mice were used for analysis. Of interest, similar trends were observed when 3H1-DC-immunized mice splenocytes were stimulated in vitro with 3H1, and cytokine release was determined by flow cytometry (data not shown). However, if mice received the CEA-DC vaccine, the production of IL-2, IFN-γ, and TNF-α decreased as evidenced by lower percent positive cells (Fig. 3B), although the difference was not significant (P > 0.09). Of note, analysis of naive mice splenocytes resulted in baseline expression of intracellular cytokines (Fig. 3C).

Ag-Specific CTL Activity in CEA.Tg Mice. We tested whether DC immunizations of CEA.Tg mice could also generate CEA-specific T-cell cytotoxicity. Splenic lymphocytes were isolated from different groups of immunized mice and stimulated in vitro in the presence of CEA or 3H1 along with recombinant human IL-2 for 5 days, and these cells were used as effector cells. CTLs from mice that received immunization with 3H1-pulsed DC proved to be most effective, inducing up to 75–80% lysis of the CEA expressing murine colon carcinoma cells, C15 (Fig. 4A) compared with 40–50% lysis by CTLs obtained from mice immunized with CEA-pulsed DC (Fig. 4B) irrespective of stimulants used. The difference in lysis was significant at all E:T ratios tested (P < 0.005). In contrast, only background lysis was observed with splenocytes obtained from control mice immunized with 1A7-pulsed DC (Fig. 4C), DC alone (Fig. 4D), or PBS (data not shown). Tumor cell lysis was Ag specific, because the use of MC-38 parental tumor cells lacking CEA expression as targets resulted in background lysis when incubated with CTLs from 3H1-DC-immunized mice (Fig. 4A) or CEA-DC-immunized mice (Fig. 4B) at all E:T cell ratios tested. Lysis of C15 cells with CTLs from all 3H1-DC-immunized mice were significantly greater than that obtained with control targets (P < 0.0001). Similar results were obtained when CEA-DC-immunized mice were used (P < 0.006). Spleen cells from additional nine immunized mice from each individual group produced similar results. The nonspecific killing activity was measured by lysis of natural killer-sensitive YAC-1 cells (data not shown). Immunization of mice with DC alone induced some nonspecific lytic activity, which is typically seen with bone marrow-derived dendritic cells as reported previously (27). Subsequent experiments were performed to establish the Ag specificity of the CTL responses induced in vaccinated mice. Spleen cells from vaccinated mice were stimulated in vitro with 1A7 and tested for lytic activity against C15 or MC-38 tumor cells. Only background lysis of C15 or MC-38 targets occurred when incubated with spleen cells that were exposed to 1A7 in vitro (Figs. 4, A and B).

Additional experiments were carried out to assess whether DC pulsed with protein could process the Ag for MHC class I presentation in immunized mice. In these experiments, DC were used as target cells and immunized mice splenocytes stimulated in vitro with 3H1 or CEA were used as effector cells. As summarized in Fig. 4E, CTLs obtained from

Table 1  Polarization of helper T cells by vaccination with DC" pulsed with 3H1 or CEA"

<table>
<thead>
<tr>
<th>Immunized with</th>
<th>mIL-2</th>
<th>mIFN-γ</th>
<th>mTNF-α</th>
<th>mIL-4</th>
<th>mIL-10</th>
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<tr>
<td>3H1-DC</td>
<td>1760 ± 40</td>
<td>4600 ± 75</td>
<td>720 ± 30</td>
<td>36 ± 8</td>
<td>128 ± 16</td>
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<td>CEA-DC</td>
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<td>1050 ± 25</td>
<td>230 ± 15</td>
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<td>88 ± 12</td>
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<td>1A7-DC</td>
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<td>410 ± 20</td>
<td>90 ± 10</td>
<td>20 ± 4</td>
<td>72 ± 8</td>
</tr>
<tr>
<td>Unpulsed DC</td>
<td>160 ± 8</td>
<td>380 ± 20</td>
<td>80 ± 10</td>
<td>16 ± 4</td>
<td>80 ± 8</td>
</tr>
<tr>
<td>3H1-DC</td>
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<td>4200 ± 80</td>
<td>600 ± 25</td>
<td>36 ± 8</td>
<td>128 ± 16</td>
</tr>
<tr>
<td>CEA-DC</td>
<td>880 ± 20</td>
<td>2175 ± 35</td>
<td>320 ± 15</td>
<td>32 ± 6</td>
<td>104 ± 16</td>
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*DC, dendritic cells; CEA, carcinoembryonic antigen; mIL, mouse interleukin; mTNF, mouse tumor necrosis factor.*

