TREATMENT BY LIMITED SURGERY AND SPECIFIC IMMUNIZATION OF GUINEA PIGS WITH STAGE II EXPERIMENTAL CANCER

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A majority of guinea pigs, each with a growing syngeneic tumor implant in the skin and no other outward sign of malignancy (clinical stage I disease) but with tumor cells in the regional lymph nodes, was cured by tumor-specific immunization after limited surgery consisting of excision of the dermal tumor (1, 2). Animals treated by limited surgery alone were not cured; they developed palpable tumors in the draining lymph nodes, and at autopsy, 70-80% of the animals were found to have pulmonary metastases (3). We now report the results of studies to test the efficacy of limited surgery and tumor-specific immunization in the treatment of animals, each with a dermal tumor and a palpable draining lymph node (clinical stage II disease). We also tested limited surgery and immunization for the treatment of animals with clinical stage II disease and pulmonary tumors that had been implanted intravenously. Information from these studies may be useful in the design of clinical investigations to evaluate proposed treatments for humans with cancer.

Materials and Methods

Animals. Male Sewall Wright strain 2 guinea pigs 3-4 mo old and weighing 500-550 grams were obtained from the Laboratory Aids Branch, Division of Research Services, National Institutes of Health (NIH) Bethesda, Md., and from the Experimental Animal Breeding Facility of the National Cancer Institute, Frederick Cancer Research Center, Frederick, Md. Caged in groups of six, the animals were maintained on a diet of NIH guinea pig ration and filtered tap water, which were available to the animals at all times.

Tumor Line. We used a transplantable, syngeneic hepatocellular carcinoma—designated line 10 (L10)1—in transplant generations 9-19. This diethylaminoinduced tumor has been converted to ascites form and is passed intraperitoneally in weanling strain 2 guinea pigs. Inoculation of 106 L10 tumor cells intradermally in the flank 2 cm posterior to the axillae of animals that are left untreated leads to progressive intradermal tumor growth, metastasis of tumor cells to draining lymph nodes, and death. At autopsy, 30-40% of the animals are found to have gross, visible pulmonary metastases (3). Limited surgery designed to remove the dermal tumor and to leave microscopic lymph node metastases in place is not curative, but prolongs survival of the guinea pigs; at autopsy, the majority of them are found to have pulmonary as well as lymph node metastases (3).

Lymph Nodes. In the descriptions that follow, the lymph nodes are listed in the order of their...
drainage pattern. The superficial distal axillary (SDA) lymph node is the lymph node closest to the intradermal tumor and is located in a fat pad posterior to the humeral scapular joint superficial to the latissimus dorsi muscle and in an angle described by the triceps brachii muscle ventrocranially and the trapezius muscle dorsocaudally. The proximal axillary (PA) lymph nodes are in a fat pad at the apex of the axilla alongside the axillary vein at the caudomedial border of the teres major muscle, near the insertion of the latissimus dorsi muscle. The first rib axillary (FRA) lymph node is ventral to the first rib and dorsal to the pectoral muscles near the termination of the first rib and dorsal to the pectoral muscles near the termination of the axillary vein. The superficial dorsal cervical (SDC) lymph node is in a fat pad immediately anterior to the cranial border of the scapula. The superficialinguinal lymph nodes are located just beneath the skin at the junction of the abdomen and medial aspect of the hindlimb (4).

Staging. A staging system was devised based on similar systems in use for human cancer. Animals with established intradermal tumors and palpable axillary metastases, but without gross evidence of distant metastases, were considered to be at clinical stage II. In some experiments, pulmonary tumor deposits were established by intravenous injection of 1 ml of a suspension of L10 cells into the lateral vein of the penis of guinea pigs anesthetized with methoxyflurane (Metafane; Pitman-Moore, Inc., Washington Crossing, N. J.).

Preparation of Cell Walls of Mycobacterium bovis, Strain Bacillus Calmette-Guerin (BCG CW) in Oil-in-Water Emulsion. BCG CW (lots 286 and 276), prepared as described previously (5), were obtained from Dr. Edgar Ribi (Rocky Mountain Laboratory, Hamilton, Mont.). Oil-in-water emulsions of BCG CW were prepared as described (1) to contain (per ml) 1.87 mg BCG CW, 30 µl oil, and 2 µl Tween 80.

