Efficacy of Tumor Cell Extracts in Immunotherapy of Murine EL-4 Leukemia

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ABSTRACT

The ability of water-soluble tumor-associated antigens, in combination with chemotherapy, to cause regression of established EL-4 lymphocytic leukemia in C57BL/10 mice was determined. Extracts of EL-4 (G-) cells obtained by solubilization with 3 M KCl (SAE:EL-4) contained tumor-associated antigens as measured by immunodiffusion and inhibition of complement-dependent cytotoxicity using either rabbit or C57BL/10 anti-EL-4 sera.

Tumors were established by s.c. injection of 2 to 5 × 10⁴ ascites-grown EL-4 tumor cells. Twenty-five- or 100-μg quantities of mitomycin C were administered by intraleisional injection 7 days after implantation when the tumors were about 4 mm in diameter. Intraleional injection 2 days later of oil-in-water emulsions containing 150 or 300 μg SAE:EL-4 caused complete regression of tumors in 50 to 80% (p < 0.01 to < 0.001) of treated animals. Administration of 25 or 100 μg mitomycin C without immunotherapy cured 14 of 70 (20%) and 16 of 60 (27%) of treated animals, respectively. Conversely, oil emulsions of SAE:EL-4 when tested without chemotherapy were ineffective. Essentially without value (less than 20% cures) were treatments which included chemotherapy followed by immunotherapy with emulsions containing 3 M KCl extracts of C57BL/10 spleen cells, L1210 tumor cells, or SAE:EL-4 without being incorporated into oil droplets but merely dissolved in phosphate-buffered saline (0.15 M NaCl and 0.01 M NaH₂PO₄-NaHPO₄, pH 7.3). Animals cured with combination therapy rejected a retransplant of EL-4 cells but not a transplant of B-16 tumor cells. High cytotoxic activity against EL-4 cells was detected with intratumor chemotherapy, to cause regression of tumors in 50 to 80% (p < 0.01 to < 0.001) of treated animals. Administration of 25 or 100 μg mitomycin C without immunotherapy cured 14 of 70 (20%) and 16 of 60 (27%) of treated animals, respectively. Conversely, oil emulsions of SAE:EL-4 when tested without chemotherapy were ineffective. Essentially without value (less than 20% cures) were treatments which included chemotherapy followed by immunotherapy with emulsions containing 3 M KCl extracts of C57BL/10 spleen cells, L1210 tumor cells, or SAE:EL-4 without being incorporated into oil droplets but merely dissolved in phosphate-buffered saline (0.15 M NaCl and 0.01 M NaH₂PO₄-NaHPO₄, pH 7.3). Animals cured with combination therapy rejected a retransplant of EL-4 cells but not a transplant of B-16 tumor cells. High cytotoxic activity against EL-4 cells was detected with intratumor chemotherapy.

INTRODUCTION

It is widely accepted that neoplasms, regardless of etiology, have surface membrane components that are antigenically distinct from those of host cells in which they arise, namely, TAA (25, 35). The immune response elicited by these antigens has been postulated to play a major role in the resistance of the host to malignant disease (8). TAA may be either highly immunogenic, which results in the spontaneous rejection of tumors before they become detectable (15), or weakly immunogenic, which results in little immunity, if any, as evidenced by progressive tumor growth and death following an injection of low numbers of tumor cells (24). Several studies have demonstrated an increase in immunogenicity of tumor cells after treatment with neuraminidase (5, 27, 43). Administration of these modified tumor cells elicits tumor-specific immunity and when combined with nonspecific adjuvants, BCG, causes complete regression of small tumors. Therefore, the isolation, purification, and characterization of TAA are important, since purified antigens may be useful in the treatment of cancer.

With the use of techniques developed for the isolation and partial purification of histocompatibility antigens, solubilized membrane fractions from chemically or virus-induced neoplasms have been shown by various in vivo and in vitro assays to possess tumor specificity (3, 7, 13, 30). However, these chemical and physical extraction procedures usually yield soluble tumor antigens in low concentration which, when tested in vivo, require large amounts of antigen to obtain an effective immune response (14). One may speculate that the molecular properties of soluble antigens obtained by these extraction procedures are altered in such a manner that the recovered materials have diminished antigenicity.

