Preparation of Immunotherapeutic Autologous Tumor Cell Vaccines from Solid Tumors

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ABSTRACT

Comparisons of mechanical and various enzymatic methods of disaggregation of solid tumor were made for the purpose of producing a tumor cell suspension, which would be effective as an immunotherapeutic, autologous tumor cell vaccine when admixed with Bacillus Calmette-Guérin (BCG). Transplantable L10 hepatocarcinoma, syngeneic in strain 2 guinea pigs, grows in either ascites or solid form. The solid tumor grown in the muscle was used for evaluation of dissociation procedures. Trypsin, collagenase type I, collagenase type III, collagenase type I combined with hyaluronidase, and mechanical procedures were used under various conditions, and the cell suspensions were compared for cell yield per g tumor, percentage of viability, and membrane tumor-specific antigen. Of the methods tested, the enzymatic procedure utilizing collagenase type I exhibited the greatest potential to dissociate solid tumor with the least destruction or loss of tumor cell surface antigen. A vaccine of BCG admixed with irradiated solid tumor dissociated L10 cells had a therapeutic effect on subclinical, disseminated micrometastasis. Inbred strain 2 guinea pigs given i.v. injections of either 10^4, 10^5, or 10^6 syngeneic L10 hepatocarcinoma cells were treated with the BCG plus tumor cell vaccine after metastatic foci were established in the lung parenchyma. The results show that under defined conditions of preparation and administration, the nontumorigenic vaccines of BCG and tumor cells dissociated from solid tumors effect a highly significant survival rate in animals bearing otherwise lethal visceral metastasis.

INTRODUCTION

The success of immunotherapy in experimental animals and in humans is limited by tumor stage. Immunotherapy is most effective when administered as an adjunct to surgery of the primary tumor and when there is only minimal regional lymph node metastases and micrometastatic disease in visceral organs. Nonspecific immunomodulation to enhance immune reactivity toward disseminated minimal residual cancer has been attempted clinically with such microbial vaccines as Mycobacterium bovis strain BCG. The unsuccessful or equivocal results of these problematic, although feasible clinical protocols may be partly attributable to the fact that most positive trials of nonspecific immunotherapy in animal systems involved relatively antigenic transplantable tumors, whereas human cancers may be less antigenic.

More fundamental information is needed on the efficacy of induced, specific cell-mediated tumor immunity for treatment and control of minimal residual cancer. Active specific immunotherapy, although recognized as biologically sound, has been burdened by technical limitations which must be overcome to be practical in the laboratory or in the clinical setting. Also, the basic insights into active specific immunotherapy should be derived from an experimental animal model based not on prevention of metastasis but on elimination of established micrometastasis.

Utilizing the syngeneic L10 hepatocarcinoma in inbred strain 2 guinea pigs, we have demonstrated that a BCG-plus-irradiated ascites L10 tumor cell vaccine can induce systemic immunity capable of eliminating disseminated visceral micrometastasis (6, 7). The vaccine is effective in both normal and BCG-immune guinea pigs and will boost BCG-induced specific tumor immunity in cured tumor-resistant guinea pigs. We have shown that autogenous tumor immunity, developed in the absence of immunopotentiators (a more clinically applicable condition), can also be boosted with the BCG plus tumor cell vaccine. Vaccines of BCG alone or admixed with an antigenically distinct syngeneic tumor (L1) were ineffective against the L10 tumor, indicating that the therapy achieved in this experimental model is the result of tumor-specific immunity.

Significant and reproducible immunotherapy is achieved when the BCG-plus-tumor cell vaccines are prepared under defined conditions. Frozen L10 ascites cells are as effective as were freshly harvested L10 cells only so long as they are cryobiologically preserved, stored, and thawed, with a final vital-stain membrane integrity after X-irradiation, approximately that of the unmanipulated ascites L10. Although 20,000 R destroy the tumorigenicity of the L10 cell, the cell retains its level of immunogenicity upon i.d. injection with BCG. Tumor cell metabolism and limited cell division continue for several days after i.d. injection. This metabolic potential may be vital for a potent vaccine and may account for the poor efficacy of nontumorigenic low-viability tumor cell vaccines (2).

Although the efficacy of the vaccine prepared from ascites cells is extremely encouraging, most human tumors are available in solid form. The purpose of the present study was to develop methods of dissociating solid tumor that would yield the largest number of viable cells per g tumor without destroying the immunogenic potential of the cells.