Irradiation. L10 tumor cells (3 X 10⁷/ml) were irradiated with a dose of 10,000 rad gamma (¹³⁷Cs or ⁶⁰Co) or x radiation. The cells were centrifuged and resuspended in fresh medium 199 to a concentration of 62.5 X 10⁶ cells/ml. After irradiation, the concentration and viability of the cells were determined by the trypan blue exclusion test; no decrease in viability was observed after irradiation. No ascites tumors formed after intraperitoneal injection of 10⁸ irradiated L10 cells.

Tumor Cell and Adjuvant Mixture. Irradiated tumor cells and BCG CW were prepared immediately before use. A suspension of washed, irradiated L10 cells (5.0 X 10⁸ cells) was centrifuged at 140 g in a 17- X 100-mm polypropylene test tube (2059; Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.), and the cell pellet was resuspended by dropwise addition of 2 ml of BCG CW emulsion with continuous mixing.

Limited Surgery for Stage II Disease. Guinea pigs were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg) supplemented with inhalation of methoxyflurane. The dermal tumor in each animal was excised with a 1-cm margin of skin; the SDA lymph node and all of the associated fat pad were dissected free from underlying tissues and removed. Hemostasis was maintained with cautery, and the incision was closed with closely spaced metal wound clips.

Extensive Surgery. In some experiments, the dermal tumor, fore limb, and scapula including the SDA and PA lymph nodes were removed in one piece. In addition, the FRA and SDC nodes were removed by dissection. Hemostasis was maintained by ligation with 4-0 silk and cautery. The surgical wounds were irrigated by washing with a solution of cephaloridine (Eli Lilly & Co., Indianapolis, Ind.) containing 2 mg of antibiotic/ml of phosphate-buffered saline, and the incision was closed with closely spaced metal wound clips.

Immunotherapy. 1 or 2 d after surgery and again 6 or 7 d later, animals received four intradermal injections each time of a mixture of irradiated tumor cells (2.5 X 10⁷ per injection site) and adjuvant (188 µg BCG CW per injection site). Injections were given in the flank in a line extending from back to belly. The first treatment was given on the left (ipsilateral) flank and the second on the contralateral flank 7 d after the first. The ipsilateral injections were given so that one injection site was above the incision line, one was just below the incision line, and two were in the axilla. Contralateral injections were made along a vertical line ~2.5 cm posterior to the SDA lymph node. Each animal received a total of 2 X 10⁸ irradiated tumor cells and 1,500 µg of BCG CW.

Detection of Living Tumor Cells in Lymph Nodes. Lymph nodes were disaggregated in medium 199 by mincing them with scalpel blades. Each disaggregated lymph node was transferred
quantitatively to an individual weanling strain 2 male recipient by i.p. injection through a 13-gauge trocar. The presence of tumor cells in the donor lymph node was indicated by the progressive growth of ascites tumor in the recipient. A single, living L10 ascites tumor cell injected intraperitoneally will multiply and the recipient will die within ~42 d (J. T. Hunter, unpublished observations).

Measurements. Animals were examined at weekly intervals after surgery to detect growth of tumors. Measurements were made of the size of PA lymph nodes, because this was invariably the first site of palpable tumor growth after limited surgery for clinical stage II disease. For 3–4 wk after immunization, it was not possible to distinguish between lymphadenopathy resulting from immunostimulation and that resulting from tumor growth. Enlargement of the PA lymph nodes detected 4 wk after immunization was a reliable indicator of tumor progression. Animals with disease remaining after surgery died 60–90 d after tumor cell injection with widespread lymph node metastases (axillary, inguinal, and cervical lymph nodes) and anasarca. At autopsy, about one-half of the animals were found to have pulmonary metastases as a consequence of the dermal implant. There were no recurrences of tumors at the site of excision. The date of death was recorded. The animals were observed for a minimum of 120 d after injection of tumor cells.

Statistics. The significance of differences in tumor incidence was assessed with the Fisher exact test (one tailed). Differences in survival time were assessed with the Mann-Whitney U test.

