ABSTRACT

Interleukin-2 (IL-2) has been an effective immune modulator in several active-specific immunotherapy experimental protocols using either viral or oncolysate-based vaccines. In this report, data indicate that IL-2 administration can appreciably augment the therapeutic effect of a single immunization of a recombinant vaccinia virus carcinoembryonic antigen (rV-CEA) vaccine using a CEA-expressing syngeneic experimental murine model system. A single rV-CEA immunization of C57BL/6 mice bearing palpable CEA-positive colon adenocarcinoma tumors results in complete tumor regression in approximately 20% of the mice. The addition of a course of low-dose IL-2 results in complete tumor regression in 60–70% of the mice. Moreover, the combination of rV-CEA and IL-2 induces systemic immunity, which protects those tumor-free mice from subsequent rechallenge with the CEA-expressing tumor cells. No such tumor regression or protection was observed in those mice immunized with the wild-type vaccinia vaccine (V-Wyeth) alone or with IL-2 administration alone. Cellular immune assays revealed that the addition of IL-2 to rV-CEA immunization significantly increased the CEA-specific T-cell proliferative responses as well as the cytolytic T-cell responses when compared with rV-CEA immunization alone. The enhanced CEA-specific immune response, coupled with the improved experimental therapeutic outcome following IL-2 administration, suggests that treatment with this cytokine may effectively substitute for multiple rV-CEA immunizations in active-specific immunotherapy clinical protocols directed at CEA-expressing tumors.

INTRODUCTION

IL-2 is a 15,000 glycoprotein cytokine secreted principally by T helper cells that plays an integral role in the homeostasis of the immune system (1). The ability of IL-2 to promote the proliferation and differentiation of T and B cells and to generate and enhance cytokytic activity of natural killers and lymphokine-activated killer cells is well documented (2–6). IL-2 can mediate significant antitumor activity in some patients with renal cell carcinoma, melanoma, and leukemia (7–9). IL-2 administration alone or in combination with an infusion of tumor-infiltrating lymphocytes to some melanoma and renal cell carcinoma cancer patients has been associated with an improved antitumor response that is presumably related to induction of tumor-specific T cells (9, 10). Concomitant studies have been and are currently being carried out to investigate whether IL-2 administration could play a role as part of active immunotherapeutic protocols. The results have shown IL-2 to be a potent activator of immune responses that improve the protective as well as the therapeutic potential of the vaccine (11, 12). In particular, IL-2 has been a potent immune modulator in many viral vaccines, including inactivated rabies; bovine respiratory, foot, and mouth disease; and malaria peptide vaccines (13–16). In those studies, IL-2 administration was shown to improve the protective effects of each vaccine by increasing both humoral and specific cell-mediated cytotoxic responses (11, 17–19). In another study, patients were shown to have a heightened protective response to the influenza virus when influenza vaccines were coupled with subsequent IL-2 administration (20).

Recently, investigators have shown that IL-2 administration can also augment active-specific immunotherapy with vaccinia virus-modified tumor cell lysates (21–23). When using a vaccinia colon oncolysate in combination with either exogenously administered IL-2 (21, 22) or the IL-2 gene encoded as a recombinant vaccinia virus (23), a reduction in the number of hepatic colon metastases as well as an increase in the survival of mice was observed. In a clinical trial that combined a virus-modified tumor vaccine with IL-2, clinical responses were observed in approximately 33% of patients diagnosed with breast or ovarian carcinoma (24). In another murine model in which the tumor expresses β-galactosidase, immunization with β-galactosidase-expressing recombinant vaccinia viruses in combination with IL-2 improved its therapeutic effectiveness (25).

We have previously described a recombinant vaccinia virus that expresses human CEA, designated rV-CEA. CEA is a Mr 180,000 glycoprotein expressed by human colorectal, breast, and lung carcinomas (26). In an experimental model, it was shown previously that three immunizations with rV-CEA protect mice from tumor challenge as well as induce regression of established syngeneic murine colorectal tumors expressing CEA (27–29). Immunization with rV-CEA also induces CEA-specific T-cell responses in mice (30), nonhuman primates (31), and patients with late-stage colorectal carcinoma (32, 33). The present study investigates the CEA-specific T-cell responses in mice immunized with rV-CEA in combination with IL-2 treatment and whether that treatment can mediate the regression of established CEA-expressing murine colorectal carcinomas.