Utilizing the solid L10 tumor grown i.m., we designed studies to test various modes and conditions of solid tumor disaggregation. The resulting cell suspensions were monitored for: (a) sterility of preparation; (b) cell yield per g

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tissue dissociated; (c) percentage of viability by vital stain; (d) cryobiological preservation and recovery; (e) metabolic activity and viability of tumor cells after 20,000 R X-irradiation; (f) cell surface antigen utilizing tumor-specific antibody and immunofluorescence; and (g) immunotherapeutic potential in vivo as a nontumorigenic vaccine used to immunize animals with established disseminated micrometastasis.

MATERIALS AND METHODS

**Animals.** Inbred male Sewall Wright strain 2 guinea pigs were obtained from the Frederick Cancer Research Center Animal Production Area, Frederick, Md. These guinea pigs were shown to be histocompatible by skin grafting. They were housed 7 to 8 per cage and fed Wayne chow and kale; they weighed 400 to 500 g at the beginning of the experiments.

**Tumors.** The L10 hepatocarcinoma was induced in strain 2 guinea pigs by diethylnitrosamine feeding as described previously (16). The antigenic and biological properties of the transplantable ascites tumors developed from the primary hepatocarcinomas have also been described (20). To establish disseminated micrometastasis in various visceral organs of the experimental animals, freshly harvested ascites cells were washed 3 times in HBSS, and 1-ml cell suspensions containing 10⁴, 10⁵, or 10⁶ L10 cells/dose were injected into the dorsal penile vein. Injections of 10⁴ cells were fatal in 70 to 80% of the animals, whereas 10⁵ and 10⁶ cells were fatal in all animals. The time of death varies with the dose. All animals died from metastases in the lung, in the mediastinal and tracheobronchial lymph nodes, and in the visera. For the production of L10 solid tumor, 5 × 10⁶ L10 ascites cells in 0.5 ml volume were injected into the gastrocnemius muscle of adult guinea pigs.

**BCG.** *M. bovis* strain BCG (Phipps strain TMC 1029) was obtained from the Trudeau Institute (Saranac Lake, N. Y.). Preparations of BCG, stored in the gas phase of a LR-301A Linde liquid nitrogen freezer, were rapidly thawed in a 37°C water bath, diluted to proper concentrations, and administered within 2 h.

**Enzymes.** Collagenase type I (190 units/mg), type III (720 units/mg), DNase (1790 Kunitz units/mg), and hyaluronidase (300 units/mg) were purchased from Sigma Chemical Co., St. Louis, and trypsin, twice-crystalline (>10,000 BAEE units/mg) was purchased from Grand Island Biological Co., Grand Island, N. Y.

**Disaggregation of L10 Solid Tumor.** Tumors were surgically excised when they were approximately 1 to 3 cm in diameter. The guinea pigs were killed by CO₂ asphyxiation, and the flank bearing the solid tumor was shaved. The cadaver was soaked first in Wescodyne, then soaked in 70% ethyl alcohol, and placed on a sterile drape in a laminar flow hood. All surgical procedures were sterile. The tumors were carefully excised and transferred immediately to 150-mm Petri dishes containing CMF-HBSS or HBSS. Excessive tissue was removed; the tumor was minced in Petri dishes held on a tray of crushed ice covered with autoclaved foil. Care was taken that scalpel and forcep pressure was not excessive. Using 2 curved-edge scalpels (Becton-Dickinson No. 20 Disposable) in a cross-cutting motion, the tumor was sliced into pieces approximately 2 to 3 mm across their greatest dimension. The fragments were thoroughly rinsed in CMF-HBSS or HBSS (pH 7.0 to 7.4).

The enzymes used in these experiments had pH optima in or near the physiological range. The enzymes were weighed, added to media (CMF-HBSS or HBSS) containing 500 Kunitz units (0.03%) DNase per ml, and then filtered through 0.8- and 0.2-μm Nalgene membrane filters. Before being added to the tumor tissue, the enzyme media were warmed to room temperature or 37°C. A 37°C water bath and immersible magnetic stirrers were used for 37°C tissue dissociations.