Results

Stage II Metastatic Tumor: Spread of Disease and Host Immunity. In two experiments, 10⁶ L10 tumor cells were injected i.d. into each of 11 guinea pigs. 19–20 days later, when the dermal tumors had grown to an average diameter of 11.3 ± 0.4 mm and the draining SDA lymph nodes were palpable with an average diameter of 6.2 ± 1.0 mm, animals were killed and the draining lymph nodes beyond the SDA were excised from each animal. Each lymph node was minced and transferred intraperitoneally to a normal weanling guinea pig. 10 of the 11 excised PA, 2 of 11 FRA, and 1 of 11 SDC lymph nodes were found to contain living tumor cells. Another group of 12 guinea pigs with clinical stage II disease was treated by excision of the dermal tumor and the palpable draining lymph node, and the next day, each of them as well as control animals received an intradermal challenge of 10⁵ L10 tumor cells. Controls were 12 animals with stage II disease not treated by surgery and 7 animals that received no tumor initially and no surgery. 9 of the 12 animals treated by limited surgery, 3 of the 12 animals not treated by surgery, and none of the seven normal controls rejected the tumor challenge. All 10 animals in a group treated by extensive surgery were cured, and 6 of them rejected the dermal tumor challenge (Table I).

The results of these experiments indicated that when palpable lymph node metastases developed in the SDA lymph node, tumor cells were present in the PA lymph nodes, and to a lesser extent in more distant lymph nodes. Though some degree of anti-tumor immunity was generated at or after the time of challenge, it was evidently insufficient to prevent the progressive growth of micrometastases remaining after limited surgery.

Post-surgical Immunotherapy for Stage II Disease: Micrometastases in the PA Lymph Nodes. We have previously shown that micrometastases in the SDA lymph nodes (i.e., clinical stage I disease) could be eradicated by excision of the primary tumor followed by a single immunization with living tumor cells plus BCG CW (1, 2). Preparations containing 30 × 10⁶ live L10 cells that were reproducibly effective in treating micrometastases in SDA nodes of guinea pigs with stage I disease (2)
significantly prolonged survival of animals with stage II disease, but were ineffective in preventing growth of PA node metastases (results not shown). The incidence of tumors growing at the immunization site (12/24) was higher than we found with identical preparations used to treat less advanced (stage I) disease (2).

Preparations containing irradiated L10 and BCG CW were reproducibly effective for treatment of animals with residual stage I disease and were not tumorigenic (2). This approach was used to treat PA lymph node metastases remaining after limited surgery for stage II disease, with the difference that two treatments were given: the first on the ipsilateral flank followed 1 wk later by a booster treatment on the contralateral flank. This treatment prevented growth of residual lymph node metastases in 29 of 38 animals treated (Fig. 1 A and B). In the groups treated by surgery alone (excision of the primary tumor and SDA lymph node), metastases in the PA node grew in all but 2 of the 30 animals, and more than one-half of the animals had pulmonary metastases at death.

**Immunotherapy of Animals with Intravenously Implanted Microscopic Tumor Deposits in the Lung.** Microscopic tumor deposits established in the lungs of healthy animals by intravenous injection of L10 cells could be eradicated by immunization with a mixture of irradiated L10 cells and live BCG (6). Preparations containing irradiated L10 and BCG CW instead of living bacteria were also effective in preventing the growth of these micrometastases. Two treatments were given, the first on the left flank 2 d after intravenous injection of the L10 cells, and the second on the right flank 1 wk after the first treatment. Treatment with a preparation containing $10^8$ irradiated L10 cells and 750 µg of BCG CW protected animals that had received $10^4$, $10^5$, or $10^6$ L10 cells i.v. (Fig. 2 A, B, and C). Significant protection and prolongation of survival was obtained even when treatment was delayed for as much as 7 d after injection of $10^6$ L10 cells intravenously (Fig. 3).

**Immunotherapy of Animals with Lymph Node Micrometastases and Intravenously Implanted Microscopic Pulmonary Tumor Deposits.** The efficacy of limited or extensive surgery with or without active specific immunization was evaluated in animals with clinical stage II disease, with and without the added burden of pulmonary tumor deposits implanted
FIG. 1. Postsurgical immunotherapy of animals with occult metastases in the second draining (PA) lymph node in two experiments (A and B). All animals received $10^6$ L10 cells intradermally on day 0. The dermal tumor and palpable SDA lymph node were excised on day 19 (A) or 20 (B). ●, no postsurgical treatment. ○, intradermal injection of 750 μg BCG CW + $10^9$ irradiated L10 cells ipsilaterally 2 d after surgery followed 7 d later by contralateral treatment with the same vaccine. The fractions indicate the number of animals alive and tumor free/number treated.