*Splenocytes (2 × 10^7/well) obtained from groups of immunized mice were cultured in the presence of 1.0 μg of 3H1 (A) or CEA (B) in 96-well flat-bottomed microtiter plates in a final volume of 200 μl. Cell-free supernatants were harvested, and cytokine levels were measured by ELISA. Data represent mean ± SE of triplicate cultures. Results are representative of two independent experiments.
3H1-DC-immunized mice could not lyse DC pulsed with 1A7 effectively; however, DC were lysed if pulsed with 3H1 (P < 0.005) or CEA (P < 0.005). Similar specific killing was also observed when DC were pulsed with CEA (P < 0.0001) or 3H1 (P < 0.005) and were incubated with in vitro stimulated spleen cells from CEA-DC-vaccinated mice (Fig. 4F). These data clearly demonstrate that DC vaccination can induce CTL responses in CEA.Tg mice.

DC Vaccination Induced MHC Class I Ag-restricted CTL Response against CEA Expressing Colon Carcinoma Cells. We next determined the effector cell phenotype responsible for specific killing of C15 tumor cells. In these experiments, 3H1-DC- or CEA-DC-immunized mice splenocytes were stimulated in vitro with 3H1 or CEA along with recombinant human IL-2, respectively, and were used as effector cells. CTL activity against C15 cells was significantly inhibited by preincubation of effector cells with anti-CD8 but not anti-CD4 mAb (Fig. 5A). This inhibition ranged from 73 to 88% at all E:T cell ratios tested compared with isotype-matched control antibody (P < 0.0001). The inhibition of CTL activity with anti-CD4 mAb ranged between 12 and 18% (P < 0.005) or CEA expressed CD25 when splenocytes obtained from 3H1-pulsed DC-immunized mice exposed to CEA (Fig. 5B). Of note, 3H1-DC-immunized mice splenocytes exposed to CEA also lysed C15 target cells by MHC class I Ag-restricted manner (data not shown).

Subsequent experiments were performed to determine the MHC class I Ag-restricted CTL specificity for C15 target cells. The data depicted in Fig. 5B demonstrate MHC class I Ag restriction of this CTL response because the presence of mAb directed against I-A^b MHC class II Ags did not inhibit cytolyis. The inhibition of CTL activity with anti-I-A^b mAb ranged between 12 and 18% (P < 0.1). Similar results were obtained by splenocytes from CEA-DC-immunized mice (Fig. 5D). Of note, 3H1-DC-immunized mice splenocytes exposed to CEA also lysed C15 target cells with MHC class I Ag-restricted manner (data not shown).

Up-Regulated Expression of Activation Molecules on T Cells after Anti-Id Pulsed DC Vaccination. We have observed a correlation between the ability of 3H1-pulsed DC vaccine to enhance T cell-dependent immune responses and the increase in expression of T-cell activation molecules. This was evident by increase in expression of CD69, CD25, and CD28 on T cells. The data shown in Fig. 6A demonstrate that 3.3% of CD4^+ and 16.4% of CD8^+ T cells expressed CD69, whereas 2.3% of CD4^+ and 10% of CD8^+ T cells expressed CD25 when splenocytes obtained from 3H1-pulsed DC-immunized mice were stimulated in vitro with 3H1 and used for analysis. Also, 5.7% of CD4^+ T cells were positive for CD28 expression. Expression of activation molecules on both CD4^+ and CD8^+ T cells was similar when 3H1-pulsed DC-immunized mice splenocytes were exposed to CEA in vitro (Fig. 6B). In contrast, expression of CD69, CD25, and CD28 on CD8^+ T cells was significantly lower (36–54%) when splenocytes obtained from CEA-pulsed DC-immunized mice were used for analysis (Fig. 6C).