The efficiency of the immune response to soluble antigens may be increased by giving adjuvants; e.g., Granger et al. (17, 18) have reported heightened delayed-type hypersensitivity and inflammatory reactions in guinea pigs given i.d. injections of oil-in-water emulsions containing P3 mixed with protein antigens, or BCG cell walls associated with oil droplets. Conversely, no increase in immunogenicity was observed when these components were associated with incomplete Freund's adjuvant or Tween:0.5 M NaCl. Based on these observations, the present experiments were designed to evaluate the therapeutic value of oil-in-water emulsions containing solubilized EL-4 tumor-associated antigens (SAE:EL-4) in the regression of tumor nodules. Our results demonstrate that a single i.t. injection of SAE:EL-4, with or without P3 and associated with oil droplets, was effective in the regression of established tumors after mice bearing EL-4 tumors were treated with chemotherapy. Furthermore, the results indicate that tumor-specific immunity was elicited in animals cured with chemoinmunotherapy, as measured in vivo by their ability to reject a lethal challenge of EL-4 cells and in vitro by the presence of tumor-specific antibodies.

MATERIALS AND METHODS

Mice. Male C57BL/10 (hereafter called B10) (H-2b) mice
(10 to 20 animals per experimental group) were obtained from the specific-pathogen-free production colonies of the Rocky Mountain Laboratory. The mice were 8 to 12 weeks old and weighed more than 18 g on entry in the experiments.

**Tumor Cells.** Although the mouse lymphoma EL-4 was originally induced in a C57BL/6J (H-2b) mouse with 7,12-dimethylbenz(a)anthracene by Gorer (16) in 1945, we have shown that the B10 mouse is a suitable host for this tumor (see "Results"). EL-4 (G-) cells used in this study were provided by Dr. B. Chesbro, Rocky Mountain Laboratory, who originally obtained EL-4 (G-) from Dr. R. Herberman, National Cancer Institute, Bethesda, Md. EL-4 (G-) tumor cells have been shown to be negative for both C-type virus and cell-surface antigens (19).

Tumors were maintained in the ascites form by serial i.p. transfers in B10 males. Tumor cells were harvested from the peritoneal cavity and washed with 20 ml PBS. RBC were lysed with 10 ml of 1% ammonium oxalate. After 30 sec, physiological osmolarity was restored by the addition of 40 ml PBS. Cells were pelleted by centrifugation, and the cell dose was adjusted so that a s.c. inoculum of 2 to 5 × 10^6 cells was administered in 0.1 ml of PBS.

Suspension cultures of EL-4 cells were grown in MEM supplemented with 10% heat-inactivated fetal calf serum and antibiotics (Flow Laboratories, Inc., Rockville, Md.). SAE:EL-4 was prepared by a modified procedure using 3 M KCl extraction according to the method of Meltzer et al. (32). Briefly, 3 M KCl in PBS was added to a cell pellet of live EL-4 cells at a concentration of 5 ml/× 10^6 cells. The suspension was stirred for 18 to 24 hr at 4°C and then centrifuged at 100,000 × g for 2 hr at 4°C, after which the pellets were discarded and the supernatant fluid was dialyzed against distilled water for 24 hr at 4°C. The dialysate was finally centrifuged at 100,000 × g for 2 hr at 4°C to remove the fine gelatinous precipitate that formed after dialysis. The soluble material was then lyophilized. Soluble antigen extracts were also prepared in a similar manner from B10 spleen cells (SAE:B10) and L1210 tumor cells (SAE:L1210).

**Antiserum.** For preparation of heterologous antisera to EL-4 cells, rabbits were given initial s.c. injections of 10^7 cells emulsified in complete Freund's adjuvant into multiple sites on the back. Booster immunizations consisting of 1 to 5 × 10^7 cells in 0.5 ml NaCl were given i.v. at 3-week intervals for 3 months. Animals were bled by cardiac puncture at 7 and 14 days after the last immunization. The blood was allowed to clot at 25°C for 1 hr and then at 4°C for 2 hr. The sera were separated, pooled, and heated to 56°C for 30 min. This antiserum was absorbed 8 to 10 times with equal volumes of freshly prepared and packed normal mouse erythrocytes and spleen cells. After absorption, no agglutinin or cytotoxic activity remained for mouse erythrocytes and spleen sera were separated, pooled, and stored at —70°C.