i.v. on the day of surgery. The data presented in Table II show the following: In animals with regional disease and no intravenously implanted tumor: (a) All animals treated with limited surgery died with extensive lymph node metastases, and a majority had grossly evident lung metastases. (b) Extensive surgery cured a significant proportion of the treated animals. (c) Limited surgery plus active immunization also cured a significant proportion of treated animals. Active specific immunotherapy plus limited surgery was no more effective than extensive surgery. In animals with regional disease and intravenously implanted pulmonary tumor: (a) Animals treated either by limited or extensive surgery died. (b) A significant proportion of animals treated by active immunization after limited or extensive surgery survived.

Discussion

The purpose of these experiments was to develop an immunization procedure to eliminate occult metastases in lymph nodes and viscera. Such metastases may be present in cancer patients after all the detectable tumor has been removed surgically. If a systemic specific immune response to the tumor could be generated by active immunotherapy, malignant cells not removed at surgery might be eliminated. Several animal models have been used to demonstrate the efficacy of immunization with tumor cell vaccines in the treatment of animals with subcutaneous, intradermal, or intravenous transplants of live tumor cells (3, 7–10). Living bacillus Calmette-Guérin (BCG) as well as BCG CW on oil droplets have been shown to be effective adjuvants in generating systemic immunity to L10 (2, 6, 7, 11). In this and previous reports, we have used the L10 tumor as a model for postsurgical immunotherapy of animals with lymph node metastases. In those studies, BCG CW on oil droplets was found to be an effective adjuvant (1, 2).
Fig. 2. Therapy of pulmonary tumor deposits with vaccines containing irradiated L10 cells. Animals received $10^4$ (A), $10^5$ (B), or $10^6$ (C) L10 cells intravenously on day 0. Solid lines: no treatment after L10 injection intravenously. Broken lines: Treated with 750 μg BCG CW and $10^6$ irradiated L10 cells on day 2 (left flank) and day 9 (right flank). The fractions indicate the number of animals alive and tumor-free/number tested. (Two treated animals in A died tumor-free: one on day 118 and one on day 120, and have been excluded from the analysis. The other two treated animals that died in A and B were not necropsied to confirm the presence of tumor. Necropsies were also not performed on three untreated animals [two in B and one in C]. All other animals that died had multiple lung tumors. In addition, seven had penile tumors [two in B and five in C].)

In previous experiments, immunotherapy was given after excision of the primary dermal tumor at a stage when there were micrometastases only in the first draining (SDA) lymph node and no detectable immunity to tumor challenge (1, 2, 12). In the present report, we tested immunotherapy after the development of palpable metastasis in the first lymph node and dissemination of microscopic metastases to more distant sites. Residual micrometastases grew even though at this stage of the disease, tumor rejection immunity was detectable. The detection of tumor rejection immunity at this stage of disease confirms previous reports (12, 13, 14). Occult metastases could be eradicated in most treated animals when surgery was followed by two treatments with BCG CW mixed with $10^6$ irradiated L10 cells.

There was some indication that animals that had developed palpable lymph node metastases had impaired ability to respond to therapy. Thus, we found, they were afforded little protection by a single treatment with vaccines that were previously shown to be effective against micrometastases in the SDA lymph node (2). They were also less able to resist the outgrowth of tumor cells from injection sites containing a mixture of L10 cells and adjuvant. Whether or not this reflects an immunological impairment of the host has not been established.
Fig. 3. Therapy of pulmonary tumor deposits with vaccines containing irradiated L10 cells. Animals received $10^6$ L10 cells intravenously on day 0, and received: no treatment (●), vaccination on day 1 and 8 (□), vaccination on days 4 and 11 (○), and vaccination on days 7 and 14 (△). Vaccination consisted of injection of 750 μg BCG CW and $10^6$ irradiated L10 cells intradermally, first on the left flank, and 7 d later on the right flank. A control group with animals treated with BCG CW only on days 1 and 8 (■) was included. (There were 10 animals per group. All control animals that received tumor intravenously died with multiple tumor nodules in the lungs. One animal treated with vaccine on days 1 and 8 died with penile tumors as well as multiple tumor nodules in the lung on day 103. Three animals treated with vaccine on days 4 and 7 died with ascites tumor and intra-abdominal lymph node metastases; an additional animal died tumor-free on day 102. Four animals treated on day 7 and 15 died with penile tumors as well as multiple tumor nodules in the lung. All but one of the animals treated with BCG CW alone died with penile tumors and multiple tumor nodules in the lungs. The remaining animals that died were found to have tumor nodules in the lungs only.)

