Rejection of Mammary Adenocarcinoma Cell Tumors and the Prevention of Progressive Growth of Incipient Metastases following Intratumor Permeation with Killed *Bordetella pertussis*

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SUMMARY

Syngeneic DBA/2 mice bearing 14-day-old s.c.-growing mammary adenocarcinoma cell tumors were treated with a single intratumor infiltration of killed *Bordetella pertussis* (26 $\times$ $10^9$ organisms). Thereafter, there was a rapid and lasting rejection of the tumors in all 12 animals that was completed within 7 days. These animals exhibited resistance to s.c. reinjection of large doses of cells of the same tumor line initially used, but died with growing tumors and metastases when tumor cells of an antigenically different syngeneic line were injected. Nine of the 12 animals treated by surgical excision of the tumor nodule at Day 14 died with growing pulmonary and visceral metastases. Groups of mice treated with twice-weekly injections of killed *B. pertussis* 1 cm away from and around the growing tumor exhibited inhibition of rates of tumor growth and prolonged ranges of survival. Microscopic examination of histological sections of tumors excised 4 days after intratumor infiltration revealed a massive infiltration of mast cells and macrophages and lymphocytes, while sections of untreated tumors rarely revealed the presence of these cells.

INTRODUCTION

Inhibition of tumor cell growth in experimental animals has been achieved by the intradermal inoculation of syngeneic tumor cells admixed with the BCG$^5$ strain of *Mycobacterium bovis* (33). Furthermore, animals receiving s.c. grafts of tumor cells mixed with *Corynebacterium parvum* exhibited a delayed and lasting rejection of the tumors formed at the injection site (18). In addition, intratumor infection of BCG infiltrated directly and only into growing intradermal tumors (32) or permeating doses of killed *C. parvum* injected into growing s.c. tumors (19) have not only resulted in the rejection of the transplanted tumor, but the conferred immune response also appeared to prevent the potential progressive growth of early metastases. The protected animals exhibited resistance to subsequent cell grafts of tumor cells only of the cell line originally used. In all these experiments, the action of the immunostimulant reflected a generalized stimulation of the reticuloendothelial system and phagocytosis (1, 13, 23, 24) resulting in the emergence of immunity toward the tumor used (19, 30, 33).

Recent experiments performed at this laboratory have revealed that preparations of killed *B. pertussis* were also effective in experimental cancer immunotherapy (17). Mice given s.c. injections of syngeneic tumor cells previously admixed with killed *B. pertussis* organisms formed tumors that underwent permanent rejection beginning 2 weeks later. Furthermore, these animals were protected against reinjections of large quantities of tumor cells only of the antigenic line initially used. The studies described below show that a single permeation of killed *B. pertussis* directly into the growing tumor results in the immediate and lasting rejection of the tumor. There was also evidence indicating that the procedure may have also prevented the progressive growth of metastases that were present at the time of therapy.

MATERIALS AND METHODS

Mice. Female DBA/2 mice (age, 8 to 10 weeks; The Jackson Laboratory, Bar Harbor, Maine) were used throughout these experiments. They were kept under standard laboratory conditions and fed lab chow and tap water *ad libitum*.

Tumors. Two different transplantable mammary adenocarcinoma (lines CAD$_2$ and T1699, The Jackson Laboratory) syngeneic to DBA/2 mice were used in these experiments. Both are weakly antigenic tumors (spontaneous in origin) that have been serially transplanted without loss of characteristics. The growth characteristics of the CAD$_2$ mammary carcinoma have been previously described (17–19). The T1699 mammary adenocarcinoma, originating in a DBA/2 female mouse, has a s.c. 100% lethal dose of 100 cells. The tumor has somewhat slower growing characteristics when compared to the CAD$_2$ line, and the s.c. injection of $10^6$ to $10^7$ cells results in death, with growing tumors and metastases in the range of 32 to 42 days. Spontaneous regression of formed tumors have not been observed, in our experience, and protection conferred to tumor cells of the T1699 line does not reflect to the CAD$_2$ line (and vice versa). The tumors do not grow in allogeneic mice.

