A Preliminary Study on the Effect of *Clostridium sporogenes* on the Volume of HT29 Tumor Xenografts in Immunosuppressed Mice (*Mus musculus* L.)

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An Undergraduate Thesis Submitted to the
Department of Biology
College of Arts and Sciences
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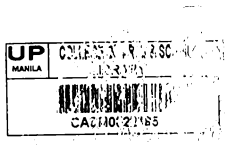

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ENDORSEMENT

The thesis attached hereto, entitled **"A Preliminary Study on the Effect of *Clostridium sporogenes* on the Volume of HT29 Tumor Xenografts in Immunosuppressed Mice (*Mus musculus* L.)"** prepared and submitted by **Rochelle L. Castillo** and **Caryl Joy P. Nonan**, in partial fulfillment of the requirements for the degree of Bachelor of Science in Biology, is hereby accepted.

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ABSTRACT

Acute bacterial infection has been known to induce regression in solid malignant tumors. This study aimed to determine if *Clostridium sporogenes* possessed antitumor activity against HT29 human colon adenocarcinoma xenografts. Endospores numbering approximately 10^6 suspended in 0.1 mL of phosphate-buffered saline solution were injected intratumorally into tumors grown for 5 days in Cyclosporine-immunosuppressed female BALB/c mice. Tumor volume was measured using a ruler once daily for 3 days. An untreated tumor, and tumors treated for 24, 48, and 72 hours were excised, cut into serial cross-sections, and stained with hematoxylin-eosin (H&E) for histopathological analysis. The results revealed that the introduction of *C. sporogenes* caused a decrease in the volume of HT29 tumors by at least 50% and at most 83.33% daily, for 3 days. The control tumor contained numerous tumor cell nests while the tumor treated for 24 hours contained no tumor cells and exhibited a high degree of acute inflammation induced by the presence of *C. sporogenes*. The tumors treated for 48 and 72 hours exhibited signs of repair and considerably less inflammation. The inflammatory response could be the reason for the observed antitumor activity of *C. sporogenes*.

INTRODUCTION

Background of the Study

Cancer is the proliferation of cells due to the loss of normal controls in cell division resulting in unregulated growth, lack of differentiation, local tissue invasion, and metastasis (Beers & Berkow, 2004). This disease can strike at any age. It kills children between ages 3 and 14 more than any other disease (Cancer Facts and Figures, 1994). Advancing age, incidentally, increases the risk of developing cancer (Bullock, 1996). Current research indicates that almost 90% of all cancers are related to lifestyle and environmental factors (Daly, 1993).

Cancer causes 6 million deaths yearly—or 12% of deaths worldwide, and more than 10 million people are diagnosed with it every year. It is estimated that there will be 15 million new cases annually by 2020. Lung, colorectal, and stomach cancer are among the five most common cancers in the world for both men and women (World Health Organization [WHO], 2004).

In the Philippines, cancer is the fourth leading cause of mortality (Department of Health, 1998). A separate study by GLOBOCAN in 2000 reported 171,023 existing cases of cancer and 115,097 deaths attributed to it.

Development of treatments for cancer continues to be a challenge for researchers. In particular, the poorly-oxygenated areas in solid human tumors have proven to be inaccessible to currently existing chemotherapeutic drugs and resistant to radiation

treatments. Novel therapeutic approaches are required that offer greater potency and selectivity toward these hypoxic areas. One such approach, the use of bacteria or their extracts, goes back more than 100 years (Chakrabarty, 2003; Jain & Forbes, 2001; Pawelek et al., 2003; Yamada et al., 2002). Tumor-targeted microorganisms with selectivity for tumor cells due to their affinity for hypoxic areas have been studied as antitumor agents, either to provide direct tumoricidal effects, deliver tumoricidal molecules, or generate potent immune responses (Bermudes et al., 2002; Agrawal; et al., 2004). *Bifidobacterium*, *Clostridium*, and *Salmonella* have all been shown to preferentially replicate within and cause lysis in solid tumors when injected from a distal site (Pawelek et al., 2003; Jain & Forbes, 2001). In particular, *Clostridium novyi* and *C. sordelii* have been shown to exhibit extensive spreading throughout the hypoxic areas of tumors, and injection of modified *Clostridium novyi* endospores devoid of the lethal toxin gene has resulted in expanded areas of necrosis and the generation of a potent immune response, leading to tumor volume regression (Dang et al., 2001; Agrawal et al., 2004). Currently, there are no rapid, reliable, inexpensive methods for screening for bacteria with tumor cytolytic activity, but members of the *Clostridium* genera appear promising (Jain & Forbes, 2001).

Statement of the Problem

Will *Clostridium sporogenes* have an effect on the volume of HT29 tumor xenografts in immunosuppressed mice?

Research Objective

To determine the effect of *Clostridium sporogenes* on the volume of HT29 tumor xenografts in immunosuppressed mice.