MATERIALS AND METHODS

Cell Culture. The MC-38 (H-2d) murine colon carcinoma cell line was supplied by Dr. Steven Rosenberg (National Cancer Institute, Bethesda, MD). The CEA-expressing MC-38 cells, designated MC-38-CEA-2, were produced by transducing the human CEA gene using the retroviral expression vector pBNC (27). That cell line was subsequently cloned and routinely examined for stable CEA expression, as measured by the cell surface binding of monoclonal antibody COL-1 (34). In the present study, >70% of the MC-38-CEA-2 cells expressed CEA, as determined by routine FACS analysis. FACS analysis also revealed strong MHC class I and the absence of any class II expression on the MC-38-CEA-2 cell surface. Both the MC-38 and MC-38-CEA-2 cell lines were routinely grown in DMEM containing high glucose and 10% heat-inactivated fetal bovine serum.

Recombinant Vaccinia Virus. rV-CEA was produced by Therion Biologics Corp. (Cambridge, MA). The virus has been shown to express human CEA and have safety features, immunogenicity, and antitumor characteristics similar to the recombinant rV-CEA described initially (29, 30). Briefly, the new rV-CEA was produced by homologous recombination of a plasmid (provided by Therion Biologics Corp.) containing the human CEA gene inserted into the HindIII M site of the Wyeth strain of vaccinia virus. The differences between this rV-CEA and that described previously (29) is that CEA expression is now driven from the vaccinia virus early promoter, P0. The new construct does not contain the Escherichia coli Lac Z gene. The analysis of the recombinant virus
was performed as described previously, and CEA was detected by Western blot analysis using the murine monoclonal antibody COL-1 (34). V-Wyeth was obtained from Therion Biologics and was used as the wild-type control.

Both the recombinant rV-CEA and the purified wild-type vaccinia virus (V-Wyeth) (10⁶ pfu) were administered in 10 μl by tail scarification. Immunizations were carried out at either 7 or 10 days after MC-38-CEA-2 tumor cell inoculation. In selected studies, C57BL/6 mice were given repeat immunizations of 10⁷ pfu of rV-CEA or V-Wyeth at 2-week intervals.

In Vivo Studies. Six- to 8-week-old female C57BL/6 (B6; H-2b) mice were purchased from Taconic Farms ( Germantown, NY) and received s.c. injections of 3 × 10⁵ MC-38-CEA-2 (CEA-expressing) cells. At the time of tumor cell inoculation, the MC-38-CEA-2 cells were checked by FACS for the percentage of CEA-positive cells. Using COL-1 reactivity, routine analysis consistently revealed 60–70% of the MC32A cells expressed surface CEA. Mice were checked weekly for tumors. Tumors were measured using micropalcers, and individual tumor volumes were calculated using the following formula:

\[
\text{tumor volume} = (\text{width}^2 \times \text{length})/2
\]

In all experiments, mice bearing tumors >2 cm³ were sacrificed by cervical dislocation for humane reasons, and the day of death was recorded.

IL-2. Recombinant human IL-2 (Proleukin) was generously provided by Dr. Martin Giedlin of Chiron Corp. (Emeryville, CA) and stored at −70°C until use. Specific activity was 1.6 × 10⁷ IU/mg protein, which was rechecked at 3- to 6-month intervals using the HT-2 proliferation assay (27). IL-2 units were calculated as outlined previously (35). Prior to injection, IL-2 was reconstituted with double-distilled water and further diluted to the appropriate concentration with saline containing 1% mouse serum. One hundred μl were administered i.p. In a previous study, results showed that IL-2 was most effective in augmenting specific cytotoxicity when administered 2–5 days after antigen challenge (36). In that regard, IL-2 was administered beginning 72 h after rV-CEA immunization and continued for 5 consecutive days.