**Bacterial Components.** Extraction and purification methods of P3, isolated from the wax D fraction of Mycobacterium bovis strain AN5, were performed as previously described (1). P3 was dissolved in chloroform:methanol (95:5) at a concentration of 10 mg/ml before preparation of the emulsions.

**Preparation of Oil Droplet Emulsions.** Oil droplet emulsions containing SAE:EL-4 with or without P3 were prepared as described in detail by Ribi et al. (40). Briefly, varying amounts (2.1 to 0.2 mg) of dry SAE:EL-4 were combined with 0.7 mg of chloroform:methanol-solubilized P3. Following evaporation of the chloroform:methanol, 25 μl of Drakeo 6-VR mineral oil (Pennsylvania Refining Co., Butler, Pa.) were added, and the mixtures were ground in a tissue homogenizer with a Teflon pestle until a uniform paste was obtained. For preparation of emulsions containing SAE:EL-4 alone, the oil was added directly to the lyophilized extract. The paste was then homogenized to form an emulsion with 2.8 ml of 0.5 M NaCl and 0.02% Tween 80 detergent (Emulsion Engineering, Inc., Melrose Park, Ill.) in 50 mM NaHPO4-NaH2PO4, pH 7.4.

**Design of Chemotherapy-Immunotherapy Experiments.** EL-4 tumor cells (2 to 5 × 10^6) in 0.1 ml of suspension were inoculated s.c. into male B10 mice on Day 0. Tumor controls received no further treatment. On Day 7, at which time the tumors were approximately 4 mm in diameter, mice were given a single i.l. injection of mitomycin C (Sigma Chemical Co., St. Louis, Mo.). Mitomycin C was diluted to the appropriate concentrations (500 to 25 μg/mouse) in sterile PBS immediately prior to use and administered in 0.2-ml volumes. Some of the mice were randomized into groups to receive emulsions containing SAE:EL-4, P3, or combinations of both agents. These emulsions were given as a single 0.2-ml i.l. injection 48 hr after mitomycin C treatment. Control groups received an i.l. injection of 0.2 ml of oil:Tween:0.5 M NaCl. Additional groups of mice received immunotherapy, SAE:EL-4 alone or in combination with P3, on Day 9 without prior treatment with mitomycin C. Animals in each group were observed daily to determine percentage survival and/or MST.

**Skin Grafting.** Orthotopic full-thickness skin was grafted and scored as previously described (9). Graft recipients were unbandaged on the 7th postoperative day, and the grafts were scored every 3 days for the first 30 days and weekly thereafter.

**Immunodiffusion.** Immunodiffusion was performed according to a modified Ouchterlony technique (36). Ten ml of 1% agarose solution were poured into 60-× 15-mm plastic Petri dishes (Falcon Plastics, Oxnard, Calif.) and allowed to solidify at 4°C. Antiserum and antigen samples were then applied to prepunched wells (2 mm in diameter), and the reaction was allowed to develop at room temperature for 18 to 24 hr.

**Cytotoxicity Test.** Cultured EL-4 tumor cells were labeled with 32P and reacted with heterologous or syngeneic antisera according to the procedure of Kaliss (22). Control sera were obtained from normal rabbits and B10 mice under identical conditions. The target cells, consisting of 10^6 EL-4 cells in 1 ml of MEM containing 10% fetal calf serum, were incubated for 60 min at 37°C with 100 Ci of 32P as sodium chromate. The cells were washed 5 times and finally suspended in serum-free MEM at a concentration of 10^6 cells/ml. Serial dilutions of 0.1-ml volumes of the antisera were prepared in tissue culture plates (Microtest II; Falcon Plas-
tics), and 0.05 ml of labeled EL-4 cells \((5 \times 10^4)\) was added to each well. The plates were covered and incubated at \(37^\circ\) for 30 min. As a source of complement, 0.1 ml of normal rabbit serum, 8-fold diluted with serum-free MEM, was added to each well. The plates were covered and incubated at \(37^\circ\) for 2 hr. The plates were then centrifuged at 1000 rpm for 10 min, and 0.1-ml samples of the supernatant fluid from each well were used to measure the radioactivity released into the medium. The results are expressed as the percentage of specific \(^{51}\text{Cr}\) released using the following formulation:

\[
\% \text{ specific lysis} = \frac{\text{test sera counts} - \text{control sera counts}}{\text{maximum} \ ^{51}\text{Cr} \text{ released} - \text{control sera counts}} \times 100
\]