Significance of the Study

While *Clostridium sporogenes* has been shown to successfully target and proliferate in various animal tumors, no known study has been done to test its antitumor properties on tumors induced by HT29 colon cancer cells. This study will determine whether or not *C. sporogenes* possesses antitumor activities against this particular cell line. This has far-reaching implications in the field of cancer therapy, as the past several years have seen renewed interest in the treatment of cancer with live microorganisms, based on the observation that some microorganisms display selective replication or preferential accumulation in the tumor microenvironment.

Scope and Limitations

Only one strain of *Clostridium sporogenes* was utilized for this experiment and no comparisons were made with other strains or species. The cell line HT29 was the only tumorigenic line available for study at the time.

Injection of *C. sporogenes* endospore suspension was administered after 5 days of tumor growth. Histopathological studies were performed to confirm the presence of HT29 cancer cells and to observe changes in tumor morphology after treatment. Gram staining of sections of the tumors to determine the distribution of the germinated bacteria

within the tumors was not performed. Serum cytokine and degradative enzyme (protease, lipase) assays, antibody titering, and rechallenge experiments to establish the presence and specificity of the immune response were neither carried out.

REVIEW OF RELATED LITERATURE

Existing Therapies for Cancer

The development of novel cancer therapies that are selective for cancer cells with limited toxicity to normal tissues is a challenge for oncology researches. Cancer treatments are divided into four main groups: a. surgery, b. radiation therapy (including photodynamic therapy), c. chemotherapy (including hormonal therapy), and d. biologic therapy (including immunotherapy, differentiating agents, and agents targeting cancer cell biology) (Sausville & Longo, 2001).

Surgery is the most effective means of treatment, curing about 40% of cancer patients today (Sausville & Longo, 2001). Hyperthermia (HT) is known to cause direct cytotoxicity and also acts as a radiation and chemosensitizer. The mechanisms of action of HT are complementary to the effects of radiation; when used in combination with chemotherapy, it increases drug uptake into cells and at least partially reverses drug resistance (Dewhirst et al., 2003).

Cancer gene therapy offers a potential replacement or augmentation of traditional cancer treatments, which use invasive or toxic protocols. Suicide genes that encode an enzyme that activates a prodrug into a toxic molecule, or genes that induce apoptosis, have been or are currently being tested in clinical trials for their efficacy in cancer therapy (Unfer et al., 2003). Antigen-specific cancer immunotherapy and antiangiogenesis represent two other attractive approaches to cancer treatment. Activation

of antigen-specific T cell–mediated immune responses allows for killing of tumors associated with a specific antigen while inhibition of angiogenesis controls neoplastic growth by sequestering neoplastic cells from an adequate blood supply (Cheng, et al., 2001).

The use of complementary and alternative medicine (CAM) is practiced extensively by some 42% of cancer patients usually along with conventional therapy. Examples of CAM include herbals, vitamins, organics, chemicals, diet, massage, acupuncture, and body-mind therapy. Overall, there is little clinical evidence to suggest that complementary therapies cause harm or interact unfavorably with regular medications, although ingested or injected CAM might pose risks to some patients by causing liver failure in the case of chaparral tea or hypertension in the case of Ma huang (Rheingold, 2001).

Tumor Hypoxia

Tumors or neoplasms are abnormal masses of proliferating cells that do not respond to normal controls on cell division (Marieb, 2002). In a normal cell population, inhibitory controls slow or stop reproduction, whereas stimulating factors cause the process to proceed more rapidly. Neoplastic cell populations ignore normal growth limitations and enter the cell cycle at different rates. They may be either benign (does not invade the surrounding tissues and does not metastasize) or malignant (invades

surrounding tissues and has the ability to metastasize to receptive tissues). Malignant tumors are cancerous (Bullock, 1996).

Solid human tumors grow within a unique microenvironment characterized by abnormal vasculature which leads to an insufficient supply of oxygen and nutrients to the tumor cells (Wouters et al., 2002). This condition is termed hypoxia. Experimental evidence suggests that tumor cells exist under hypoxic conditions at a distance of 300–400 μm from blood vessels, indicating that hypoxic conditions are generally established in tumors measuring only 0.5 mm in diameter (Duffy et al., 2003). Current chemotherapeutic approaches to cancer are in part limited by the inability of drugs to destroy neoplastic cells within these poorly-vascularized compartments of tumors (Dang et al., 2001). The low level of oxygenation within tumors is also a major cause of radiation treatment failures, whose cell-killing effects are oxygen-dependent (Bettegowda et al., 2003). Furthermore, hypoxia in solid tumors appears to accelerate malignant progression and increase metastasis (Brown, 1999). The more malignant phenotype affects genomic stability, apoptosis, and angiogenesis (Wouters et al., 2002).