*B. pertussis*. Sterile suspensions of killed *B. pertussis* organisms in 0.9% NaCl solution (with Merthiolate, 1:10,100) were provided by Eli Lilly and Company, Indianapolis, Ind.
Tumor Cell Preparation. Using aseptic technique, tumor tissue was surgically excised from freshly killed donor animals and placed in Petri dishes containing Hanks' solution. The necrotic tissue was excised and the tissue was cut into pieces of 1 to 2 cu mm. Suspensions of tumor cells in Hanks' solution were prepared by means of mild manipulation on surgical tantalum gauze, washed 3 times with Hanks' solution, (800 X g for 10 min), and resuspended at concentrations of 5 X 10^8 living cells per ml. Whenever necessary, preparations of irradiated cells were prepared by exposure to 4000 R (10 min).

Tumor Implantation. Approximately 10^7 CAD2 cells (0.2 ml Hanks' solution) were injected s.c. into the lateral posterior flank of 84 previously unsensitized DBA/2 female mice. Fourteen days later, the growing tumors reached a mean diameter of 0.9 cm and the animals were separated into 7 groups (12 each). At the time, each mouse of 1 group received permeating injections of killed B. pertussis (approximately 26 X 10^9 organisms; volume, 0.3 ml) directly and only into the growing tumor. Surgical excision (anesthesia: sodium phenobarbital, 5 mg/100 g body weight) of the tumor nodule was performed on each mouse of a 2nd group. The remaining 5 groups of mice were treated with twice-weekly injections (volume, 0.2 ml) of one of the following: (a) intratumor-permeating injections of 0.9% NaCl solution containing Merthiolate, 1:10,000 (b) 5 X 10^5 heat-killed (65°, 1 hr) formalin-treated (5% formalin in Hanks' solution for 24 hr, washed 4 times with Hanks' solution at 800 X g for 10 min) CAD2 cells; (c) killed B. pertussis (26 X 10^9 organisms) injected s.c. 1 cm away from and around the growing periphery; or (d) the same amount of killed B. pertussis injected either i.p. or s.c. at the side opposite the tumor. The (mean) diameters along 3 axes of each growing tumor were measured at twice-weekly intervals until the time of death.

RESULTS

The results summarized in Chart 1 revealed that, of the 7 groups of mice, only the group of animals treated with intratumor B. pertussis survived. Following intratumor infiltration with B. pertussis, the tumors in these animals became hypergenic, regressed in size, and disappeared in 12 of 12 mice within 7 days. Prior to the disappearance of each tumor, an eschar formed at the tumor site, followed by replacement of the eschar with scar tissue. Nine of 12 mice of the group treated by surgical removal of the tumor nodule died within 50 to 60 days. Autopsies performed on these animals revealed the presence of many metastatic nodules in the pulmonary and peritoneal organs. Tumor growth was not observed at the site of surgical excision of the tumor nodule.

All animals of the remaining 5 groups died with growing tumors and metastases. Of these, the group of mice treated with twice-weekly injections of killed B. pertussis 1 cm away...
from and around the growing tumor nodule exhibited an inhibition of the rates of tumor growth and died within 50 to 60 days, with growing tumors and metastases. The groups of mice treated with either intratumor 0.9% NaCl solution or killed B. pertussis injected at the side opposite (contralateral to) the growing tumor died within 28 to 35 days with growing tumors and metastases. Accelerated rates of tumor growth were observed in the group of mice receiving i.p. injections of killed B. pertussis.

The experiment was repeated, and similar results were observed. (In the group of mice treated with surgical excision of the tumor nodule at 14 days, 8 of 12 animals died with metastatic pulmonary and visceral nodules.) An additional group of mice was inoculated s.c. with 10⁷ CAD₂ cells and sacrificed 14 days thereafter. These animals not only developed tumors at the site of injections but also contained microscopic metastases in the pulmonary, splenic, hepatic, and other peritoneal organs. The surviving 24 mice from the above-described experiments and 24 previously unsensitized mice of similar ages (controls) were then separated into 3 groups (8/groups, 4 mice from each experiment). Each animal of these control and experimental groups then received s.c. injections of either 10⁷, 5 × 10⁷, or 10⁸ living CAD₂ cells. The results revealed that the 3 groups of mice treated previously with intratumor pertussis were protected following injections of the 3 different cell doses of CAD₂ cells. These animals exhibited induration within 48 hr at the site of CAD₂ injection but did not develop tumors, and they have now survived tumor free for 8 to 11 months. The mice (7 total from both experiments) surviving after surgical removal of the 14-day-old tumor, as well as 7 control mice, died with growing tumors and metastases following s.c. inoculation with 10⁷ CAD₂ cells.