Tumor fragments were placed into 75-ml trypsinizing flasks into which 20 to 40 ml of enzyme medium was then added. The tissue was stirred for 20 to 30 min at a speed which caused tumbling of the tissue but did not produce foaming. After incubation, the flask arm was flamed and free cells were decanted through 3 layers of rinsed, sterile, medium-wet nylon mesh (166T; Martin Supply Co., Baltimore, Md.) into a 50-ml centrifuge tube. The tube was then filled by rinsing the filter with either cold HBSS or CMF-HBSS. The cells were spun at 1250 rpm or 225 × g in a refrigerated centrifuge for 10 min. The supernatant was removed by suction, and the cells were resuspended in 5 to 10 ml of DNase, 0.03% in HBSS or CMF-HBSS for 5 min. The test tubes were again filled with the appropriate chilled media and spun, the supernatant was removed, and the cells were resuspended in 10 ml of HBSS containing 10% heat-inactivated FBS. The above disaggregation process was repeated for 2 to 5 incubations.

Tumor fragments that were dissociated mechanically were placed in a tissue sieve (E. C. Apparatus, St. Petersburg, Fla.), which was partially submerged in medium, and pressed through the stainless steel mesh with the glass plunger of a syringe. The cells were filtered and processed in the same way as the enzyme-dissociated cells.

Cells were counted from each digest. Those cells excluding trypan blue (0.2% in HBSS) or those that were positive when stained by fluorescein diacetate (17) were defined as viable or metabolically active.

**Stability Test.** Three-ml samples were taken from all media used in the dissociation process, including enzyme and freezing reagents. Also, samples of supernatants of the final tissue wash and the final pooled tumor cell wash were taken. These samples were injected into Vacutainer tubes containing 20 ml of peptone broth-supplemented culture medium (Becton-Dickinson, Rutherford, N. J.). These peptone broth samples were microbiologically evaluated for the presence of any bacteria or fungi. Any organisms present were identified. Of 16 separate enzyme dissociations of solid tumors, 14 were uncontaminated. The 2 contaminated samples were from final cell washes of dissociated cells, and were contaminated with Group D streptococcus.

**Quantitative Immunofluorescent Measurements of Tumor Antigen on L10 Cells.** Rabbit anti-L10 antiserum was kindly provided by Dr. T. Borsos, National Cancer Institute, Bethesda, Md. This serum was first absorbed with guinea pig RBC, then absorbed in vivo in 3-day-old guinea pigs, and finally absorbed 3 times with L1 ascites cells. Normal rabbit serum, which was used as a control serum, was also...
absorbed as described above. Serum specificity tests were
done using an indirect immunofluorescence assay, and
relative fluorescence intensities were measured in a micro-
scope cytophotometer. L10 cells (1 x 10^8) were fixed lightly
with 1% formalin in CMF-HBSS for 5 min at 5° and washed
twice with HBSS. The cells were resuspended in 0.1 ml of
HBSS and incubated with 0.1 ml of a 1:4 dilution of
absorbed rabbit anti-L10 serum for 30 min at 5°. The
samples were washed twice and then incubated with a 1:50
dilution of fluorescein-conjugated goat anti-rabbit IgG
(Cappel Laboratories, Inc., Downington, Pa.) for 30 min at
5°. The samples were washed twice and fixed with 1%
formalin for 5 min at 5°, and then washed and resuspended
in 3 ml of HBSS. The samples were examined visually in a
Zeiss photomicroscope III equipped with a photometer, and
relative fluorescence intensities of 100 to 200 cells were
measured. The rest of the sample was analyzed in a cyto-
fluorograph (Ortho Instruments; Model 4800A, S/N 1238)
where fluorescence intensities of about 10,000 cells were
measured. The data were recorded directly on tape and
histograms of the median log channel were determined.
The histograms obtained were analyzed statistically. Re-
corded in this manner, relative fluorescence increases as a
function of channel number. Approximately 1 x 10^8 total
cells are recorded in each run through the cytofluorometer;
thus, the median log channel times the reciprocal of per-
centage of fluorescent cells per total cell population is used
to determine relative fluorescence per total population of
cells. Control ascites cells and normal rabbit serum were
used as controls for every run. All samples were checked
microscopically for clumping and fluorescence intensities.
The results of the specificity tests are seen in Table 1.