Efforts that are underway to develop therapies that exploit the tumor micro-environment can be categorized into three groups. The first includes agents that exploit the environmental changes that occur within the micro-environment such as hypoxia and reduced pH. This includes bioreductive drugs that are specifically toxic to hypoxic cells, as well as hypoxia-specific gene delivery systems. The second category includes therapies designed to exploit the unique properties of the tumor vasculature and include

both angiogenesis inhibitors and vascular targeting agents. The final category includes agents that exploit the molecular and cellular responses to hypoxia. In line with these strategies, the use of anaerobic bacteria which have the capacity to grow expansively within the avascular compartments of tumors has been studied extensively (Wouters et al., 2002).

Bacteria in the Treatment of Cancer

The use of bacteria or their extracts in the treatment of cancer goes back more than 100 years. Since Edward Jenner first showed in the 1790's that bacteria could be employed to stimulate the immune system, bacteria have become front-line treatment for some cancers (Cheadle & Jackson, 2002). In the 1890's, William B. Coley observed that a fraction of cancer patients who developed postoperative bacterial infections went into remission and were cured of their tumors. Coley stated that daily injections should be given, if the patient could bear it, as discontinuing the vaccine even for a few days would often lead to regrowth of residual tumor—suggesting that specific antitumor immunity was not a primary mechanism of bacterial infection (Hoption Cann et al., 2003). Coley's work was thereby a major stimulus to the burgeoning field of tumor immunology (Agrawal et al., 2004). The activation of the immune system by making it respond to an iatrogenic infection as Coley did remains one of the principles underlying contemporary cancer immunotherapy (Bickel et al., 2002). Attenuated *Mycobacterium bovis* BCG vaccine, and recombinant *Salmonella typhimurium* and *Listeria monocytogenes* tumor

vaccines continue to be successfully applied in this context (Cheadle & Jackson, 2002). A study by Agrawal et al. in 2004 showed that a significant fraction of immunocompetent animals treated with *C. novyi*-NT spores exhibited complete tumor regression in the absence of any additional chemotherapy or radiation by generating a potent immune response.

Over the past 60 years, several strains of anaerobic bacteria have been shown to localize within and cause cell lysis of experimental animal tumors (Theys et al., 2003). Attenuated *Salmonella*, *Clostridium*, and *Bifidobacterium* were found to be capable of multiplying selectively in tumors and inhibiting their growth, representing a new approach to cancer treatment (Bermudes et al., 2002). However, very little is known about the detailed mechanism of tumor regression by such pathogens (Yamada et al., 2002). When *Clostridium novyi*-NT spores were administered together with conventional chemotherapeutic drugs, extensive hemorrhagic necrosis of tumors resulted in significant and prolonged antitumor effects, a strategy that has come to be known as combination bacteriolytic therapy (COBALT) (Dang et al., 2001). *C. novyi*-NT has also been shown to markedly improve the efficacy of radiotherapy when administered together (Bettegowda et al., 2003). Separate studies have shown that *Clostridium acetobutylicum* and *C. oncolyticum*, as well as *Bifidobacterium longum* may be used in gene therapy as possible vectors to transfer a specific gene product into the extracellular microenvironment of tumors (Lambin et al., 1998; Yazawa et al., 2001). Clostridia species have also been found to be potentially useful in tumor-specific delivery of high

therapeutic doses of anticancer agents to tumors (Nuyts et al., 2002; Minton, 2003). A novel approach to cancer gene therapy involves the use genetically of engineered anaerobic bacteria as tumor-specific vectors for the delivery of antitumor genes to hypoxic/necrotic areas (Liu et al., 2002). In bacteria-directed enzyme prodrug therapy (BDEPT), targeting bacteria are engineered to produce enzymes that can activate prodrugs within the tumor (Jain & Forbes, 2001).

Description of *Clostridium sporogenes*

The *Clostridium* genera are obligate anaerobes ranging in size from 3 to 8 micrometers. Obligate anaerobes are unable to use molecular oxygen for energy-yielding reactions. They use oxygen atoms in their cellular materials but these are sourced from water (Tortora et al., 1995). Over one hundred species have been differentiated on the basis of physiology, morphology, and toxin formation; pathogenic species produce destructive exotoxins or enzymes (Dorland's, 2004). Different species are found in soil, in water, and in the intestinal tracts of humans and other animals (Tortora et al., 1995).

The *Clostridium* genera are also known to have endospores, specialized "resting" cells formed when essential nutrients are depleted or when water is unavailable. Unique to bacteria, these are highly dehydrated cells with thick walls and additional layers contributing to their resistance to desiccation and heat, enabling them to remain dormant even for thousands of years. They do not carry out metabolic reactions but they do

contain an enzyme (dipicolinic acid) responsible for resuming their metabolic activity (Tortora et al., 1995).