Maximum \(^{51}\text{Cr}\) release = counts obtained by lysis of \(5 \times 10^4\) target cells with freezing and thawing (approximately 85% of the total counts was released). For determination of the degree of inhibition of cytotoxicity by soluble antigen extracts, SAE:EL-4 and SAE:B10 were serially diluted and mixed with an equal volume (0.05 ml) of the syngeneic or heterologous antisera which had been appropriately diluted to lyse 45 and 95% of the labeled cells, respectively. After incubation for 1 hr at room temperature, labeled target cells and complement were added to the mixture in accordance with the procedure described above.

RESULTS

B10-EL-4 (G−) Tumor Model. The tumorigenicity of EL-4 cells in B10 mice was determined by administering an i.p. injection of \(10^2\) to \(10^8\) viable tumor cells (Chart 1). All of the animals died of progressive tumor growth when inoculated with as few as \(10^2\) cells. The median survival time, which was inversely proportional to the logarithm of the number of cells injected, ranged from 23 to 14 days after an injection of \(10^6\) to \(10^8\) EL-4 cells, respectively.

To determine whether minor histocompatibility differences exist between B10 (H-2\(^d\)) and C57BL/6 (H-2\(^d\)) that are strong enough to induce allograft rejection (9), normal B10 recipients that were immunized with 3 weekly injections of mitomycin C-treated EL-4 (G−) cells, and had rejected a challenge of \(10^6\) viable EL-4 cells, accepted C57BL/6 skin grafts for 120 days (Fig. 1B). Thus, one may speculate that whatever minor histocompatibility differences exist between B10 and C57BL/6 mice are either not expressed on EL-4 cells or are too weak to induce cell-mediated immunity. From these data, we feel that the B10-EL-4 (G−) tumor-host system is a suitable model for the studies described herein.

Soluble TAA in Extracts of EL-4 Tumor Cells. When reacted against specific rabbit anti-EL-4 antiserum, soluble antigen extracts of ascites-grown EL-4 tumor cells (SAE:EL-4) developed one distinct line of precipitation as measured by immunodiffusion (Fig. 2). This single line formed a line of identity with precipitin lines formed by the interaction of rabbit anti-EL-4 serum with concentrated EL-4 culture supernatant and ascites fluid from mice with progressively growing EL-4 tumors. Conversely, no precipitin lines could be detected when SAE:B10 or normal B10 sera were tested against the absorbed rabbit anti-EL-4 serum (data not shown).

Additional studies were done to confirm that the precipitin lines detected by immunodiffusion were due to the interaction of TAA with antitumor antibodies. As shown in Chart 2, rabbit anti-EL-4 serum absorbed with normal B10 cells retained strong cytotoxic activity against \(^{51}\text{Cr}\)-labeled EL-4 tumor cells in the presence of rabbit complement but was not cytotoxic for labeled B-16 tumor cells or normal murine spleen cells. Thus, the cytotoxic activity of absorbed rabbit anti-EL-4 serum was specific. The inhibitory effects of SAE:EL-4 and SAE:B10 on the cytotoxic activity of the rabbit anti-EL-4 sera were assessed. The estimated percentages of inhibition are shown in Chart 2. SAE:EL-4 at a concentration of 10 mg/ml inhibited (64%) the cytotoxic effect of the specific antisera on tumor cells to a statistically significant degree \((p < 0.001)\). The magnitude of inhibition was dependent on the concentration of the extract. No significant inhibition of antibody cytotoxicity was detected with similar concentrations of SAE:B10 when tested under...
identical conditions. Moreover, no inhibition of cytotoxicity was observed when SAE:EL-4 was incubated with allogeneic B10 anti-DBA/2 (H-2\textsuperscript{d} anti-H-2\textsuperscript{j}) antisera (data not shown). Thus, the inhibition induced by SAE:EL-4 was specific and did not inhibit all antibody plus complement cytotoxicity per se. Collectively, these results demonstrate that soluble TAA were present in the 3 M KCl extract of EL-4 tumor cells.