The following experiments were performed in order to investigate the nature of protection associated with intratumor B. pertussis. Ninety-six previously unsensitized mice were separated into 8 groups of 12 animals each. Of these, each animal of 3 groups received s.c. injections of 10⁷ CAD₂ cells, and 3 groups of mice received similarly 10⁷ T1699 cells (per mouse). At the same time, of the remaining 2 groups of mice, one received 10⁷ irradiated CAD₂ cells and the other received irradiated T1699 cells. At Day 14, the groups of mice receiving living tumor cells alone exhibited tumor growth, whereas the groups of mice inoculated with irradiated tumor cells did not exhibit tumor growth at the injection site. At this time, 4 groups (CAD₂ tumor. 2 groups; T1699 tumor. 2 groups) with growing tumors received a single intratumor infiltration with killed B. pertussis (26 × 10⁹ organisms/mouse). The remaining 2 groups of mice bearing CAD₂ (1 group) and T1699 (1 group) tumors served as controls. Thereafter (Table 1), all mice receiving intratumor infiltration with pertussis vaccine exhibited tumor rejection as previously described. All mice in the groups receiving either irradiated T1699 or CAD₂ cells survived without developing tumors. The untreated group(s) of mice bearing CAD₂ and T1699 tumors died predictably with growing tumors and metastases. Thirty days after the experiment commenced, the groups of mice received s.c. injections of 10⁷ or 10⁸ tumor cells of the line originally used, or of the antigenically different line. The 2 groups of mice sensitized to irradiated tumor cells were protected and did not develop tumors when challenged s.c. with 10⁵ or 10⁶ tumor cells of the line originally used, but died with growing tumors and metastases following transplantation of either 10⁵ or 10⁶ tumor cells of the antigenically different line. The 2 groups of mice sensitized to irradiated tumor cells were protected and did not develop tumors when given injections of 10⁷ tumor cells of the line originally used, but died with growing tumors and metastases following inoculation of 10⁷ tumor cells of the line initially used. The 4 groups of controls receiving either 10⁷ or 10⁸ tumor cells were not protected and died with growing tumors and metastases. These experiments have been repeated and similar results were observed.

Subsequently, 2 groups of mice (8/group) received 10⁷ CAD₂ cells s.c. Fourteen days later, each mouse of a group received intratumor infiltration (volume, 0.3 ml) with either killed B. pertussis (26 × 10⁹ organisms) or 0.9% solution (with 1:10,100 Merthiolate). Four days thereafter, the animals were sacrificed, and the tumors were dissected and placed in 10% formalin in Hanks' solution. Microscopic evaluation of histological sections (Giemsa stain) from tumors of the B. pertussis.
pertussis-treated group were compared with those obtained from the 0.9% NaCl solution-treated group. Examination of the tumors from the B. pertussis-treated mice revealed an intense infiltration of mast cells, macrophages, and lymphocytes, whereas those from the 0.9% NaCl solution-treated group revealed a few macrophages, and only very rarely mast cells and lymphocytes. These experiments were repeated and the same results were observed. Autopsies on the above-described animals revealed the presence of pulmonary and peritoneal metastases.

**DISCUSSION**

The studies described above demonstrated that there was a lasting rejection of s.c.-growing tumors following intratumor infiltration with pertussis vaccine. There was also evidence suggesting that the response following intratumor treatment with killed B. pertussis prevented, in a certain percentage of mice, progressive growth of potential metastases present at the time of therapy. This is suggested by the finding that 17 of 24 of the mice treated by surgical removal of the tumor nodule died with growing pulmonary and visceral metastases. The fact that 7 of 24 animals survived following surgical excision of the tumor nodules suggested either that metastases were absent at the time of extirpation or that the conferred immune response was effective in the prevention of progressive growth of the decreased tumor cell mass. Tumor rejection following intratumor infiltration with killed B. pertussis was probably not due to the direct cytotoxic action of pertussis (17), but appeared to involve a participation of the host immune defenses. The method of therapy conferred an augmented tumor-specific host resistance that protected the animals against subsequent challenge injections of 10^6 tumor cells (many times the dose that was lethal to 100% of the animals). Evidence for the stimulation of tumor-specific immunity was observed in these animals, as the protection conferred was restricted only to the antigenic line of tumor cells originally used. The studies presented here also suggested that optimal suppression of tumor growth required close contact between tumor cells and pertussis, as tumor-bearing mice treated with i.p. injections of B. pertussis exhibited accelerated rates of tumor growth. However, inhibition of tumor growth was observed in mice treated with pertussis in the region of the growing tumor. These observations suggest that a local reaction in situ or a draining lymphoid organ to pertussis may have had a reaction to tumor cells, as inhibition of tumor growth rates was not observed in mice treated with killed B. pertussis at the side opposite the growing tumor. Yet, some contact between tumor cells and pertussis organisms may have occurred directly or via the regional lymphatics.