Clostridium sporogenes is a Gram positive, proteolytic, catalase-negative, motile bacillus which is highly distributed in nature and in the intestines of animals. It is a saprophyte causing gas gangrene with oval endospores which are centrally or subterminally located (eMedicine, 2004). It is also associated with a foul odor. Its endospore may survive boiling for periods ranging from 15 minutes to 6 hours. It forms small- to medium-sized flat colonies with a raised yellow-grey center and a flattened periphery ("Medusa head" colony). The colonies are opaque with a matte surface and are adherent to the agar (Microbiology Lab Notes, 2004).

Studies involved in the toxicity of *C. sporogenes* in various animals and cultured cells have suggested that the haemorrhagic toxin of *C. sporogenes* exerts its effects in rabbits but not in mice, rats, or guinea-pigs, through direct action on endothelial cells (Hara-Kudo et al., 1997).

The Use of *Clostridium sporogenes* in Cancer Biotherapy

It has been known for several decades that certain species of anaerobic bacteria, of the genus *Clostridium*, can selectively germinate and grow in the hypoxic regions of solid tumors after intravenous injection (Lemmon et al., 1997). These hypoxic regions are characteristic of solid tumors in rodents and occur with high frequency in many human tumors. The Clostridia are often absent or have no discernible antitumor effect in small

metastatic lesions (Sznol et al., 2000). The ability of intravenously-injected Clostridial endospores to infiltrate, then selectively germinate in the hypoxic regions of solid tumors seems to be a totally natural phenomenon, which requires no fundamental alterations and is exquisitely specific (Minton, 2003).

Tumor pathology resulting from Clostridia infection is manifested as a liquid necrotic center surrounded by a rim of viable tumor cells, from which the tumor would eventually regrow. Some animals may become ill and die during the peak of oncolysis, presumably from the toxic and/or systemic inflammatory effects of bacteria, their by-products, or necrotic tumor debris (Sznol et al., 2000).

Of the Clostridial species, *C. sporogenes* has the highest reported tumor colonization efficiency (Liu et al., 2002). An early clinical trial on 49 patients in 1978 showed that *C. sporogenes* endospores injected intracarotidally could liquefy vascular glioblastomas completely in 1 week, a process then termed "oncolysis" (Heppner & Mose, 1978). A study by Liu et al. (2002) reported the successful transformation of *C. sporogenes* with the *E. coli* cytosine deaminase (CD) gene and showed that systemically injected endospores of these bacteria express CD only in the tumor. This enzyme can convert the nontoxic prodrug 5-fluorocytosine (5-FC) to the anticancer drug 5-fluorouracil (5-FU). Since most human solid tumors have hypoxic and necrotic areas, this vector system has considerable promise for tumor-selective gene therapy.

Principles of Xenografting

Xenografting is the transplanting of tissue from one species to another. The tissue that is transferred is known as a xenograft. The first experiment with this technology occurred in 1963, when scientists and doctors took several kidneys from chimpanzees and transplanted them into human patients. Presently, the most common animal used for xenografting is the pig, although primates and rodents are also used often. The largest setback to this medical procedure is hyperacute rejection where the body instantly realizes that the tissue transplant is foreign and the immune system attacks it (Schaeffer, 2002).

Xenografting human tumor tissue into immunodeficient test organisms provides samples enriched for neoplastic cells that are optimal for molecular analysis. Xenografted tumor DNA has been shown to remain stable in relation to the primary lesion's DNA. Studies using this technique have thus far led to important discoveries of genetic changes underlying colorectal (*i.e.*, SMAD2) and pancreatic (*i.e.*, SMAD4) cancers (Yustein et al., 1999).

Cyclosporine as an Immunosuppressant

Immunosuppression refers to the pharmacologic suppression of the immune system. Immunosuppressants have been used extensively to decrease the rejection phenomenon in transplanted tissue (Bullock, 1996).

The most effective immunosuppressant against the rejection of organ transplant is cyclosporine. It is a cyclic peptide composed of 11 amino acids isolated from the fungus *Tolypocladium inflatum*. It was discovered to be immunosuppressive when it was observed to suppress antibody production in mice during the screening of fungal extracts (Trubek, 1997). It acts as a specific suppressor of T_H cells by preventing these cells from producing interleukin-2 and interleukin-2 receptors. Thus, the immune reaction against the graft is inhibited because the necessary helper step is prevented. Furthermore, it does not significantly affect suppressor T or B lymphocytes. Hence, it is a selective immunosuppressive drug without the cytotoxicity characteristic of most other immunosuppressive drugs. Since cyclosporine works only in the primary (afferent) immune phase, it must be administered before exposure to the attacking antigen (Gennaro, 2000).