In Vivo Prevention of CEA-Expressing Tumors in Immunized Mice. It was important to determine whether the induction of CEA-specific host immunity could protect CEA.Tg mice against a challenge with CEA-expressing tumors. Mice were immunized three times with either 3H1-DC, CEA-DC, 1A7-DC, or DC alone every other week and...
challenged with CEA-transfected C15 (Fig. 7A) or nontransfected parental MC-38 (Fig. 7B) tumor cells 2 weeks after the final immunization. Tumor appearance and subsequent growth were monitored regularly. As shown in Fig. 7A, immunization of mice with 3H1-pulsed DC resulted in rejection of C15 tumor growth in 19 of 20 mice (95%) with long-term survival. In contrast, 40% (6 of 15) of mice immunized with CEA-pulsed DC were protected from C15 tumor growth and remained tumor-free till end of study. Of the nine CEA-DC-immunized mice that developed tumors, tumor appeared late and grew slowly when compared with controls (P < 0.05). Mice (n = 15) immunized with 1A7-pulsed DC developed progressively growing tumors, and these mice were sacrificed by week 6. This was similar to the other control group of mice (n = 10) where immunizations were performed by unpulsed DC (Fig. 7A). These results clearly demonstrate that s.c.-injected DC do not induce tumor immunity by Ag-independent mechanisms in this model. Of interest, mice (n = 10) immunized with 3H1-DC, CEA-DC, or control vaccine were not protected from challenge with nontransfected parental MC-38 cells (Fig. 7B), and all of the mice died within 38 days. These data suggest that protective immunity was Ag specific, depending on CEA expression by the target cells.

**DISCUSSION**

One of the major concerns of active specific immunotherapy is to overcome the immune tolerance against tumor self-Ags and the induction of effective antibody and CTL responses that can eradicate tumor metastases and generate long-lasting memory responses to prevent tumor recurrence (28, 29). CEA is one of several self-Ags expressed by human carcinomas that have been identified as potential targets for specific immunotherapy. In this study, we have documented that bone marrow-derived dendritic cells pulsed with an anti-Id antibody, 3H1 can break peripheral T-cell tolerance against CEA and induce protective immune responses, which resulted in rejection of CEA-expressing murine colon carcinoma cells in almost 100% of experimental mice. Under the experimental conditions used, we have documented that in this DC-based vaccination strategy, 3H1 was more effective than its nominal Ag, CEA, in overcoming peripheral tolerance and mediating tumor rejection. Immunization of CEA.Tg mice with 3H1-pulsed DC induced anti-CEA IgG antibody (Fig. 1A). Ab3 induced in these mice inhibited the Ab1-Ab2 binding, and binding of Ab1 to CEA was also inhibited, suggesting that true Ab1-like antibody has been developed by 3H1-DC vaccinations. The anti-CEA antibody response in 3H1-DC-immunized mice was mainly directed against the specific epitope on CEA recognized by the parental mAb 8019 against which mAb 3H1 was raised, whereas the anti-CEA antibody response in CEA-DC-vaccinated mice apparently lacked reactivity against this particular epitope. In comparison to our previous studies in humans, the antibody responses were relatively weak. In our clinical studies, 3H1 was presented after absorption onto...
Fig. 5. MHC class I antigen restriction of the CTL response. Splenocytes from mice immunized with 3H1-pulsed dendritic cells (A, B) or CEA-pulsed dendritic cells (C, D) were harvested 2 weeks after the third immunization and stimulated in vitro with 3H1 or CEA along with recombinant human interleukin-2, respectively, for 5 days. Cytotoxic activity was then measured by 6-h $^{51}$Cr release cytotoxicity assay using C15 as target cells. A and C, antibody-blocking experiments were performed in the presence of anti-CD4, anti-CD8, or isotype-matched control mAb. B and D, antibody-blocking experiments were performed in the presence of anti-MHC class II mAb (I-Ab), anti-MHC class I mAb (H-2K$^b$/H-2D$^d$), or isotype-matched control mAb. Each experiment included six mice. Results are representative of two experiments performed. CEA, carcinoembryonic antigen; mAb, monoclonal antibody.