We studied the effect of specific immunization on intravenously established pulmonary tumor deposits by intravenous injection of L10 tumor cells. Metastasis from dermal L10 tumors occurs primarily by the lymphatic route, although hematogenous lung metastases occur after extensive growth of lymph node metastases (3). Hanna and Peters (6) and Bartlett et al. (15) have shown that the outgrowth of intravenously injected L10 can be prevented by immunization with irradiated tumor cells plus live BCG. In this study, we found that immunization with a mixture of x-irradiated L10 cells and BCG CW as adjuvant could eradicate pulmonary tumor deposits implanted intravenously in the lungs of normal guinea pigs. Immunization was also effective in postsurgical therapy of animals with pulmonary tumor deposits and microscopic PA lymph node metastases.

The therapeutic efficacy of surgery and active immunization for animals with stage II and III disease offers some hope that analogous treatment may be effective for humans with stage III cancer, provided that human malignant neoplasms contain appropriate antigens. The hope for therapy of stage III disease must be tempered because in this and other studies (6), pulmonary tumor deposits were introduced at a time when animals were at clinical stage I or stage II and, therefore may have been in a better state of health than would have been animals with stage III disease arising as a consequence of the dermal implant. Experiments are in progress in which the therapeutic efficacy of surgery and active immunization is being tested for animals at stage III without intravenously implanted tumor deposits.
TABLE II

Postsurgical Immunotherapy of Microscopic Lymph Node and Lung Metastases

Table

| Tumor implantation | Surgery (day 2?) | Number alive and tumor-free/total |
|--------------------|-----------------|----------------------------------|
|                    |                 | BCG CW + irradiated L10§ (days 22 and 29) |
| Day 0              | Day 21          | No vaccine                       |                                |
| None               | L10 i.v.        | None                             | 0/11                           | 11/12§§ |
| L10 i.d.           | None            | Limited                           | 6/12                           | 4/11§§ |
| L10 i.d.           | None            | Radical                           | 8/11**                         | Not tested |
| L10 i.d.           | L10 i.v.        | Limited                           | 0/12                           | 6/12§§ | 7/12¶¶ |
| L10 i.d.           | L10 i.v.        | Radical                           | 0/11                           | 10/11 |

* Experiment was observed for 220 d after intradermal (i.d.) injection of L10. §10⁶ L10 cells injected intraperitoneally or 10⁵ cells injected intravenously (i.v.). ¶§ 750 μg BCG CW + 10⁶ irradiated L10 cells given ipsilaterally on day 22 and contralaterally on day 29.

† Exclusion of dermal tumor and SDA lymph node.

‡ Exclusion of dermal tumor, fore limb, and the SDA, PA, FRA, and SDC lymph nodes.

** One animal died tumor-free 73 d after i.d. tumor implantation.

†† One animal died tumor-free 106 d after i.v. injection of tumor.

$$$ One animal died tumor-free 124 d after i.d. tumor implantation. P = 0.037 vs. controls receiving no vaccine.

¶ One animal died tumor-free 98 d after i.d. implantation of tumor. P = 0.007 vs. controls receiving no vaccine.

¶¶ Vaccine divided between all four quadrants on both days 22 and 29. P = 0.002 vs. controls receiving no vaccine.

Summary

The malignant disease produced in guinea pigs by intradermal inoculation of line-10 was allowed to progress to stage II, at which time the dermal tumor and the first draining lymph node were grossly evident. At that stage, the external appearance of the next draining lymph node was normal, but it contained tumor cells. Limited surgery consisting of excision of the dermal tumor and first draining lymph node was not curative; palpable metastases developed in the second and other draining lymph nodes, and at autopsy, some animals were found to have gross, visible lung metastases.

Immunization of guinea pigs with a mixture of irradiated syngeneic tumor cells plus mycobacterial cell walls in an oil-in-water emulsion eradicated tumor cells remaining in lymph nodes after limited surgery for stage II experimental cancer and prevented progression of the disease to stage III. Tumor intravenously implanted in the lungs of animals after limited surgery for stage II disease was also eliminated by immunization.

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