Cyclosporine has shown usefulness in both animal and human transplantation, and has been used in xenograft studies involving implantation of ewe mammary tissue into virgin female mice, human skin into Lewis rats, lamb hearts into goats, and human glioma into male cats (Daniel et al., 1991; Biren et al, 1986; Bailey et al., 1985; Krushelnycky et al., 1991). In a study involving the implantation of human carcinomas under the kidney capsule of mice, conventional mice treated daily with 60 mg of cyclosporine A per kg were comparable to nude mice as hosts which supported the long-term persistence and growth of the human tumor implants. Such mice could therefore provide an alternative to nude mice as hosts in which chemosensitivity assays could be

carried out against growing human tumors at a considerable saving in cost and convenience (Bennett et al., 1985).

Characterization of HT29 Colon Cancer Cells

The HT29 cell line of human colon adenocarcinomas was established from the primary tumor of a 44-year-old Caucasian woman with colon adenocarcinoma in 1964. It is described to be heterotransplantable and xenograftable in nude mice and steroid-treated hamsters, forming well-differentiated grade II tumors (Cell Line Data Base [CLDB], 2004). According to the WHO tumor classification and grading, grade II tumors are slow-growing tumors that have poorly-defined margins or diffuse spread which often preclude complete surgical resection. It is commonly used in experimental studies on antitumor testing, tumorigenicity, differentiation, pharmacodynamics, cloning, cytotoxicity, cell biology, antineoplastic agents sensibility, and virology (Interlab Cell Line Collection [ICLC], 2004). HT29 tumors xenografted into nude mice reach a mean target window size of 100-200 mg in 4-6 days and a mean tumor size of 1000 mg in 35-40 days (Charles River Laboratories, 2002).

MATERIALS AND METHODS

Source of Chemicals

Cyclosporine was purchased from Novartis, normal saline solution (NSS) from Mercury Drug, and the depilatory lotion from PCX. Malachite green dye powder and the reagents needed to prepare the phosphate-buffered saline solution (PBS) were obtained from the Department of Biology (DB) stockroom. All the chemicals used to maintain, subculture, and count the cancer cells were provided by the Bacteriology Laboratory of the National Institutes of Health (NIH).

Source and Preparation of *Clostridium sporogenes* Endospores

Clostridium sporogenes was purchased from the Philippine National Collection of Microorganisms (PNCM) at the University of the Philippines - Los Baños (UPLB). The culture was sporolated by heating it in a water bath at boiling temperature for 45 minutes and then incubating it at 37°C for 24 hours. This was repeated 3 times after which the culture was centrifuged at 2500 revolutions per minute (rpm) for 10 minutes to separate the endospores from the culture media and other cellular components. The supernatant was discarded and the remaining pellet which consisted of the endospores was saved and suspended in 1 mL of PBS. The endospore density (number of endospores per mL of PBS) was determined using direct microscopic count method with Schaeffer-Fulton staining (Appendix B).

Test Animals

Female BALB/c mice 6-8 weeks of age were used as test organisms in all treatments and were purchased from the NIH. The mice were maintained at the NIH for the entire duration of the experiment. They were housed 3-5 to a cage containing sterile wood-chip bedding and were provided with chow pellets and distilled water *ad libitum*. Tagging of animals for easier identification was accomplished by marking the tails with different-numbered bands using non-toxic permanent markers. The dorsal posterior surface of the mice was kept hairless throughout the study by constant depilation as necessary to facilitate injection procedures and the detection of tumor growth and changes in tumor volume. Depilation was carried out by liberally spreading depilatory lotion over the body surface. After 4 minutes, the lotion with the removed hairs was scraped off. At the end of the experiment, the mice were sacrificed for use in histopathological analysis.

Source of and Maintenance of Cancer Cell Line

HT29 human colon adenocarcinoma cells were purchased from the Research and Biotechnology Division (RBD) of St. Luke's Medical Center in Quezon City. The cells were confined in a flask containing 10 mL of culture media consisting of 8.6 mL Roswell Park Memorial Institute (RPMI) medium supplemented with 1 mL fetal bovine serum, 0.1 mL penicillin-streptomycin, 0.1 mL fungizone, and 0.2 mL L-glutamine and maintained at 37°C and 5% CO_2 using a CO_2 incubator. The cells were initially split into

two cultures (split ratio 1:2) and subcultured and harvested inside a laminar flow hood every 2 days.

Harvesting, Subculturing, and Counting of Cancer Cells

To harvest and subculture the HT29 cells, the culture media in the flask was first discarded. Five mL of 0.25% trypsin-0.01% ethyldiaminetetracetic acid (EDTA) solution was introduced into the flask to dislodge the cells, which adhere to the bottom of the flask. The flask was then returned to the CO_2 incubator for 5 minutes after which the trypsin-EDTA solution now containing the dislodged HT29 cells was resuspended in 5 mL RPMI, transferred into a conical tube, and centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded and the pellet consisting of the cells was resuspended in 1 mL RPMI. Equal distribution of the cells in the medium was facilitated by vigorous shaking of the conical tube. Half of the medium was returned to the flask for subculturing while the other half was harvested for counting and eventual injection into the immunosuppressed mice.