**Effect of Chemotherapy on Established EL-4 Tumors.** In the following studies, mitomycin C was used to minimize the burden of progressively growing tumor nodules in B10. Table 1 shows the percentage cured and the MST of mice given 2 to 5 x 10\textsuperscript{4} EL-4 cells, followed on Day 7 by an i.t. injection of varying doses of mitomycin C when the tumors were about 4 mm in diameter. The majority of untreated mice (37 of 38) died of progressive tumor growth within 23 days after tumor inoculation. High doses of mitomycin C (500 \mu g/animal) significantly decreased the MST when compared to that of untreated mice (p < 0.0002). Similarly, although not significantly, the MST of mice given 250 \mu g of mitomycin C was shorter than that of control animals. Low doses of mitomycin C (25 or 100 \mu g), on the other hand, delayed the onset of death and induced regression in 20 and 27% of the animals, respectively. Similar results were observed with an i.t. injection of 50 \mu g mitomycin C. It is very likely that the deaths induced by 500 \mu g of mitomycin C were due to the toxicity of the drug and not to progressive tumor growth per se.

**Effect of Immunotherapy on Established EL-4 Tumors.** Experiments were done to evaluate the therapeutic value of solubilized TAA (SAE:EL-4) in the regression of EL-4 tumor nodules. One way the efficacy of immunotherapeutic agents can be dramatized is by applying initial chemotherapy which increases MST and cures a small percentage of recipients (24). Therefore, mice with established tumors were treated with 25 or 100 \mu g of mitomycin C and then given an i.t. injection of oil droplet emulsions containing SAE:EL-4 alone or in combination with P3 2 days after drug therapy. The pooled results of 6 experiments are summarized in Table 2. It is clear from this table that soluble membrane preparations derived from EL-4 tumor cells possess high tumor regressive potency. Mice given both immunotherapy (SAE:EL-4 with or without P3) and chemotherapy responded more favorably than did control animals receiving chemotherapy. A significant increase (p < 0.01) was observed in the percentage of chemotherapy-treated mice cured with emulsions containing only SAE:EL-4 when compared to animals given the drug alone. Conversely, no increase in survival (10 to 20% cured) was observed in animals treated with chemotherapy and emulsions containing P3 alone or in combination with extracts of syngeneic spleen cells (SAE:B10) and allogeneic tumor cells (SAE: L1210). Although the percentage of animals cured with 15 \mu g SAE:EL-4 + 50 g P3, in combination with 25 \mu g mitomycin C, was not significantly different from that of the chemotherapy controls, i.t. injections of this dose of SAE:EL-4 + P3 and 100 \mu g mitomycin C were more effective (p > 0.05) than was mitomycin C alone. Maximum percentages of tumor regressions (70 to 80%; p = 0.01) were observed in animals given 100 \mu g of mitomycin C in combination with 150 or 300 \mu g of SAE:EL-4 with or without P3.

Tumor-specific immunity was assessed in animals cured with chemotherapy in combination with 150 or 300 \mu g SAE:EL-4 + P3 by challenging with either 10\textsuperscript{4} EL-4 cells or 10\textsuperscript{6} B-16 tumor cells 2 months after therapy. All of the cured animals (15 of 15) challenged with EL-4 cells rejected the tumor cells, whereas none of the animals (15 of 15) given B-16 cells survived.

Concomitant groups of animals were given immunotherapy without prior drug therapy in order to determine whether the immunostimulants alone would be of therapeutic value. Groups of tumor-bearing mice were given a single dose of mitomycin C.
Regression of EL-4 tumors in mice treated with immunostimulants with or without chemotherapy

All animals were given a s.c. injection of 2 to 5 × 10^4 EL-4 cells on Day 0 followed by chemotherapy on Day 7 and/or immunotherapy on Day 9. Groups 2 to 7 were given 0.5 M NaCl on Day 7. Tumor regression