Lymphoproliferative Assay. The complete details of the lymphoproliferative assay was published previously (30). The lymphoproliferative response to CEA as well as other stimuli was analyzed 21 days following immunization with rV-CEA or V-Wyeth ± IL-2. At that time, spleens were removed from three mice per treatment group, the cells were mechanically dispersed through 70-μm cell strainers, and the resulting single cell suspensions for each treatment group were combined. Cells were centrifuged over a Ficoll-Hypaque gradient to remove dead cells and erythrocytes, and the T-cell population was enriched by two successive passages over nylon wool columns. Flow cytometric analyses of those cells revealed the following T-cell phenotype: 87% CD3⁺, 32% CD8⁺, and 48% CD4⁺. T lymphocytes were resuspended in complete medium that consisted of RPMI 1640, 15 mM HEpes (pH 7.4), 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 50 μg/ml gentamicin, and 250 μM β-mercaptoethanol. The assay was carried out by incubating the T lymphocytes (1.5 × 10⁵ cells/well) in the presence of 5 × 10⁵ irradiated, naive syngeneic splenic T cells, used as antigen-presentation cells, with various stimuli in 96-well, flat-bottomed plates. In vitro stimulators included UV-inactivated V-Wyeth (2 × 10⁷ pfu/ml), CEA (50–62.5 μg/ml; Vitro Diagnostics, Littleton, CO), and ovalbumin (100 μg/ml; Sigma Chemical Co., St. Louis, MO). After 5 days in culture, the cells were pulsed with [³H]thymidine (1 μCi/well) for 18–24 h. Cells were collected with a Harvester 96 Mach II harvester (Tomtec, Inc., Orange, CT), and the incorporated radioactivity was measured with a 1205 Betaplate liquid scintillation counter (Wallac, Gaithersburg, MD). Results from triplicate wells were averaged and reported as stimulation index (SI) as calculated:

\[
\text{stimulation index} = \frac{\text{cpm(antigen-stimulated cells)}}{\text{cpm(untreated cells)}}
\]

Cytokine Production Assays. T lymphocytes from B6 mice immunized with rV-CEA or V-Wyeth alone or in combination with 0.1 μg or 1.0 μg IL-2 were isolated and grown in vitro in the presence of antigen-presentation cells and stimuli as outlined for the lymphoproliferative assay. Stimuli included 50 μg/ml purified CEA, 50 μg/ml ovalbumin, or media alone. Supernatants from designated wells for each treatment group were harvested at 24, 48, and 72 h and IL-2, IFN-γ, and IL-4 levels were measured. Each cytokine was measured using the appropriate ELISA assay (Endogen, Inc., Cambridge, MA).

Cytotoxicity Assay. Mice were immunized with 10⁷ pfu of either rV-CEA or V-Wyeth alone or followed with a 5-day IL-2 treatment as described previously. The cytotoxic assay used was a modification of a previously described method (30). Approximately 6 days after the final IL-2 treatment, spleens from the different groups of mice were harvested, and T cells were isolated as described previously. The phenotype (i.e. CD3/CD4/CD8) of those T cells was the same as that stated previously for the T cells analyzed in the lymphoproliferative assay. The parental MC-38 and the CEA-positive MC-38-CEA-2 cell lines were prepared for use as targets, and 4 × 10⁶ cells were radiolabeled with 50 μCi ¹¹¹In-oxyquinoline (Amersham, Chicago, IL) for 30 min at 37°C. Target cells were subsequently washed to remove the unincorporated radioactivity. T cells and target cells were combined at different E:T cell ratios in 96-well, U-bottomed plates (Costar, Cambridge, MA) and incubated for 18–24 h at 37°C. Supernatants were then collected with a Supernatant Collection System (Skatron, Inc., Sterling, VA), and the radioactivity was measured in a gamma counter (Cobra Autogamma; Packard Instruments, Downers Grove, IL). Spontaneous ¹¹¹In release was determined by incubation of target cells alone, whereas maximum ¹¹¹In release was measured in supernatants from cells treated with 0.1% Triton X-100. Results from triplicate wells were averaged and reported as the percentage of specific lysis as determined by:

\[
\% \text{ specific lysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100
\]

Statistical Analysis. The differences in the growth rate of the MC-38-CEA-2 tumors that were reflected in the tumor volumes within each treatment group were compared using the Mann-Whitney U test. In studies that compare the survival in different treatment groups, the statistical analysis was done using Kaplan-Meier survival plots and the logrank test. All other statistical analyses were based on Student’s two-tailed t test. All P values reported are two-sided and have not been adjusted for the multiplicity of evaluation performed on the data.