**Vaccine Preparation.** Tumor cell vaccines were prepared
either from the dissociated solid L10 tumor or from L10
ascites cells. For the latter, at the time of maximum ascites
(approximately 10 to 12 days after injection), the cells were
aseptically removed with 19-gauge needles, placed into 30-
ml syringes, washed 3 times in HBSS, and used either fresh
or frozen-thawed. For the solid tumor vaccine, the tumors
were taken at 2 weeks after i.m. transplantation, a time
when the tumor is well established and sufficiently vascu-
larized with little necrosis. The tumor was dissociated with
pronase, trypsin, interstitial fiber-specific collagenase, and
collagenase with hyaluronidase. The potential alteration of
cell surface moieties and particularly

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<th>Table 1</th>
<th>Indirect immunofluorescence assay of rabbit antiserum</th>
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<td>Cells</td>
<td>Serum</td>
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<td>L10 ascites</td>
<td>Anti-L10</td>
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<td>L10 asces</td>
<td>Normal rabbit</td>
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<tr>
<td>L1 ascites</td>
<td>Anti-L10</td>
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*Mean ± S.E.*

have been discussed recently (2, 11). On the day of vacci-
nation, the vials were rapidly thawed in a 37° water bath.
Frozen-thawed cells were diluted slowly to 50 ml in HBSS,
ashed once, and resuspended in preparation for X-irradia-
tion. Suspensions of fresh and/or frozen-thawed cells were
X-irradiated in 50-ml beakers on ice at 500 R/min for a total
of 20,000 R. X-irradiation was performed with a Phillips MG-
301 X-irradiation unit. Cell viability counts were performed
after X-irradiation using the trypan blue dye exclusion test.
The cells were diluted to 50 ml, centrifuged, and resus-
pended at 10^6 viable cells/ml. BCG (10^6 viable organisms/ml)
was added in equal volumes for a vaccine ratio of 10:1
viable organisms to viable cells. A vaccination consisted of
an i.d. injection of 0.2 ml. All vaccinations were performed
less than 2 hr after the BCG-tumor cell mixtures were
prepared.

**Test for Immunotherapeutic Efficacy of Vaccine Prepara-
tions.** On Day 0, 135 male strain 2 guinea pigs (400 to 500
) were randomized and housed 7 to 8 per cage. The
animals were divided into 3 groups of 45 each and were
iven i.v. injections of 1-ml doses of either 1, 10^5, or 10^6
L10 ascites cells, respectively, into the dorsal penile vein to
produce artificial metastases. On Day 1, each group was
subdivided into 3 groups of 15 guinea pigs each. Each of
these groups of 15 guinea pigs was given an i.d. injection
(0.2 ml) of 10^8 BCG viable organisms admixed with 10^7
irradiated L10 ascites cells or 10^8 BCG viable organisms
admixed with 10^7 irradiated L10 dissociated solid tumor
cells, or no vaccination, respectively. The vaccine was
given on the upper right quadrant and repeated on Day 7
on the upper left quadrant. The guinea pigs were held and
monitored for mortality, and survival differences were sta-
tistically analyzed by the 2-tailed Fisher’s exact test.

**RESULTS**

**Mechanical Disaggregation of L10 Solid Tumors.** Me-
chanical disaggregation of L10 solid tumors is a rapid
procedure. Although the yield of cells per g tumor was
relatively high compared to most enzymatic procedures, the
viability of cells as measured by trypan blue exclusion
was 10% (Table 2). This eliminates mechanical dissociation
as a practical procedure for tumor cell vaccine prepara-
sion, since yields of approximately 4 x 10^7 cells/g would be
limiting when vaccines require 10^6 cells/vaccination. Also,
cell suspensions of such low viability would probably re-
quire gradient separation to isolate the viable cells.

**Enzymatic Alteration of L10 Tumor Cell Surface Anti-
gens.** Among the enzymes commonly used in dissociation
of solid tumor are pronase, trypsin, interstitial fiber-specific
collagenase, and collagenase with hyaluronidase. The po-
tential alteration of cell surface moieties and particularly
Comparison of mechanical and enzymatic procedures for disaggregation of L10 solid tumor

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<tr>
<th>Medium</th>
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<tr>
<td></td>
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<td>Total cells/g tumor (x10^6)</td>
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<tr>
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<td>Viability (%)</td>
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<tr>
<td>Mechanical</td>
<td>HBSS</td>
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<td>0.14% trypsin</td>
<td>CMF-HBSS</td>
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<td>0.14% collagenase type I</td>
<td>HBSS</td>
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<td>0.14% collagenase type III</td>
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<tr>
<td>0.01% collagenase type I</td>
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<td>+ 3000 units hyaluronidase</td>
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Table 2

Comparison of mechanical and enzymatic procedures for disaggregation of L10 solid tumor

