Lasting Rejection of Mammary Adenocarcinoma Cell Tumors in DBA/2 Mice with Intratumor Injection of Killed Corynebacterium parvum

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SUMMARY

Preparations of killed *Corynebacterium parvum* were injected into 14-day-old growing mammary adenocarcinoma cell tumors in DBA/2 mice. The tumors and the microscopic metastases undergo rapid and lasting rejection and the animals are protected against challenges of large quantities of tumor cells of the same line. This protection can be overcome if the challenge dose is massive. Groups of tumor-bearing animals treated with s.c. injections of killed *C. parvum* on the ipsilateral side or with i.p. injections demonstrate suppression of tumor growth and longer survival time, compared with animals receiving killed *C. parvum* s.c. on the contralateral side or intratumor injections of Hanks' solution.

INTRODUCTION

The injection of live BCG protects experimental animals against tumor growth of transplanted syngeneic cells (3, 8, 13, 14). Generally speaking, BCG was most protective when injected directly and only into the growing tumor or when inoculated admixed with the transplanted tumor cells (17, 18). Those animals that had been protected were almost always immunized against subsequent injections of tumor cells of the same line. Similar results are also obtained when preparations of killed *C. parvum* are used (9, 11, 15). When tumor cells are injected, admixed with preparations of killed *C. parvum*, the formed tumors undergo rapid and permanent rejection beginning 14 days later. These animals are subsequently protected against challenges of tumor cells of the same line (12). In all these experiments, the action of the immunostimulants reflects a generalized stimulation of phagocytosis and activation of the reticuloendothelial system (1, 10, 13, 14, 16), resulting in the emergence of immunity toward the tumors used (16–18).

The studies described below demonstrate a total and lasting rejection of tumors with intratumor injections of killed *C. parvum*. Furthermore, the host animals failed to develop tumors when they were challenged by inoculations of massive cell doses of the same tumor line. The nature of this permanent remission strongly suggests that the formed tumors were rejected as the consequence of a primary immunological response.

MATERIALS AND METHODS

Animals. Female DBA/2 mice (age, 10 to 12 weeks; The Jackson Laboratory, Bar Harbor, Maine) were used throughout these studies. They were kept under normal laboratory conditions and were fed Purina laboratory chow and tap water *ad libitum*.

Tumor. Mammary adenocarcinoma (CAD2; The Jackson Laboratory) is a spontaneous tumor, originating in a DBA/2 female mouse, that has been serially transplanted in syngeneic DBA/2 female mice without evidence of loss of antigens. In unimmunized mice, the s.c. 100% lethal dose is 10^5 CAD2 cells; animals usually die 50 to 60 days after inoculation. A s.c. inoculation of approximately 10^7 CAD2 cells will kill all recipients in 28 to 35 days. The tumor has metastatic properties that are grossly evident around 14 days after transplantation, and spontaneous regressions have never been observed. The tumor is also weakly immunogenic and does not grow in allogeneic mice.

*C. parvum*. NaCl solution (0.9%) suspensions of heat-killed (65°, 1 hr), formalin-treated (2%, 24 hr) organisms were obtained from Institut Merieux, Lyon, France, in 4-mg ampuls.

Tumor Implantation. By means of an aseptic technique, tumor tissue was removed from freshly killed tumor-bearing animals. Skin and necrotic material were removed before the tumor material was transferred to Petri dishes containing Hanks' solution. The tumor tissue was cut into pieces of approximately 2 cu mm, and suspensions of CAD2 cells were obtained by mild manipulation on surgical tantalum gauze. The cells were then washed 3 times with Hanks' solution and resuspended therein at a concentration of 10^6 CAD2 cells per ml prior to transplantation.

Preparations of killed tumor cells were obtained by first heating tumor cell suspensions at 65° for 30 min and then placing them in 2% formalin in Hanks' solution for 24 hr.

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3 The abbreviation used is: BCG, *Bacillus Calmette-Guérin*.

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The cells were then centrifuged at 800 x g for 10 min, washed 3 times, and stored in Hanks' solution at 4°.