RESULTS

Antitumor Effects of rV-CEA ± IL-2. It was reported previously that three immunizations at 2-week intervals with 10⁷ pfu rV-CEA can induce complete regression of palpable MC-38-CEA-2 tumors in 80–90% of B6 mice (30). We were interested in establishing a suboptimal immunization protocol that would allow the evaluation of combining rV-CEA with IL-2 administration for the treatment of mice bearing syngeneic, CEA-expressing MC-38-CEA-2 tumors. Table 1 illustrates the effects of a single immunization of MC-38-CEA-2A tumor-bearing mice with either rV-CEA or V-Wyeth alone or followed with a 5-day administration of IL-2. Mice bearing palpable MC-38-CEA-2A tumors were immunized with 10⁷ pfu of either rV-CEA or V-Wyeth 7 days after tumor inoculation. As shown, a single immunization with 10⁷ pfu rV-CEA resulted in complete MC-38-CEA-2A tumor regression in 20% of the mice (i.e., 2 of 10 mice; Table 1). The remaining eight mice had progressively growing tumors, however, with a mean tumor volume not statistically different (P = 0.33) from that of the untreated mice. The addition of a 5-day, 1.0 μg IL-2 treatment beginning 3 days after rV-CEA increased the percentage of mice that had complete MC-38-CEA-2A tumor regression. Specifically, treatment of mice with rV-CEA and 1.0 μg IL-2 resulted in complete MC-38-CEA-2 tumor regression in 60% of the mice. Furthermore, the tumor volumes of the remaining four MC-38-CEA-2A tumors were significantly smaller than those of the untreated (P < 0.005) or rV-CEA (P < 0.05) immunized mice. Treatment with a higher IL-2 dose (i.e., 5 μg/injection) revealed no further improvement in the treatment of palpable MC-38-CEA-2A tumors as measured by tumor regression or delay of tumor growth. Immunization with 10⁷ pfu V-Wyeth with or without IL-2 or IL-2 treatment alone also had no comparable reduction on either MC-38-CEA-2A tumor incidence or tumor growth (Table 1).
**R**V-CEA immunization ± IL-2. Table 3 summarizes the lymphoproliferative responses to purified CEA, UV-inactivated V-Wyeth and purified ovalbumin obtained from splenic T cells isolated from untreated mice or mice immunized with rV-CEA or V-Wyeth alone or in combination with IL-2. A specific response to purified CEA was measured using purified splenic T cells isolated from mice treated with rV-CEA, rV-CEA + 0.1, or 1.0 µg IL-2. T cells isolated from mice treated with rV-CEA + 0.1 µg IL-2 had approximately a 2.6-fold higher stimulation index (P < 0.05) when compared with splenic T cells isolated from mice immunized with rV-CEA alone. As expected, splenic T cells from all the mice groups immunized with either rV-CEA or V-Wyeth proliferated when incubated in the presence of UV-inactivated vaccinia virus. Splenic T cells from mice treated with rV-CEA ± IL-2 or V-Wyeth ± IL-2 did not proliferate when incubated in the presence of ovalbumin, indicating specific T-cell responses for CEA as well as vaccinia viral antigens.

**Cytokine Profiles.** It is well established that functionally specific populations of CD4+ T cells secrete *in vitro* distinct sets of cytokines in response to antigen. Th1 cells produce IFN-γ and IL-2, whereas Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 (37). Therefore, IFN-γ, IL-2, and IL-2 were administered to each group i.p. twice/day for 5 days. Weekly tumor measurements were carried out. and mice with tumor volumes >2 cm³ were sacrificed.

**Table 3** Lymphoproliferative responses to purified CEA

<table>
<thead>
<tr>
<th>Group</th>
<th>rV-CEA (10⁷ pfu)</th>
<th>± IL-2</th>
<th>(µg/injection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previously treated</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Previously treated</td>
<td>1 µg</td>
<td>+</td>
<td>± IL-2</td>
</tr>
<tr>
<td>+ ± IL-2</td>
<td>10/10 (100%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* All rV-CEA-treated B6 mice (10/10) bearing MC-38-CEA-2 tumors had progressively growing tumors (see Fig. 1); therefore, none were rechallenged with MC-38-CEA-2 tumor cells. N/A, not applicable.

*b* Represents appropriate age-matched control mice.

**Fig. 1.** Tumor-free survival of B6 mice following treatment with rV-CEA or V-Wyeth alone or in combination with 1.0 µg IL-2. B6 mice (10/group) were administered 3 × 10⁷ MC-38-CEA-2 cells s.c. on day 0. On day 7, mice bearing palpable MC-38-CEA-2 tumors were randomly placed into groups that were then immunized with 10⁷ pfu of either rV-CEA or V-Wyeth via tail scarification. Ten additional mice received no immunization and were used as the untreated, control group (dashed line). IL-2 (1.0 µg/injection) was given i.p. twice/day beginning on day 10 and continuing for 5 days. Weekly tumor measurements were carried out, and mice with tumor volumes >2 cm³ were sacrificed. Results are from a representative experiment; three separate experiments with similar outcomes were performed.