Tumor rejection following intratumor infiltration with killed B. pertussis was probably related to the immunostimulant adjuvant properties which have been optimally observed when these organisms are suspended with sensitizing antigen prior to injection (5, 7, 20, 23). Augmentation of the immune responses were maximally observed in experimental animals when the sensitizing antigen was administered in close contact with pertussis vaccine (2, 6, 34). Mice receiving injections of killed B. pertussis not only reflect a profound lymphocytosis (lymphocyte count of 200,000 cu mm or more) but also express an intense stimulation of the reticuloendothelial system and phagocytosis (8, 27) events that have been observed to be associated with the induction of the cellular and humoral immune responses. Of these, the cell-mediated immune response has been shown to provide a major contribution toward tumor rejection; other mechanisms may have been associated with B. pertussis-mediated tumor rejection. This was evidence by the large quantities of mast cells observed in tumors undergoing rejection following intratumor B. pertussis treatment. These cells, which already are suspected of playing a role in host defenses against cancers (4), contain large quantities of cytoplasmic granules laden with vasoactive substances and also contain membrane receptors for Fc fragments or reaginic antibodies (3, 11, 14, 22). Mast cells “primed” with specific reaginic antibodies release “large quantities of these vasoactive substances following interaction with sensitizing antigen, thereby providing a basis for allergic injury.

Experimental animals can be induced to produce extremely high liters of reaginic antibody within 4 days after administration of sensitizing antigens mixed with pertussis vaccine (22, 28, 29). Similarly, the close association between tumor cells and B. pertussis (following intratumor administration of pertussis vaccine) may result not only in an augmented tumor-specific cell-mediated immune response, but also may induce an augmented humoral response consisting of tumor-specific reaginic antibodies, thereby providing a basis for tumor rejection by means of an acute tumor-specific cell-mediated hypersensitivity reaction. Recent experiments performed at this laboratory have been designed to study tumor cells interactions with peritoneal mast cells separated from mice expressing tumor rejection following intratumor B. pertussis treatment. Preliminary results revealed that these mast cells not only released large quantities of histamine and serotonin following in vitro contact with the sensitizing tumor cells, but also inhibit tumor growth when administered to unsensitized animals inoculated s.c. with lethal doses of the sensitizing tumor cells.

The group of tumor-bearing mice treated with i.p. injections of pertussis organisms exhibited accelerated rates of tumor growth, findings that are comparable with previous reports (10, 21). These observations may correlate with the depression of specific humoral and cell-mediated immune reactions toward sensitizing antigens injected via separate routes and with time to administration of pertussis vaccine (9). However, mammary adenocarcinomas in mice have been observed to have strong organospecific antigenic substances that may confer immune responses associated with immunological enhancement. Enhancement of tumor growth has been previously reported with similar experiments that used BCG and its extracts (15, 16, 30), and the same mechanisms may be associated with B. pertussis.

The studies presented here have been repeated with other transplantable syngeneic tumors in mice (pulmonary carcinoma, SAD2 sarcoma and B16 melanoma) and rats (13762 and DMB14 mammary adenocarcinomas and Shay chloroma) and comparable results were observed (although, in some cases, weekly tumor infiltrations were necessary to obtain optimal therapeutic results). These studies compare favorably with similar experimental approaches using C. parvum.
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although the mechanisms associated with tumor rejection appear to be different. They also compare favorably with those of Sinkovics et al. (26), who have reported that there is a suppression of adult leukemogenesis in AKR mice that were given neonatal injections of pertussis vaccine. Although nonspecific augmentation of host resistance with the use of \( B. \) pertussis has been attempted in patients with cancer (12), the effectiveness of this method of specific cancer immunotherapy remains to be evaluated.

References

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