Seventy-two previously unimmunized mice received s.c. inoculations of 10⁷ CAD₂ cells and then were separated into 6 groups of 12 animals each. Fourteen days thereafter, the formed tumors reached a mean diameter of 0.9 cm. At this time, 1 group of 12 mice each received a single permeating injection of killed C. parvum (400 µg total dose in 0.2 ml Hanks' solution) directly and into at least 6 different points of each growing tumor simultaneously. Commencing at the same time, 1 of the remaining 5 groups of mice received twice weekly injections (volume, 0.2 ml) of either (a) intratumor permeation of Hanks', (b) intratumor permeation of 5 x 10⁵ killed CAD₂ tumor cells, (c) killed C. parvum (400 µg) injected s.c. at sites 1 cm from and around the growing tumor periphery, (d) the same amount of killed C. parvum injected s.c. in the side contralateral to the tumor, or (e) the same amount of killed C. parvum injected i.p. The largest diameter of each growing tumor was measured twice weekly, and the injections were continued until the time of death.

RESULTS

The results of the experiments summarized in Chart 1 reveal that, of the 6 groups of mice, only the animals receiving intratumor injections of killed C. parvum survived. Within 24 hr after injection, the tumors in these animals became extremely hyperemic. Thereafter these tumors regressed in size and permanently disappeared within 7 days in 12 of 12 mice. There was only minimal pinpoint ulceration at the site of injection, and a large eschar formed prior to total disappearance of the tumor.

The remaining 5 groups of mice had tumor-induced deaths within different ranges of time. Of these, the group of mice receiving intratumor injection of Hanks' solution exhibited expected rates of tumor growth, with the range of survival approximately 28 to 35 days after inoculation of tumor cells. Similar rates of tumor growth with similar survival times were also observed in the groups of mice that received twice weekly injections of killed C. parvum on the side opposite the tumor, or twice weekly intratumor injections of killed tumor cells. The groups of mice treated with i.p. injections of killed C. parvum exhibited an inhibition of tumor growth and prolonged survival times in the range of 48 to 60 days. Similar results were observed in the group of mice that received s.c. injections of killed C. parvum in the proximity of the growing tumors. Examination of mice at death revealed gross metastases to the viscera and pulmonary systems. The entire experiment was repeated with 24 mice in each group with the same results. The surviving 36 mice from Experiments 1 and 2 were separated into 3 groups (12 each) to include 4 mice from Experiment 1 and 8 mice from Experiment 2. Three groups of previously unimmunized mice of similar ages (12 mice/group) served as controls. A group of control mice and surviving experimental mice each received s.c. injections of either (a) 10⁷, (b) 5 x 10⁷, or (c) 10⁸ live CAD₂ cells. All mice in the control groups died from tumors progressing from the site of injection. The mice from Groups a and b that had previously rejected tumors did not develop tumors and now have survived tumor-free from 8 to 10 months. The mice in Group c that had also rejected tumors and received 10⁸ CAD₂ cells remained tumor-free for an average of 20 days after injection. These mice then developed tumors that grew progressively from the site of injection and tumor-induced deaths were incurred from 30 to 50 days thereafter.

In another experiment, a group of 12 mice rejected 14-day-old tumors arising from an intratumor injection of killed C. parvum. These animals each received a s.c. injection of live mammary adenocarcinoma cells from an antigenically different line (T1699, The Jackson Laboratory). These mice were not protected and died due to a tumor progressing from the 2nd injection site.

Chart 1. The effect of killed C. parvum on the growth of CAD₂ mammary adenocarcinoma in DBA/2 mice. Groups of mice received an initial s.c. inoculum of 10⁷ CAD₂ cells. The tumors formed at 14 days, when treated with an intratumor injection (1) of 400 µg of killed C. parvum (■), undergo rapid and lasting rejection. Tumor growth is suppressed in animals treated with twice weekly injections of killed C. parvum (400 µg) on the ipsilateral side (▲) and also is suppressed in animals receiving similar injections i.p. (▲). Similar rates of tumor growth are observed in animals receiving intratumor injections of killed 5 x 10⁵ CAD₂ cells (O) when these rates are compared to similar injections of Hanks' solution (●) without affecting the range of tumor-induced deaths (- - - -). The mean tumor growth of the group of mice receiving a single intratumor injection of killed C. parvum is significantly different from that of each of the other groups, with p < 0.001 by unpaired t test.
Histological sections of CAD₂ tumors excised 4 days after a single intratumor injection of killed C. parvum were compared with tumors injected with Hanks' solution. The tumors injected with Hanks' solution revealed infiltration of mild to moderate numbers of macrophages and lymphocytes near the tumor periphery. Those injected with killed C. parvum revealed an immense infiltration of macrophages and lymphocytes throughout the tumor, with some necrosis.