Material injected into Dose Cured/ % cured p° p'^
Total tumors (pg) total
100 SAE:EL-4 + P3 15 + 50 6/10 60 <0.05 <0.01
100 SAE:EL-4 + P3 150 + 50 29/39 74 <0.001 <0.001
100 SAE:EL-4 + P3 300 + 50 24/30 80 <0.001 <0.001
25 SAE:EL-4 + P3 15 + 50 4/10 40 >0.05 >0.05
25 SAE:EL-4 + P3 150 + 50 15/20 75 <0.001 <0.001
25 SAE:EL-4 + P3 300 + 50 8/10 80 <0.001 <0.001
100 SAE:EL-4 150 17/20 81 <0.001 <0.001
100 SAE:EL-4 300 20/28 71 <0.001 <0.001
100 P3 50 4/20 20 >0.05 >0.05
25 SAE:EL-4 150 10/20 50 <0.01 <0.01
25 SAE:EL-4 300 8/10 80 <0.001 <0.001
25 SAE:B10 + P3 300 + 50 2/10 20 >0.05
25 SAE:L1210 + P3 300 + 50 1/10 10 >0.05
100 None 16/60 27
25 None 14/70 20
None Oil:Tween:saline 3/60 5

a Animals were given an i.t. injection of mitomycin C on Day 7.
b Material was emulsified with oil:Tween:0.5 M NaCl and given i.t. on Day 9.
c Statistical evaluations were determined by χ² distribution for difference with mice given the appropriate dose of mitomycin C.
d Statistical evaluations were determined by χ² distribution for difference with mice given mitomycin C combined with SAE:B10 + P3 and SAE:L1210 + P3.

Table 3
Regression of EL-4 tumors in mice treated with immunostimulants

All animals were given a s.c. injection of 2 to 5 × 10^4 EL-4 cells on Day 0 followed by chemotherapy on Day 7 and/or immunotherapy on Day 9. Groups 2 to 7 were given 0.5 M NaCl on Day 7. Tumor regression

Material injected into Dose Cured/ % cured p°
Total tumors (pg) total
100 mitomycin C 150 7/10 70 <0.001
100 mitomycin C and SAE:EL-4 150 7/10 70 <0.001
2 SAE:EL-4 + P3 150 + 50 0/20 0 NS
3 SAE:EL-4 150 0/30 0 NS
4 P3 50 0/30 0 NS
5 SAE:B10 300 0/10 0 NS
6 SAE:L1210 300 0/10 0 NS
7 Oil:Tween:0.9% NaCl solution 3/60 5

a KCl extracts and/or P3 were emulsified in oil:Tween:0.5 M NaCl and given i.t. Group 1 served as a positive control.
b In Group 1, statistical evaluations were determined by χ² distribution for difference with mice given 100 μg mitomycin C only (see Table 1).
c NS, not significant.

Table 4
Regression of EL-4 tumors in mice treated with immunostimulants solubilized in PBS

All animals were given a s.c. injection of 2 to 5 × 10^4 EL-4 cells on Day 0 followed by chemotherapy on Day 7 and/or immunotherapy on Day 9. Tumor regression

Material injected into Dose Cured/ % cured p°
Total tumors (pg) total
1 100 μg mitomycin C and SAE:EL-4 150 7/10 70 <0.001
2 SAE:EL-4 150 0/20 0 NS
3 100 μg mitomycin C and SAE:EL-4 150 2/20 10 >0.05
4 PBS 0/10 0

a SAE:EL-4 and/or P3 were emulsified in oil:Tween:0.5 M NaCl (Group 1) or solubilized in PBS (Groups 2 and 3) and given i.t. Group 1 served as a positive control.
b In Groups 1 and 3, statistical evaluations were determined by χ² distribution for difference with mice given 100 μg mitomycin C only (see Table 1).
c NS, not significant.

To determine whether oil-in-water emulsions of SAE:EL-4 were required to induce tumor regression, groups of tumor-bearing animals were given an i.t. injection of 150 μg of SAE:EL-4 solubilized in PBS alone or in combination with 100 μg of mitomycin C. The results of such an experiment are shown in Table 4, Groups 5 and 6. No beneficial effect was observed in any of the animals treated with SAE:EL-4 dissolved in PBS.