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the elimination or destruction of tumor cell surface antigens by such enzymes are major concerns, since cell surface alterations could render the isolated cells ineffective as a specific immunogen. The first experiment shown in Chart 1 was designed to test the alteration of antigen concentration on L10 cell surfaces by various enzyme treatments. We tested twice-crystalline trypsin (greater than 10,000 BAEE units/mg at pH 8.1 and 25°C), collagenase type I (190 units/mg solid), a crude preparation containing some proteolytic contaminants and collagenase type III, a purified collagenase Fraction A (720 units/mg solid). Pronase, an enzyme of generally higher substrate activity, was not tried since there are no pronase inhibitors in serum (5, 15), and it has been shown that, in contrast to trypsin, pronase cannot be washed from the surface of kidney cells and continues to damage the cell surface (15). L10 ascites cells were used to determine parameters of enzyme treatment to be used for preparation of viable, single-cell suspension from solid tumors. The ascites cells were incubated at room temperature or 37°C for 30, 60, or 90 min in CMF-HBSS containing 0.02% EDTA and DNase (500 Kunitz units/ml). Calcium and magnesium play a role in intercellular binding (1, 9), and DNase inhibits the formation of nucleic acid gel in enzyme-dissociated cell suspensions.

The cytofluorographic analysis of 10,000 cells per enzyme treatment is shown in Chart 1. No significant difference in cytofluorographic curves was related to incubation time (30, 60, or 90 min). Also, the enzyme effect on L10 antigen for a given enzyme was the same at room temperature or 37°C. Compared to non-enzyme-treated cells, the relative immunofluorescence per cell population was increased as a function of channel number. Approximately 1 x 10^6 cells/enzyme treatment group were recorded in the cytofluorometer. The median log channel, enzyme type, and percentage of fluorescent cells per population is indicated for each curve (treatment group).

**Comparison of Enzymatic Procedures for Disaggregation of L10 Solid Tumors.** The combined data of several experiments comparing enzymatic procedures for disaggregation of L10 solid tumors are presented in Table 2. The dissociation potential of trypsin, collagenase type I, collagenase type III, and collagenase type I plus hyaluronidase was evaluated on the basis of cell yield per g tumor and percentage of cell viability. Various collagenase type I concentrations were tested, and media were modified for calcium and magnesium, protease contaminants, and temperature. The treatment of L10 cells with trypsin led to a 4-fold reduction in immunofluorescence. Relative to non-enzyme-treated cells, both collagenase type III and type I significantly reduced L10 cell surface antigens. Of the collagenase type III-treated cells, 86.3% were assessed in the cytofluorograph with a median log channel of 1.04, and 74.4% of the collagenase type I cells were assessed with a median log channel of 1.15. With respect to tumor antigens, both collagenase-treated cell populations were significantly higher than trypsin-treated population but significantly lower (2.26- and 1.73-fold) than the non-enzyme-treated cell population.
temperature. Solid tumors were removed 1 to 3 weeks after i.m. transplantation. The tissue was carefully sliced into 2- to 3-mm fragments. Scalpels were changed frequently. A 5-min predigest of the tumor fragments in 0.14% collagenase was performed with gentle stirring. The supernatant from this predigest contained mainly dead cells and was discarded. The tissue fragments were then rinsed thoroughly with medium. For the purpose of quantitation, 2-g samples were digested. Except where otherwise indicated 4, 20- to 30-min digests in approximately 30 ml of enzyme medium per digest were performed. Evaluations are based on total cell yield per g tumor and viability of cells in suspension.

Predigested tumor fragments incubated at 37° with 0.14% trypsin in CMF-HBSS containing DNase and EDTA yielded 52 × 10^6 cells/g tumor with 96% of the cells viable. A 0.5-g tumor dissociated with trypsin would yield ~2 × 10^7 viable cells, thus providing the minimum number (assuming intact cell surface antigen) of cells required for active specific immunotherapy in this experimental model. With collagenase type I, tumor fragments from the same pool and under the same disaggregation conditions yielded 39 × 10^6 cells/g with a 94% cell viability. With collagenase type III, the yield was 18 × 10^6 cells/g and cell viability was 67%. Although both cell yield per g tumor and percentage of viable cells were much less with collagenase type III than with trypsin, the difference between collagenase type I and trypsin was small. However, for cellular immunity the 4-fold reduction effect of trypsin on ascites cell surface antigen, as compared to collagenase type I, technically abrogates the marginally superior dissociation potential of trypsin.