**Table 2** Lymphoproliferative responses to purified CEA

<table>
<thead>
<tr>
<th>Group</th>
<th>rV-CEA (10⁷ pfu)</th>
<th>± IL-2</th>
<th>(µg/injection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previously treated</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Previously treated</td>
<td>1 µg</td>
<td>+</td>
<td>± IL-2</td>
</tr>
<tr>
<td>+ ± IL-2</td>
<td>9/10 (90%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* All rV-CEA-treated B6 mice (10/10) bearing MC-38-CEA-2 tumors had progressively growing tumors (see Fig. 1); therefore, none were rechallenged with MC-38-CEA-2 tumor cells. N/A, not applicable.

*b* Represents appropriate age-matched control mice.

**Table 1** IL-2 effects in combination with a single rV-CEA/V-Wyeth immunization (10⁷ pfu) on the growth of syngeneic MC-38-CEA-2 tumors in B6 mice

<table>
<thead>
<tr>
<th>Immunization</th>
<th>± IL-2 (µg)</th>
<th>No. of mice</th>
<th>Tumor-free mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>10</td>
<td>None</td>
</tr>
<tr>
<td>rV-CEA</td>
<td>0.1</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>rV-CEA</td>
<td>1.0</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>rV-CEA</td>
<td>5.0</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>V-Wyeth</td>
<td>0</td>
<td>10</td>
<td>None</td>
</tr>
<tr>
<td>V-Wyeth</td>
<td>0.1</td>
<td>10</td>
<td>None</td>
</tr>
<tr>
<td>V-Wyeth</td>
<td>1.0</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>V-Wyeth</td>
<td>5.0</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>None</td>
<td>0.1</td>
<td>10</td>
<td>None</td>
</tr>
<tr>
<td>None</td>
<td>1.0</td>
<td>10</td>
<td>None</td>
</tr>
<tr>
<td>rV-CEA (3 times)</td>
<td>5.0</td>
<td>10</td>
<td>8</td>
</tr>
</tbody>
</table>

*a* Female B6 mice were injected s.c. with 3 × 10⁴ MC-38-CEA-2 cells. Seven days later when the MC-38-CEA-2 tumor volumes ranged from 41-55 mm³, the mice were randomized into the groups listed above and immunized with 10⁷ pfu of either rV-CEA or V-Wyeth via tail scarification. Three days after immunization, the indicated amounts of IL-2 were administered to each group i.p. twice/day for 5 days. b Mean and range values represent the measurable MC-38-CEA-2 tumor volumes on day 28.

**Fig. 1.** Tumor-free survival of B6 mice following treatment with rV-CEA or V-Wyeth alone or in combination with 1.0 µg IL-2. B6 mice (10/group) were administered 3 × 10⁷ MC-38-CEA-2 cells s.c. on day 0. On day 7, mice bearing palpable MC-38-CEA-2 tumors were randomly placed into groups that were then immunized with 10⁷ pfu of either rV-CEA or V-Wyeth via tail scarification. Ten additional mice received no immunization and were used as the untreated, control group (dashed line). IL-2 (1.0 µg/injection) was given i.p. twice/day beginning on day 10 and continuing for 5 days. Weekly tumor measurements were carried out, and mice with tumor volumes >2 cm³ were sacrificed. Results are from a representative experiment; three separate experiments with similar outcomes were performed.

**Table 2** Rechallenge of B6 mice with MC-38-CEA-2 (CEA*) tumor cells

<table>
<thead>
<tr>
<th>Group</th>
<th>rV-CEA (10⁷ pfu)</th>
<th>± IL-2</th>
<th>(µg/injection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previously treated</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Previously treated</td>
<td>1 µg</td>
<td>+</td>
<td>± IL-2</td>
</tr>
<tr>
<td>+ ± IL-2</td>
<td>10/10 (100%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* All rV-CEA-treated B6 mice (10/10) bearing MC-38-CEA-2 tumors had progressively growing tumors (see Fig. 1); therefore, none were rechallenged with MC-38-CEA-2 tumor cells. N/A, not applicable.