Fig. 6. Expression of activation molecules by cytotoxic T cells. Splenocytes harvested from 3H1-dendritic cells immunized mice (A, B) or CEA-dendritic cells immunized mice (C) were stimulated with 3H1 (A) or CEA (B, C) along with recombinant human interleukin-2 for 5 days. At the end of the culture, cells were stained and analyzed for the expression of CD4, CD8, CD69, CD25, and CD28 by flow cytometry. Results are a representation of three independent experiments. CEA, carcinoembryonic antigen.
groups (P3H1-pulsed DC or CEA-pulsed DC was significantly longer compared with that of control functions of CD4, MHC class II Ags, costimulatory (CD86, CD28), and activation (CD25) molecules. In addition, the findings that blocking of CD40 inhibits the proliferation, indicates that Ag-specific CD4+ T cells might have been involved in the activation of antigen-presenting cells and thereby priming of CTLs. Similar results were obtained when CEA-DC-immunized mice were considered for analysis. The significant elevation in the production of Th1-associated cytokines induced by our DC vaccination suggests that T-cell activation took place in the secondary lymphoid organs and was involved in the development of cell-mediated immune responses (Table 1). Intra-cellular cytokine analysis reflects that DC vaccination was effective for activation of CD8+ T cells. Nonetheless, the production of pro-inflammatory cytokines by both CD4+ and CD8+ T cells indicates the important role played by these cells in the regulation of effector function.

Previously we demonstrated that vaccination of naïve C57BL/6 mice with 3H1-pulsed DC-induced MHC class I-Ag restricted anti-CEA CTL responses (23). In this study, we demonstrate the induction of anti-CEA CTL responses in CEA-transgenic mice after immunization with DC pulsed with either 3H1 or CEA (Fig. 4). The CTL activities can be blocked by anti murine Kβ antibody. These results suggested the presence of cross-reacting peptide epitopes in 3H1 and CEA capable of binding to murine Kβ molecule. We identified a number of peptides in 3H1, which have linear homology to CEA (3). One of these peptides (LCD-2) contains weak murine Kβ-binding motif (35). Whether LCD-2 or any of these peptides constitute the CTL epitope in this system is currently under investigation. It is important to point out that the overall immune response to CEA in the CEA.Tg mice after CEA-DC vaccination is relatively weak when compared with that generated in mice immunized with 3H1-DC. These differences seem to be inherently predictable, as an anti-Id antibody is an “internal image Ag,” which is expressed in a different molecular environment and may overcome the immunosuppression in the host by stimulating silent clones and/or by allowing T-cell help to become active, making the overall immune response stronger (36). Also, we used intact 3H1 immunoglobulin for pulsing the DC. DC can express a number of Fc receptors (37), which presumably bound the constant chain of immunoglobulin 3H1 and mediated effective endocytosis of the molecule. 3H1 protein was internalized and degraded to peptides by DC. The degraded peptides bound to the MHC molecules were presented to T cells by DC. T cells with appropriate receptors were expanded and constituted the anti-3H1 cytotoxic, helper, and memory cells. Another reason for the increased tumor protection by 3H1 over CEA may rely on the nature of the immunogen per se. 3H1 mimics a specific and protective epitope of CEA, and the predictability of fine specificities of vaccine-induced immune responses to tumors is higher for anti-Id vaccine 3H1 than for Ag vaccine CEA. CEA is an extremely heterogeneous molecule, and CEA-based vaccines presenting multiple epitopes could potentially invoke a mixture of counter-productive immune responses. Similar phenomenon was observed when intact HER-2/neu Ag was used as the immunogen. Lastly, we had some concern about the possible autoimmune side effects of immunotherapy with 3H1/CEA, but within the context of the present study, CEA.Tg mice immunized with DC-based vaccines appeared healthy and maintained normal weight when compared with mice mock-vaccinated with saline. Other reports have also shown that autoimmunity was not detected in CEA.Tg mice treated with different immunogenic forms of CEA (19, 20).

In summary, our studies have shown that anti-Id 3H1-pulsed DC can induce both humoral and cellular immune responses in CEA.Tg mice with protective antitumor immunity. Vaccination with 3H1-pulsed DC was advantageous because it was able to achieve activation of CD4+ T cells and induction of Ag-specific CTL responses. In the clinical situation, vaccination protocols that can elicit cellular and
humoral responses are expected to be more efficient for tumor therapy. Taken together, our results demonstrate that an anti-id-pulsed DC vaccination approach represents a potentially useful method for active immunotherapy of CEA-positive tumors in human cancer patients.

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Dendritic Cells Pulsed with an Anti-Idiotype Antibody Mimicking Carcinoembryonic Antigen (CEA) Can Reverse Immunological Tolerance to CEA and Induce Antitumor Immunity in CEA Transgenic Mice


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