Dissociations at 37° and room temperature were compared for both collagenase type I and type III. This approximately 10° reduction in temperature reduced cell yield per g tumor more than 50% for both enzymes with a corresponding decrease in percentage of cell viability.

Collagenase type I contains some protease and peptidase activity, while collagenase type III is substantially free of these contaminants. Dissociations with both collagenase type I and type III were performed with 10% FBS (a protease inhibitor) in the medium. Cell yield per g was reduced 21 × 10^6 for collagenase type I and 7 × 10^6 for collagenase type III.

In general, collagenase type III was much less effective than collagenase type I with respect to total cell yield per g tumor and percentage of viability, and although collagenase type III had less effect on cell surface antigen than did collagenase type I as determined by cytofluorometric measurements, the lower dissociation potential of type III rendered the enzyme inappropriate for use in these experiments.

CMF medium and a chelating agent (EDTA) are commonly used in enzyme tissue dissociations (1, 9). However, conflicting reports exist as to the applicability of these conditions when collagenase is the enzyme being utilized (3, 10). We therefore compared collagenase type I dissociation of L10 solid tumor at 37° in CMF-HBSS with EDTA or HBSS. No significant difference was seen in cell yield per g or percentage of cell viability; however, the number of digests required for tumor fragment dissociation for equivalent cell yield per g was reduced by one-half in HBSS; 2 as compared to 4 incubations. There were fewer cell clumps in the HBSS preparation, and the addition of FBS did not inhibit tumor dissociation under these calcium- and magnesium-augmented conditions. Utilizing this more catalytic system (HBSS medium and 37° temperature), an attempt was made to determine the lower limits of concentration of collagenase type I. As shown, 0.1% collagenase type I was as effective as 0.14%; however, there was a decrease in tumor cell yield per g as well as percentage of viability of cells when the enzyme concentration was less than 0.1%. For this L10 hepatocarcinoma, the addition of hyaluronidase to the lower collagenase concentration was ineffective.

Chart 2 shows the cytofluorometric analysis of a cell suspension yielded from collagenase type I dissociation of L10 solid tumor and L10 ascites preparation. Both cell suspensions were frozen and thawed prior to cytofluorometric analysis. The L10 ascites preparation had a median log channel of 1.66 with 98.9% of the cells recorded in the cytofluorograph. This dissociated cell preparation had a median log channel of 1.3 with 84.6% of the cells scored in the cytofluorograph, a 2.65-fold reduction in antigenicity as measured by immunofluorescence. It is interesting to note that the median log channel of 1.3 for the dissociated solid tumor cell suspension is identical to the median channel for the L10 ascites control incubated, with stirring, in trypsinizing flasks for 90 min as shown in Chart 1, thus indicating that a major loss of antigen in any dissociated solid tumor preparation may be attributed to the mechanical damage inflicted by stirring in the trypsinizing flasks rather than solely the effect of the enzyme.

Efficacy of BCG-plus-Ascites L10 Cell Vaccines and BCG-plus-Dissociated Solid Tumor L10 Cell Vaccine for Therapy of Micrometastasis. Guinea pigs were given i.v. injections of 10^6, 10^7, or 10^8 L10 cells and treated at 1 and 7 days by i.d. immunizations with BCG-plus-L10 tumor cell vaccines. Fifteen animals from each tumor burden group were immunized with either 10^6 BCG admixed with 10^7 irradiated ascites L10 cells or 10^6 BCG admixed with 10^7 irradiated L10 cells dissociated from solid tumor. The L10 ascites cells used for vaccine were a combination of both frozen and freshly harvested cells, with a trypan blue
exclusion index after irradiation of 97 and 95%. The L10 solid tumor-dissociated cells used for vaccines were a combination of 5 lots of frozen cells with a trypan blue exclusion index after irradiation of 82% and 84%. There were a few small clumps of aggregated cells and some cellular debris in these inocula. Results of survival in these treated and untreated guinea pigs are shown in Chart 3.

In animals given i.v. injections of $10^4$ L10 cells, 80% of the untreated controls died by 156 days after injection. In contrast, all animals immunized at 1 and 7 days with either $10^8$ BCG plus $10^7$ irradiated L10 ascites cells or $10^8$ BCG plus $10^7$ irradiated L10 solid tumor dissociated cells survived. Thus, there was significant protection in both treatment groups as compared to the untreated controls ($p = 0.0001$ and $<0.0001$, respectively). Although solid tumor vaccine gave fewer survivors (10 of 15) than the ascites vaccine (14 of 15), this difference was not significant.