Cytotoxic Activity of Sera from Cured Animals. The pooled sera from groups of mice cured with chemotherapy or chemoimmunotherapy were evaluated for cytotoxic activity in vitro against 51Cr-labeled EL-4 cells. Chart 3 plots...
DISCUSSION

Several approaches to immunotherapy have been attempted; however, active immunotherapy using specific or nonspecific immunostimulants has been the most successful. Nonspecific immunostimulants have consisted primarily of intact microorganisms such as viable BCG and formalin-killed Corynebacterium parvum (12, 20, 42), or components isolated from these and other microbes (1, 38, 40). Specific immunostimulants used in immunotherapy, however, have been confined to the use of modified or altered tumor cells (5, 27, 43). The principle of specific immunotherapy is based on the observation that tumor cells possess antigens which the host recognizes as foreign and on the immune response elicited by these antigens (2).

Based on the observation that TAA, solubilized from a variety of tumors by various extraction procedures, induce tumor-specific immunity in immunoprophylactic studies (11, 28, 33, 37), the present studies were designed to evaluate the therapeutic value of solubilized tumor-associated antigens in regressing established tumors. The EL-4 tumor model was used since TAA have been isolated by 3 M KCl extraction (6). In addition, previous studies have shown the efficacy of nonspecific immunostimulants when combined with chemotherapy in the treatment of animals with progressively growing EL-4 tumors (26). The results reported in this paper clearly demonstrate the therapeutic value of i.t. administration of soluble TAA in causing the regression of established EL-4 tumors when combined with i.t. injection of mitomycin C. The therapeutic effect of soluble antigens was dependent on pretreating tumors with mitomycin C and the association of the antigen with oil droplet emulsions.

Simmons et al. (44) reported tumor regression of tumor-bearing animals with specific immunotherapy (neuraminidase-treated tumor cells). Complete regression was limited to small tumor burdens. In other tumor-host systems, the beneficial effect of immunostimulants was dependent on reducing the tumor burden with chemotherapy (21), surgery (41), or irradiation (45). Our results are consistent with these findings in that cure rates as high as 80% (Table 2) were observed in tumor-bearing mice given chemotherapy followed by a single i.t. injection of oil-in-water emulsions containing soluble tumor antigens, whereas no tumor regression was seen in animals given immunotherapy only (Table 3). While the interval between chemotherapy and immunotherapy was evaluated at only one time point (2 days), the optimal time to administer immunostimulants in other tumor systems has been reported to be between 1 and 2.5 days (26, 27). In addition, some experimental work demonstrates that specific immunotherapy given before chemotherapy provided no therapeutic benefit (12) or resulted in enhanced tumor growth (29). Studies designed to evaluate the optimal time interval between mitomycin C treatment and immunotherapy with tumor antigen emulsions are currently in progress.

The observation that animals cured of EL-4 tumors with combination chemoimmunotherapy rejected a rechallenge of EL-4 cells but not transplants of unrelated but syngeneic B-16 tumors suggests the development of tumor-specific immunity. Kollmorgen et al. (27) have reported similar results in which complete rejection of rechallenge transplants was observed in chemotherapy-treated mice cured of L1210 leukemia with neuraminidase-treated tumor cells combined with viable BCG. Similarly, guinea pigs cured of...
line 10 hepatocarcinoma with oil droplet emulsions containing nonviable microbial agents rejected a retransplant of line 10 cells but not transplants of unrelated tumors (34).

Additional evidence for tumor-specific immunity in animals successfully cured of EL-4 tumors by i.t. injections of mitomycin C alone or in combination with solubilized TAA prepared in oil-in-water emulsions was assessed in vitro by complement-dependent cytotoxicity. Cytotoxic activity against EL-4 cells was detected in the sera of animals cured with either chemotherapy or chemoimmunotherapy (Chart 3). However, the percentage of specific lysis was significantly higher in sera from animals given chemotherapy combined with high-dose immunotherapy compared to sera of animals treated with chemotherapy only. Conversely, no cytotoxic activity was detected when their sera were reacted against B-16 tumor cells. These observations are consistent with previous reports demonstrating tumor-specific, humoral immunity in mice cured of leukemia by treatment with neuraminidase-treated tumor cells in combination with chemotherapy (10).