*b* Represents appropriate age-matched control mice.
IMMUNOTHERAPY WITH A VACCINIA-CEA VACCINE AND IL-2

**T-Cell Lytic Responses following rV-CEA Immunization ± IL-2.** Splenic T cells were isolated from non-tumor-bearing B6 mice as well as from B6 mice given an MC-38-CEA-2 tumor burden (2 × 10⁵ MC-38-CEA-2 cells s.c.). In each case, mice were immunized with either rV-CEA or V-Wyeth alone or with subsequent IL-2 (0.1 µg for 5 days) administration. Six days after the final IL-2 treatment, splenic T cells were isolated and purified, and their primary lytic response was evaluated using the CEA-positive MC-38-CEA-2 and the CEA-negative MC-38 cells as targets. CEA-specific, cell-mediated lysis directed against the MC-38-CEA-2 cells was observed for lymphocytes isolated from nontumor-bearing mice treated with rV-CEA alone or in combination with 0.1 µg IL-2. IL-2 administration resulted in a consistent, however not statistically significant, increase in MC-38-CEA-2 cell lysis when compared with that found in lymphocytes from mice immunized with rV-CEA alone (Fig. 4A). Background lysis, using the non-CEA-expressing, parental MC-38 tumor cells as targets, was approximately 10% (E:T ratio, 200:1) for lymphocytes isolated from mice immunized with either rV-CEA or V-Wyeth alone or in combination with 0.1 µg IL-2 (Fig. 4B). Similar background lysis was observed for lymphocytes isolated from mice immunized with V-Wyeth alone or V-Wyeth in combination with 0.1 µg IL-2 and analyzed using both MC-38-CEA-2 (Fig. 4A) and MC-38 (Fig. 4B) as targets. In mice bearing small, palpable MC-38-CEA-2 tumors, CEA-specific cytolytic activity was detected following rV-CEA immunization (Fig. 4C). Moreover, IL-2 administration resulted in a statistically significant increase in the level of CEA-specific cytolysis (Fig. 4C; P < 0.05). No CEA-specific lytic activity was observed in tumor-bearing mice immunized with V-Wyeth alone or in combination with IL-2 administration (Fig. 4D).

**DISCUSSION**

The present study was designed to determine whether IL-2 in combination with rV-CEA immunization could enhance the CEA-

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**Table 3 Lymphoproliferative response to CEA, V-Wyeth, and ovalbumin from B6 mice immunized with rV-CEA or V-Wyeth alone or in combination with IL-2"**

<table>
<thead>
<tr>
<th>Immunization</th>
<th>± IL-2</th>
<th>CEA</th>
<th>V-Wyeth</th>
<th>Ovalbumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>rV-CEA</td>
<td>None</td>
<td>22.1</td>
<td>35.6</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>+0.1 µg</td>
<td>58.5</td>
<td>53.3</td>
<td>1.4</td>
</tr>
<tr>
<td>V-Wyeth</td>
<td>None</td>
<td>1.8</td>
<td>48.3</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>+0.1 µg</td>
<td>1.5</td>
<td>33.8</td>
<td>0.7</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>0.4</td>
<td>0.7</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>+0.1 µg</td>
<td>0.4</td>
<td>0.6</td>
<td>0.4</td>
</tr>
</tbody>
</table>

*Female mice received a single immunization with 10⁷ pfu of either rV-CEA or V-Wyeth via tail scarification. Three days after immunization, the indicated amount of IL-2 was administered to each group i.p. twice/day for 5 days. Fourteen days after the final IL-2 treatment, all mice were sacrificed, splenic T cells were isolated, and the lymphoproliferative responses to the indicated stimuli were determined as described in "Materials and Methods."*

*Stimulation indices are the means of four separate experiments with a SE of <20%.