The harvested cells were counted using the trypan blue exclusion method. Ninety μL of cell-containing RPMI was mixed with 10 μL of trypan blue stain and then transferred to a haemocytometer for counting. Under the microscope, the dead cells appeared blue and the viable cells remained colorless (see Appendix C for calculation of cell density).

Cyclosporine-induced Immunosuppression of Mice

Cyclosporine diluted with NSS was administered to each mouse intraperitoneally using a 29 gauge needle at 60 mg/kg of body weight once daily for 5 days before cancer cell injection and everyday thereafter until tumor excision. The general time of injection was 10 AM. Immunosuppression was confirmed by white blood cell (WBC) counts, which were performed on two non-immunosuppressed mice and two Cyclosporine-treated mice. Blood samples were sent to Intercon Diagnostic Laboratory to determine the WBC count. Mice having counts falling below the normal range of $5\text{-}10^6$ per mL were assumed to be sufficiently immunosuppressed.

Inoculation of Cancer Cells

Tumors were xenografted into the test organisms through the injection of 10^6 HT29 cells suspended in RPMI subcutaneously using a 29 gauge needle into the dorsal posterior surface of each mouse following 5 days of immunosuppression. The tumors were allowed to grow for 5 days after which they were injected with *C. sporogenes* endospores.

Determination of Antitumor Activity of *Clostridium sporogenes*

Administration of *C. sporogenes* was accomplished by intratumoral injection of approximately 10^6 endospores suspended in 0.1 mL of PBS using a 29 gauge needle. Injection was done to a depth estimated according to the size of the tumor. Ruler measurements of the longest and shortest dimensions of each tumor were performed once

daily to determine the tumor volume, which was calculated using the formula: longest dimension × (shortest dimension)^2 × 0.5 (Fu et al., 2004) (see Appendix E).

An untreated tumor, and tumors treated for 24, 48, and 72 hours were excised, cut into serial cross-sections, and stained with hematoxylin-eosin (H&E) for histopathological analysis. The variables considered were presence and relative abundance of tumor cells and of inflammatory cells.

RESULTS

Sufficient immunosuppression of the BALB/c mice through the injection of Cyclosporine was confirmed by their lowered WBC counts (Appendix D). Establishment and growth of tumors further verified immunosuppression.

Successful sporolation was confirmed by the appearance of endospores under the oil-immersion objective (1000X) of a light microscope, when the PBS-suspended pellet obtained from centrifugation of the sporolated stock culture was subjected to Schaeffer-Fulton staining. Intratumoral injection of the *C. sporogenes* endospores was confirmed by the presence of endospores in the histological section of the tumor treated for 24 hours (see Plates).

Successful injection of the mice with 10^6 HT29 cells and likewise tumor growth were confirmed externally by the subcutaneous growth of tumors in the area of injection at least 1 day after the cancer cells were injected, and histopathologically when tumor cells were observed after the control tumor was excised, cut into serial cross-sections, stained with H&E, and viewed under the high-power objective (400X) of a light microscope (see Plates).

Externally, all the tumors, having grown subcutaneously, took on the color of the skin and remained attached to the overlying skin tissue. They were smooth and solid to the touch and were roughly spherical in shape, except for the tumor later treated for 72 hours which was initially (prior to treatment) elongated, but which became spherical 24 hours after treatment with *C. sporogenes*. The edges of the tumors were predominantly well-defined with regions of irregularity.

The volume of the tumors regressed by at least 50% and at most 83.33% daily for 3 days. Upon histological examination, the control tumor was found to contain numerous tumor cells which formed a glandular configuration and infiltrated the wall of fibrous tissue surrounding the entire tumor mass. A central core of necrotic debris was present. The individual tumor cells exhibited moderate pleomorphism, and had enlarged nuclei, prominent nucleoli, and a high nucleus-to-cytoplasm ratio. Mitotic figures were found in the tumor cell nests, indicating active cell division. All of these support the malignancy of the tumor cells.

The tumor excised 24 hours after injection of *C. sporogenes* endospores showed a striking accumulation of inflammatory cells, mostly neutrophils, indicating the occurrence of an acute inflammatory response. No tumor cells were found in the mass, which contained a central necrotic core.

The tumor excised after 48 hours of treatment exhibited considerably less inflammation than the tumor treated for 24 hours, with the inflammatory cells consisting mostly of lymphocytes. An accumulation of fibroblasts and collagen fibers was also present. Damaged muscle tissue intermixed with necrotic debris and surrounded by a thin fibroblast wall were observed. Likewise, no tumor cells were found.