An important finding reported here was that successful immunotherapy with soluble, tumor-associated antigens depended on the use of the antigen in oil-in-water emulsions. Previous studies from our laboratory have demonstrated the efficacy of nonspecific immunostimulants (nonviable microbial agents) in the treatment of established line 10 tumors in strain 2 guinea pigs (1, 34, 38). Beneficial immunotherapy depended on combining the microbial components with P3, a nonimmunogenic, adjuvant-active component of mycobacteria, and on using these agents in a 1% oil emulsion. No antitumor activity was observed when these components were suspended in 0.5 M NaCl and administered under identical conditions. Granger et al. (18) established that P3 facilitates the binding of bovine serum albumin to oil droplets. Moreover, they demonstrated enhanced cell-mediated immunity after immunization with protein antigens (bovine serum albumin and purified protein derivative of tuberculoprotein) associated with P3 in oil-in-water emulsions. Conversely, no enhanced immunogenicity was detected when these antigens were suspended in 0.5 M NaCl or emulsified in Freund's type adjuvant.

Kelly et al. (23) reported that P3 enhanced the binding of Coxiella burnetii to oil droplets which, in turn, resulted in enhanced tumor regressive activity. Moreover, McLaughlin et al. (31) have found that P3 and similar molecules quantitatively enhanced binding of radiolabeled microbial extracts to oil droplets, thereby producing emulsions with antitumor activity. P3 analogs incapable of enhancing the binding of extracts to oil produced emulsions without antitumor activity. However, materials such as BCG cell wall will interact with oil droplets in the absence of P3 and are active in producing tumor regression, although, when combined with P3, their antitumor activity was enhanced (39).

The present study supports these earlier findings in that effective immunotherapy was observed only when the soluble antigen was emulsified in oil droplets, alone or in combination with P3. No antitumor activity was detected when the soluble tumor antigen was dispersed in PBS and administered to chemotherapy-treated animals (Table 3). However, these data differ from the earlier studies in that oil emulsions containing SAE:EL-4 had antitumor activity without being associated with P3. We found that the soluble antigen extract adhered to the oil droplets without the aid of P3, producing a characteristic granular appearance as assessed by microscopic observation (4). A similar profile was observed with oil-in-water emulsions containing soluble antigen + P3. Although the mechanism(s) of tumor regression induced by tumor antigen emulsions is not known, one may speculate that the oil droplets of the oil-in-water emulsion provide a depot for the antigen, from which it is rather slowly released. Following i.t. injection, the tumor-antigen-oil droplet complex may be retained longer at the site of injection than antigens not associated with oil droplets (4). The resulting persistent source of antigenic stimulation favors the production of tumor-specific immunity. Alternatively, the association of tumor antigen with oil droplets may enhance their immunogenicity. Whatever the mechanism, it is clear from these results that soluble TAA emulsified in oil have antitumor activity and induce tumor-specific immunity when combined with chemotherapy.

The fact that soluble tumor cell extracts were effective in the regression of established EL-4 tumors raises an important question as to the nature of the immunogen. Are the immunostimulants tumor-specific antigens, viral-associated antigens, or a combination of both? From the present study, one cannot completely rule out viral-associated antigens or tumor antigens as the specific immunogen. However, the use of extracts from EL-4 (G-) cells, which have been shown to lack C-type viruses and cell surface viral antigens (19), minimizes the possibility that viral-associated antigens are playing a major role in the regression of EL-4 tumors. Isolation and characterization of fractionated components of SAE:EL-4 should provide information on the specificity of the antigen required for successful immunotherapy.

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22. Kaliss, N. Micromethod for assayig immune cytolyis by the release of 


Fig. 1. Examples of long-surviving skin grafts between C57BL/6 donors and B10 recipients. The upper animals (A) depict 120-day-old skin grafts on normal B10 recipients. The lower animals (B) exhibit 120-day-old grafts on B10 recipients following immunization with 3 weekly injections of 10⁶ mitomycin C-treated EL-4 (G⁻) cells and rejection of 10⁵ viable EL-4 cells.

Fig. 2. Immunodiffusion patterns developed by absorbed rabbit anti-EL-4 sera (α) reacting with: 1, concentrated EL-4 culture supernatant; 2, 3 M KCl extract from EL-4 cells (SAE:EL-4); 3, ascites fluid from mice with progressively growing EL-4 tumor.
Efficacy of Tumor Cell Extracts in Immunotherapy of Murine EL-4 Leukemia

John L. Cantrell, Charles A. McLaughlin and Edgar Ribi


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