DISCUSSION

The work presented here demonstrated that the growth of s.c.-transplanted syngeneic tumor grafts in DBA/2 mice was permanently suppressed in the presence of killed C. parvum. It has been previously demonstrated that this suppression was not due to the cytotoxic action of C. parvum on tumor cells (12) but involved the participation of the immune defenses of the host. Although optimal suppression of tumor growth required close contact between tumor cells and C. parvum, some suppression of tumor growth was also seen in animals that received s.c. injections of C. parvum near the tumor site. In this case, tumor cell-C. parvum contact may have occurred through connecting lymphatic channels. The permanent rejection of transplanted tumors following a single permeation of killed C. parvum directly into the growing tumor appears to be an event related to the immunostimulative properties of C. parvum antigens.

Animals receiving injections of killed C. parvum have an intense stimulation of the reticuloendothelial system and phagocytosis (1, 2, 10). The close proximity of tumor cells and killed C. parvum may thus enable the cells to be easily processed by macrophages, with the subsequent development of both humoral and cellular immunity (5–7). The result may be an augmented response that is effective in rejecting not only the tumor but also the microscopic metastases observed in autopsies of animals sacrificed 72 hr after intratumor injection of C. parvum. The augmented antitumor response is observed especially in the ability of these mice to reject challenge doses of 10⁷ and 5 × 10⁷ tumor cells, many times the 100% lethal dose. This protection can be eventually overwhelmed if larger challenge doses are used.

Evidence for the stimulation of tumor-specific immunity is demonstrated by the observation that immunity conferred towards the CAD₂ cell line did not protect against a challenge s.c. injection of an antigenically different line of a histologically similar tumor.

Inhibition of tumor growth and prolonged survival were observed in the groups of mice receiving i.p. injections or twice weekly s.c. injections of C. parvum near the tumor site, compared with the groups of mice receiving either intratumor injections of Hanks' solution or tumor cells. The group of mice receiving s.c. injections of killed C. parvum demonstrated similar "normal" rates of tumor growth. This suggests that contact between tumor cells and C. parvum antigens may have occurred by means of the regional lymphatics.

There are many similarities between the immunotherapeutic properties of live BCG and preparations of killed C. parvum. Zbar et al. (16–18) have demonstrated that the effectiveness exists only when there is close contact between tumor cells and BCG. These effects are best observed when BCG is included as a suspension in the initial tumor cell inoculum or is injected directly into the growing tumor. Furthermore, the surviving animals demonstrate a protection against subsequent injections of tumor cells of the line initially used. The immunostimulative properties of BCG appears to be similar to that of C. parvum. However, BCG is ineffective when used as a killed preparation or in animals lacking intact immune systems (16). Also, BCG is ineffective when injected at sites different from the tumor cell inoculum or the growing tumor.

Injections of preparations of killed C. parvum have been seen to inhibit growth of transplanted tumors in experimental syngeneic animals. Similar injections have also afforded protection against runting induced by the inoculation of parental spleen cells in hybrid mice in which runting has been shown to be particularly severe (2, 4). Like BCG, C. parvum when previously admixed with the initial inoculum of tumor cells causes the delayed rejection of the formed tumors at the site of the injection (12). The work presented here established further the effectiveness of this immunostimulant. It is effective both in its killed form and when injected directly into the growing tumor; in addition, it causes suppression of tumor growth when injected in the region of the lymphatic drainage of the tumor. Furthermore, preparations of killed C. parvum when administered via the intratumor route can produce a response that affords protection against metastatic lesions.

This model can serve in the study of other variables of cancer in humans. It provides a reasonable basis for experimental cancer immunotherapy in man, as C. parvum has already been used to treat humans, and no side reactions have been observed. It is effective in its killed form and not only causes regression of established tumors but also affords protection against challenges of large doses of the same tumor cells, which may provide the basis for its use in patients for whom conventional therapy has failed.

REFERENCES

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