The tumor excised after 72 hours of treatment contained denser fibroblasts and collagen fibers and a small area consisting of necrotic debris walled off by lymphocytes and fibroblasts. A single tumor cell nest whose individual cells exhibited the same characteristics as those in the control tumor was observed.

DISCUSSION

Clostridium sporogenes was found to cause regression in the volume of HT29 tumors in immunosuppressed mice. The mechanism of regression can be attributed to both or either of two events: the destruction of viable tumor cells adjacent to the original necrotic areas by the germinated bacteria, or the activation of the host's immune system by the bacterial invasion (Dang et al., 2001; Agrawal et al., 2004).

The destruction of viable tumor cells adjacent to the original necrotic areas by the germinated bacteria has been documented. Strains of *Clostridium* are known to localize within and cause cell lysis of experimental animal tumors (Theys et al., 2003). Once germinated, the bacteria destroy adjacent tumor cells through the secretion of lipases, proteases, and other degradative enzymes (Agrawal et al., 2004).

An offshoot of this mechanism is the permissive model, which proposes the possibility that the tumor cells, being "foreign", naturally elicited an immune response during tumorigenesis that was not strong enough to eradicate all tumor cells. The lytic effects of *Clostridium sporogenes*, assuming there were any, could simply have reduced the tumor load, thereby permitting the normal immune response to deal with the few remaining tumor cells and curing the mice.

Studies proving the occurrence of bacterial lysis of tumor cells include those by Dang et al. in 2001 and Bettegowda et al. in 2003, which revealed that endospores of *C. novyi*-NT, an attenuated derivative of *C. novyi* without the lethal toxic gene, could

reduce the volume of HCT116 colon cancer cells grown in athymic nude mice by this mechanism. A study by Heppner et al. in 1978 showed that vascular glioblastomas in humans become liquefied when contaminated with spores of *Clostridium sporogenes* in a process termed "oncolysis". This mechanism could not be confirmed in this study since Gram staining of sections of the tumors to determine the distribution of the germinated bacteria within the tumors was not performed.

The second mechanism attributes tumor regression to the activation of the host's immune system by the bacterial invasion. In this study, the noted immune response to bacterial infection was acute inflammation.

Inflammatory reactions caused by bacterial infections have been known to stimulate a potent cell-mediated immune response that can destroy intact tumor cells. They may also directly contribute to the destruction of tumor cells through the production of reactive oxygen species, proteases, pore-forming agents, and tumoricidal cytokines. Further, antibody molecules involved in humoral immunity circulate in the blood and enter the tissue via inflammation. In addition to providing antitumor effects, inflammatory reactions may also serve to restrain the spread of the bacterial infection, providing a second layer of control in addition to that provided by the requisite anaerobic environment (Agrawal et al., 2004).

It has been proposed that the cell-mediated immune response, rather than the humoral response, is a key mediator of cancer regression. Yet, many of the cases of spontaneous regression as well as tumor inhibition in animal studies involved infections

that elicited a humoral immune response. However, while both cell-mediated and humoral responses are often delayed by several days to a week, tumor regression was often noted within hours of tumor injection with bacteria. Exploratory evaluation of case reports of spontaneous regression thus support the concept that infection-stimulated tumor regression generally results from a "non-specific" innate immune response as opposed to a specific antitumor one (Hoption Cann et al., 2003).

Studies that confirm the use of live bacteria as an immunotherapy for cancer have been performed as early as 1891. In that year, William Coley used *Streptococcus* injections to treat malignant tumors in humans such as lymphomas, soft-tissue sarcomas, osteosarcomas, Ewing's sarcomas, malignant melanomas, and cervical, ovarian, testicular, renal, breast, and colorectal carcinomas. Vaccines containing a live attenuated strain of *Mycobacterium bovis* are currently considered the most effective treatments for human superficial bladder cancer, and are also applied to treat malignant melanoma, prostate carcinoma, and leukemia (Hoption Cann et al., 2003; Cheadle & Jackson, 2002). The vaccine is applied directly to the tumor site and leads to a complex immunological response which brings about tumor regression (Hoption Cann et al., 2003).

A study in which both bacterial lysis and bacterial immunotherapy were found to contribute to tumor volume regression was done by Agrawal et al. (2004). Endospores of *C. novyi*-NT were observed to reduce the volume of CT26 colorectal cancer tumors grown in BALB/c mice by bacterial lysis coupled with the generation of an immune response in which the host produced cytokines such as IL-6, MIP-2, G-CSF, TIMP-1, and

KC that attracted a massive influx of inflammatory cells, initiated largely by neutrophils and followed within a few days by monocyte and lymphocyte infiltration.

SUMMARY

Clostridium sporogenes endospores injected intratumorally into HT29 tumor xenografts in Cyclosporine-immunosuppressed mice caused regression in tumor volume by at least 50% and at most 83.33% daily, for 3 days. Histopathological analysis of the tumor revealed that this regression was accompanied by the influx of inflammatory cells induced by the presence of *C. sporogenes*.

