**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-carinoembryonic 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 that cytokine may effectively substitute for multiple rV-CEA Immunizations in active-specific immunotherapy clinical protocols directed at CEA-expressing tumors.

**INTRODUCTION**

IL2-2 is a Mr 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 cytolytic 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-2b) 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 Thioneron Biologics and was used as the wild-type control.

Both the recombinant rV-CEA and the purified wild-type vaccinia virus (V-Wyeth) (10^7 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 10^7 pfu of rV-CEA or V-Wyeth at 2-week intervals.

**In Vivo Studies.** Six- to 8-week-old female C57BL/6 (B6; H-2^d) mice were purchased from Taconic Farms ( Germantown, NY) and received s.c. injections of 3 x 10^6 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 microparticle calipers, and individual tumor volumes were calculated using the following formula:

\[
tumor\ \text{volume}\ (\text{mm}^3) = (\text{width}^2 \times \text{length})/2
\]

In all experiments, mice bearing tumors >2 cm^3 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 x 10^7 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 Ficol-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 50 µg/µl β-mercaptoethanol. The assay was carried out by incubating the T lymphocytes (1.5 x 10^5 cells/well) in the presence of 5 x 10^5 irradiated, naive syngeneic splenic T cells, used as antigen-presenting cells, with various stimuli in 96-well, flat-bottomed plates. In *vitro* stimulii included UV-inactivated V-Wyeth (2 x 10^7 pfu/ml), CEA (50–62.5 µg/ml; Viro 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 Harvest 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:

\[
\text{stimulation index} = \frac{\text{cpm(antigen-stimulated cells)} - \text{spontaneous cpm}}{\text{cpm(unstimulated cells)} - \text{spontaneous cpm}} \times 100
\]

**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-presenting 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 10^7 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 x 10^6 cells were radiolabeled with 50 µCi 111In-oxyquinoline (Amer sham, 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 Supernant 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 were 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 10^7 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-2-2A tumors were immunized with 10^7 pfu of either rV-CEA or V-Wyeth 7 days after tumor inoculation. As shown, a single immunization with 10^7 pfu rV-CEA resulted in complete MC-38-CEA-2 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-2 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 10^7 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).
Tu...
IMMUNOTHERAPY WITH A VACCINIA-CEA VACCINE AND IL-2

The present study was designed to determine whether IL-2 in combination with rV-CEA immunization could enhance the CEA-specific cytolytic activity. Figure 3 shows the 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 ng/ml CEA as outlined in “Materials and Methods.” Data illustrate IFN-γ and IL-4 levels measured in each group of mice after 24-72 h incubation. Results presented are the means from a representative experiment that was repeated once; bars, SE.

DISCUSSION

The present study was designed to determine whether IL-2 in combination with rV-CEA immunization could enhance the CEA-specific cytolytic activity.
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 administrated to optimally induce tumor regression (i.e., 1.0 µg) than what 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,
transiently activate lymphocytes that upon subsequent rV-CEA immu
nization results in the detection of significant primary cytolytic ac
tivity. 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 pos
sible 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+
CTLs 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
apalpable 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, al
though CEA is a foreign antigen in our murine model system, con
stitutive 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, immuniza
zion with rV-CEA elicits a measurable CEA-specific T-cell re
sponse (32). The patient data established that T-cell proliferative re
sponse could be detected when CEA was presented to peripheral
blood lymphocytes (33). In addition, upon multiple in vitro stimula
tion 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 immu
nization with rV-CEA + IL-2 is just as effective in mediating tumor regres
sion and protecting mice from subsequent tumor rechallenge 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 evi
denced 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 in
clusion of cytokine administration in context with an immunization
schema may further elevate the desired T-cell response to a specific
stimuli resulting in overall augmentation in the antitumor response.
Subsequent experimental and clinical studies are being designed to
address those questions.

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