*P < 0.05 (versus splenic T cells from rV-CEA-immunized mice).*

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IL-4, and IL-2 production by splenic T cells were examined during stimulation with CEA, V-Wyeth, or ovalbumin following their isolation from mice immunized with rV-CEA or V-Wyeth alone or in combination with IL-2 to determine their Th phenotype. T cells from mice immunized with rV-CEA alone or in combination with either 0.1 or 1.0 µg IL-2 administration produced substantial amounts of IFN-γ (Fig. 3) and IL-2 (data not shown) during a 24–72 h in vitro incubation in the presence of soluble CEA. No comparable levels of IFN-γ or IL-2 production were observed after those same T cells were grown in the presence of ovalbumin or media alone (data not shown). Fig. 3 summarizes the IFN-γ and IL-4 levels measured from T-cell supernatants after 72 h in vitro stimulation with CEA. As shown, CEA stimulation of T cells isolated from nonimmunized mice or V-Wyeth-immunized mice immunized alone or in combination with IL-2 produced low levels of either IFN-γ or IL-4. In contrast, T cells from mice immunized with rV-CEA alone or in combination with 0.1 or 1.0 µg IL-2 produced substantial levels of IFN-γ. However, there were no differences in the amount of either IFN-γ or IL-2 produced from splenic T cells isolated from mice immunized with rV-CEA alone or in combination with IL-2 and stimulated in vitro with soluble CEA. Nonetheless, production of high IFN-γ and IL-2 levels suggest that the CEA-specific proliferation consists of CD4+ cells of the Th1 phenotype as a result of rV-CEA immunization alone or in combination with IL-2 (37).

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**Fig. 3. Analyses of cytokine production during the specific CEA-mediated proliferation of T lymphocytes isolated from either nonimmunized or immunized (i.e., rV-CEA or V-Wyeth) B6 mice and subsequently treated with IL-2. T lymphocytes were isolated from the indicated groups (2–3 mice) of B6 mice and incubated in the presence of antigen-presenting cells and 50 µg/ml CEA as outlined in "Materials and Methods." Data illustrate IFN-γ and IL-4 levels measured in T-cell supernatants from each group of mice after 72 h incubation. Results presented are the means from a representative experiment that was repeated once; bars, SD.
specific T-cell response and augment the regression of the CEA-positive MC-38-CEA-2 tumors in a syngeneic murine model. At the time of rV-CEA immunization, B6 mice had small, actively growing MC-38-CEA-2 tumors. Because previous reports suggested that a specific T-cell response to an antigen can develop 2-5 days after immunization (36), IL-2 administration began 3 days after rV-CEA immunization and continued for 5 consecutive days. Therefore, the present study compared the changes in CEA-specific T-cell proliferation, cytotoxicity, and the antitumor response of mice immunized with either rV-CEA or V-Wyeth alone or in combination with IL-2. The results clearly showed that IL-2 administration following rV-CEA immunization can induce: (a) complete regression of small, palpable MC-38-CEA-2 tumors in 60-70% of mice; (b) prolonged tumor-free survival of those mice; (c) development of systemic immunity as evidenced by the complete protection of those "tumor-free" B6 mice from subsequent MC-38-CEA-2 tumor challenge; and (d) significant delay in the growth of well-established MC-38-CEA-2 tumors. Results from the immunological analyses revealed a significant CEA-specific T-cell proliferative response following a single immunization with rV-CEA that was augmented 2.5- to 3-fold (Table 3) by exogenous IL-2 administration. The T-cell proliferative response to CEA in mice immunized with rV-CEA ± IL-2 was of the Th1 phenotype (Fig. 3). Finally, cytolytic assays indicated significantly greater lytic ability of primary splenic T cells isolated from tumor-bearing mice treated with rV-CEA + IL-2 when compared with spleen cells from mice treated with rV-CEA alone. It should be pointed out that consistently more IL-2 had to be administered to optimally induce tumor regression (i.e., 1.0 µg) than was required to optimally enhance the CEA-specific T-cell proliferative/cytotoxic responses (0.1 µg IL-2). One difference was that the antitumor data was gathered in mice bearing progressively growing MC-38-CEA-2 tumors, whereas the cellular immune response studies were performed in either naive mice or mice bearing small tumors. Although it would be attractive to speculate that the requirement of additional cytokine is required for expanding T cells in vivo and/or to overcome some tumor factor(s), the findings at this time only reinforce the dangers in extrapolating in vitro to in vivo data. In any case, IL-2 treatment following rV-CEA immunization can enhance both CEA-specific T-cell proliferation as well as T-cell-mediated cytotoxicity, which seems to coincide with the ability of rV-CEA + IL-2 to induce not only tumor regression but also systemic immunity from subsequent tumor challenge.