In animals given i.v. injections of $10^6$ L10 cells, all untreated animals died by Day 63 after injection. The 50% lethal dose was less than 50 days. The number of deaths was the same (12 of 15) for each of the immunized groups. A nonstatistically significant shift in survival curves can be noted in this graph.

Overall survival achieved with immunizations with either BCG plus L10 ascites cells or BCG plus dissociated L10 solid tumor cells was statistically significant compared to untreated controls ($p < 0.00001$ for both treatment groups), and no significant difference overall could be determined between the 2 treatment groups.

**DISCUSSION**

Using a transplantable hepatocarcinoma in syngeneic...
strain 2 guinea pigs, we have demonstrated previously that established micrometastatic tumor foci in the lung can be eliminated by specific tumor immunity induced by the systemic effect of a BCG-plus-tumor cell vaccine (6, 7). These studies demonstrated that, under defined conditions of vaccine preparation and administration, nontumorigenic preparations of BCG plus tumor cells injected i.d. can cure the majority of animals of otherwise lethal visceral metastasis. No protection was achieved, however, when another syngeneic but antigenically distinct hepatocarcinoma was used in the vaccine, thus suggesting that this was active specific immunotherapy.

This mode of active immunotherapy has great appeal as an adjunct treatment to surgical excision of primary cancers in humans because of its specificity and low toxicity. Since all of our prior animal tumor studies used a convenient ascites form of tumor, which would not be available in humans, we carried out the present studies with a surgically excised solid form of the tumor, which would be more relevant to the problem of autologous tumor vaccine preparation in humans.

For tissue culture or cytological diagnosis, proteolytic enzymes are quite useful in preparing cell suspensions without irreversible damage to viability or morphology. However, these proteolytic enzymes can alter cell membranes so that surface charge, proteins, and glycoproteins are modified or eliminated (4, 18, 19). When the immunogenic properties of cells are to be maintained, mechanical dissociation is often used instead of enzyme treatment. The present study showed that the cell yield per g of tumor is relatively high with mechanical dissociation, but the viability of the dissociated cell population is low, generally around 10%. This introduces the possibility that the viable cell population resulting from mechanical dissociation may be a unique cell population rather than a representative selection of the solid tumor with respect to cell properties and antigenicity.

On the whole, careful enzymatic digestion of the L10 solid tumor with 0.14 to 0.1% collagenase type I plus DNase in either HBSS or CMF-HBSS at 37° was an efficient dissociation procedure with respect to total viable cell yield per g solid tumor and retained level of tumor antigen per cell population. With regard to the latter, there was a 2.65-fold reduction in total tumor antigen as compared to non-enzyme-treated L10 ascites cells. Trypsin digestion caused a 4-fold reduction in detectable L10 tumor antigen. Collagenase-dissociated cells from a L10 solid tumor were still efficacious when used with BCG in a vaccine to treat animals with established disseminated micrometastasis. In animals with an initial range of tumor challenge between 10^4 and 10^6 cells, significant overall survival (p = 0.00001) was achieved when they were treated with tumor cells admixed with BCG in a designed regimen of immunization. The approximate 3-fold reduction in tumor antigen mediated by collagenase type I enzymatic digestion as compared to non-enzyme-treated L10 ascites cells was associated with some reduced immunogenicity in vivo manifested by a decreased survival of animals at the 10^6 initial tumor burden. However, this difference was not statistically significant. Nevertheless, this emphasizes the need for enzymatic dissociation procedures which, while capable of providing the quantity of cells required for immunization, are also sufficient to yield the highest quality of cells with respect to integrity of type and levels of tumor antigen. We recognize that the particular enzymatic procedure used in the present study would not necessarily apply to tumors of different histological type. Also, regardless of which of the enzymes are used in the in vitro dissociation, it is uncertain to what degree the metabolically active tumor cells may regenerate some or all of the tumor cell surface antigens during prolonged survival in the cutaneous immunization site.

Various other prerequisites for preparation profoundly influence the efficacy of the vaccine in the inbred guinea pig immunotherapy model. The 2 most important variables are dose of BCG and the viability of injected tumor cells. It was shown that significant protection can be achieved only with 2 vaccinations separated by 1 week and that the initial immunization requires 10^7 or greater viable BCG organisms admixed with 10^7 tumor cells. Tumor cell viability above 85% in the final vaccine preparation is a requirement. Thus, any manipulations of the immunogenic preparation for cryobiological preservation or X-irradiation in order to develop nontumorigenic vaccines would have to incorporate these 2 major prerequisites. Using ascites L10 cells in the guinea pig experimental model, we have demonstrated that critical cryobiological preservation and 20,000 R X-irradiation of L10 cells can be accomplished and still maintain approximately 90% tumor cell viability at the time of immunization.