Active specific immunotherapy against weakly immunogenic tumors relies on vaccines containing tumor-associated antigens that have been rendered more immunogenic than those present in the unaltered tumors. Several research groups, including ours, have developed experimental murine tumor model systems in which the expression of a foreign protein, such as CEA (27-30), β-galactosidase (25), and nucleoprotein of vesicular stomatitis virus (38), does not lead to tumor rejection when presented by tumor cells in syngeneic,
immunocompetent hosts. Those experimental model systems have provided the opportunity to examine different approaches to induce as well as augment active-specific T-cell immunotherapy directed toward those foreign antigens. In all three experimental models (25, 30, 35), introduction of a foreign gene into the murine tumor cells was unable to prime specific CTL lysis in vivo. Therefore, the question arises as to whether CEA expression by the syngeneic MC-38-CEA-2 tumors contributes anything to the ongoing immune response following rV-CEA immunization ± IL-2. Our data argue that CEA expression by the MC-38-CEA-2 tumors is insufficient to induce an effector T-cell population that can subsequently be primed by exogenous IL-2 alone. Only when CEA is presented to the immune system in an immunogenic form to nontumor and MC-38-CEA-2 tumor-bearing mice (i.e., recombinant virus) was a CEA-specific T-cell proliferation and T-cell cytotoxicity detected that was further enhanced by IL-2. That result leads one to hypothesize that constitutive CEA expression by the MC-38-CEA-2 tumor cells may transiently activate lymphocytes that upon subsequent rV-CEA immunization results in the detection of significant primary cytolytic activity. Furthermore, the administration of exogenous IL-2 further increased the level of CEA-specific CTL activity, which coincided with the ability of that treatment schema to induce complete MC-38-CEA-2 tumor regression in 60–70% of the mice. Since the CEA-positive MC-38-CEA-2 tumor cells were class II negative, it is possible that a lack of a “second signal,” such as the production of a specific cytokine(s) or expression of a costimulatory molecule(s) (39), may be responsible for the missing specific CTL. Those observations are consistent with the traditional view of differentiation of CD8+ T cells that relies on participation of CD4+ cells to provide “help” in the form of cytokines thought to be necessary for proliferation and acquisition of the lytic machinery. Subsequent studies will focus on the roles that CD4+ and/or CD8+ T cells play in the elimination of palpable MC-38-CEA-2 tumors after rV-CEA + IL-2 treatment in normal and tumor-bearing mice.

We are keenly aware of the limitations of our murine experimental model for the study of T-cell responses to CEA. However, some parallels do exist between our model and the current data on the immunogenicity of CEA in humans. As mentioned previously, although CEA is a foreign antigen in our murine model system, constitutive CEA expression by the MC-38-CEA-2 tumors themselves does not elicit a T-cell response. In both patients with late-stage colorectal cancer and mice bearing MC-38-CEA-2 tumors, immunization with rV-CEA elicits a measurable CEA-specific T-cell response (32). The patient data established that T-cell proliferative response could be detected when CEA was presented to peripheral blood lymphocytes (33). In addition, upon multiple in vitro stimulations with a CEA peptide, a measurable CEA-specific T-cell response resulted. The T-cell lines that were subsequently isolated were shown to be class I MHC restricted in the ability to lyse: (a) CEA-peptide-pulsed autologous EBV-transformed B cells; (b) autologous EBV-transformed B cells transduced with the CEA gene using a retroviral vector; and (c) human carcinoma cells expressing CEA as well as the MHC class I A2 allele (32). Our results suggest that a single immunization with rV-CEA + IL-2 is just as effective in mediating tumor regression and protecting mice from subsequent tumor challenge as the previously published data on three rV-CEA immunizations (30). Perhaps cytokine production following rV-CEA immunization is rate limiting for the optimal induction of a CEA-specific T-cell response. Therefore, exogenous IL-2 may overcome that limitation, as evidenced by significantly improving the frequency of CEA-specific T-cell responses to CEA and the ability of rV-CEA + IL-2 to induce complete tumor regression in 60–70% of mice. Multiple rV-CEA immunizations in patients may be problematical due to the anti-vaccinia antibody that inhibits virus spread (30). Therefore, the inclusion of cytokine administration in context with an immunization schema may further elevate the desired T-cell response to a specific stimulus resulting in overall augmentation in the antitumor response. Subsequent experimental and clinical studies are being designed to address those questions.

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REFERENCES


IMMUNOTHERAPY WITH A VACCINIA-CEA VACCINE AND IL-2


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