The method of cryopreservation of the tumor cells used in preparation of the vaccine is critical. In the guinea pig model, tumor cells that were frozen by an established procedure used for preservation of bone marrow in transplantation studies and assessed as an optimal procedure in several low-temperature biology studies (for review, see Ref. 12) were as effective in the vaccines as fresh ascites tumor cells. This is in contrast to results in the same model by Bartlett et al. (2) who, using a less critical cryopreservation procedure (11) and achieving lower cell viability as a consequence of freezing and thawing, induced minimal cell-mediated tumor immunity in vaccine protection studies. A clinical study which we feel emphasizes the need for critically performed cryopreservation and vaccine preparation was reported by McIlmurray et al. (13, 14). In a controlled trial of active specific immunotherapy for Stage IIIB malignant melanoma, 8 of 15 patients were treated with 1 vaccination (over multiple sites). The preparation included Glaxo BCG and autologous irradiated tumor cells. Over a 24-month observation period, 6 of the 8 vaccinated patients developed recurrent melanoma and 5 died; 5 of 7 controls had recurrences and 3 died. At the 12-month time point, there was a suggestion of tumor enhancement in the vaccinated patients. The overall results indicate that active specific immunotherapy as performed in these patients was ineffective. The procedure for preparing the autologous tumor cell vaccine differed from the one described in the present study in that the irradiated tumor cells, admixed with BCG in phosphate-buffered saline (0.05 M NaH2PO4, Na2HPO4, and 0.12 M NaCl, pH 7.4), were frozen at ~1°/min in liquid nitrogen. The vaccine was rapidly thawed at 37°, and equal doses were injected i.d. in each limb and just
below the umbilicus, each of the 5 sites receiving 10^7 tumor cells and 6 x 10^6 BCG organisms. This irradiation and freezing procedure in our hands, using the L10 cells and freezing only in phosphate-buffered saline, results in greater than 90% cell death after the vaccine is thawed. These determinations on L10 cells were made by both fluorescein diacetate and trypan blue dye exclusion. On the basis of the results in the guinea pig model, we would have to assume that the single immunization, although at multi

ple sites, and the possible lack of viability as a result of insufficient cryopreservation abrogated the immunogenic potential of the BCG-plus-melanoma vaccine.

In another clinical study, Hedley et al. (8) treated patients with Stage IIB melanoma monthly with irradiated allogeneic melanoma cells and BCG, while the control patients received only BCG. Sixteen patients in the treatment group had a median relapse-free interval of 5 months compared to 8 months in the 12 controls given chemotherapy and BCG. The authors concluded that immunotherapy composed of irradiated allogeneic melanoma cells as used in this study did not prolong survival in surgically treated patients with Stage IIB melanoma and may even have promoted early, local relapse. The guinea pig model is in agreement with this study since syngeneic tumor (L1) used in a vaccine with BCG was ineffective in protecting animals with established disseminated L10 metastasis. We have an indication then that the immunity induced by the vaccine in the guinea pig model is tumor specific. The L1 hepatocarcinoma, although syngeneic in strain 2 guinea pigs, is a regressor tumor upon i.d. challenge of 10^6 cells in contrast to the progressive growth of Lb. Since, in this tumor model the growth pattern and host response to the L1 tumor may be analogous in some respects to that seen with allogeneic tumors, some of the clinical procedures using allogeneic tumor cells should be questioned.

Our results are relevant to the major issues of active specific immunotherapy. Most human immunotherapy trials have been performed with what these results suggest are suboptimal vaccine preparations. The implication of the present study is that any negative clinical or animal experiments with suboptimal vaccine preparations should not be considered definitive and should be repeated with optimal vaccine preparations before any conclusions can be reached. The present experiments provide important leads to the development of an optimal vaccine preparation to be used for active specific immunotherapy utilizing solid tumors surgically excised under standard treatment. This experimental model in guinea pigs now lends itself to studies of combinations of modalities such as chemotherapy, surgery, and active specific immunotherapy.

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Preparation of Immunotherapeutic Autologous Tumor Cell Vaccines from Solid Tumors

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