RECOMMENDATIONS

Cancer cell lines aside from HT29 may be used to induce tumor growth. A larger sample size in terms of tumor number is recommended for more conclusive results. A vernier caliper may be used to measure tumor dimensions to improve accuracy. Other bacterial species may be administered to treat established tumors. Athymic nude mice may be used in the place of Cyclosporine-immunosuppressed BALB/c mice as hosts for human cancer cells. As an alternative to direct microscopic count, optical density may be used to approximate endospore density if a standard is available.

Histopathological studies may be extended to include quantification of parameters such as the size of the area covered by inflammatory cells and/or of necrotic areas relative to the size of the tumor mass, in order to more accurately conclude changes in tumor morphology. Gram staining of sections of the tumors may be performed to determine the distribution of the germinated bacteria within the tumors. Serum cytokine and degradative enzyme (protease, lipase) assays, antibody titering, and rechallenge experiments involving the reinjection of HT29 cells into bacteria- and surgically-cured mice may be carried out to confidently establish the presence and specificity of the immune response, if any.

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TABLE

Table 1. Volume of tumors at different time periods following treatment with *Clostridium sporogenes*

Mouse	Actual tumor volume (mm^3)	Tumor volume in mm^3 (% decrease)		
		Time period of treatment with *C. sporogenes*		
		24 hours	48 hours	72 hours
Control	8.0	-	-	-
1	8.0	4.0 (50.00)	-	-
2	13.5	6.0 (55.56)	1.0 (83.33)	-
3	12.0	6.0 (50.00)	1.0 (83.33)	0.5(50.00)

Note: - : Data unavailable because tumor was previously excised.

APPENDICES

APPENDIX A
Formulation of Solutions

A.1. Malachite Green Dye Solution

0.3 g malachite green powder
10 mL distilled water

A.2. Phosphate-Buffered Saline Solution

8.08 g sodium phosphate dibasic (Na_2HPO_4)
1.37 g potassium phosphate monobasic (KH_2PO_4)
8.50 g sodium chloride (NaCl)
1000 mL distilled water

APPENDIX B

Direct Microscopic Count with Schaeffer-Fulton Endospore Staining Procedure
(Parungao, 2004)

1. An endospore suspension at a volume of 0.01 mL was spread evenly inside an outlined square with an area of 100 mm^2 on a glass slide.
2. The suspension was air dried and fixed by heat.
3. The portion of the slide containing the dried and fixed endospore suspension was covered with filter paper and flooded with Malachite green.
4. The slide was passed over the flame of an alcohol lamp for 10 minutes, during which time the endospore smear covered with filter paper was continuously saturated with Malachite green.
5. The slide was cooled and washed in gently running water.
6. The portion of the slide containing the stained endospore smear was examined under the oil-immersion objective of a light microscope.
7. The endospore density was determined using the formula:

$$\text{Spore density}\left(\frac{\text{spores}}{\text{mL}}\right) = \frac{\text{Average number of spores}}{\text{Area of microscopic field}} \times \frac{100\,\text{mm}^2}{0.01\,\text{mL}}$$

APPENDIX C
Calculation of Cancer Cell Density

Number of viable cancer cells per mL of RPMI = N X B X 10^4

where:

N = average count of unstained cells per square of the four corner squares counted

B = the dilution factor in trypan blue

$$\text{Dilution factor} = \frac{\text{volume of cancer cell solution + volume of trypan blue dye}}{\text{volume of cancer cell solution}}$$

$$\text{Dilution factor} = \frac{90\ \mu L \text{ cancer cell solution} + 10\ \mu L \text{ trypan blue dye}}{90\ \mu L \text{ cancer cell solution}} = 1.11$$

APPENDIX D
White Blood Cell Counts for Untreated and Cyclosporine-treated Mice

Mouse	Total White Blood Cell Count (10^9) per Liter	Baseline
Untreated		5.0-10.0
1	7.2	
2	7.6	
Cyclosporine-treated		
1	4.4	
2	3.7	

APPENDIX E
Sample Illustration of Longest and Shortest Tumor Dimensions

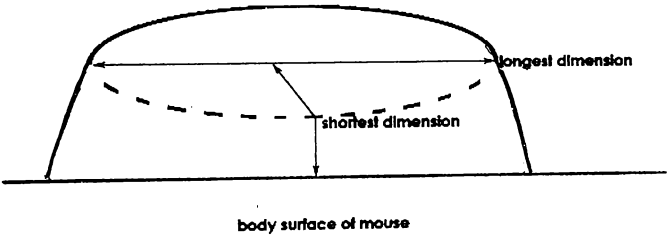

Figure 1. Diagrammatic representation of a tumor viewed from the side, showing sample longest and shortest dimensions.

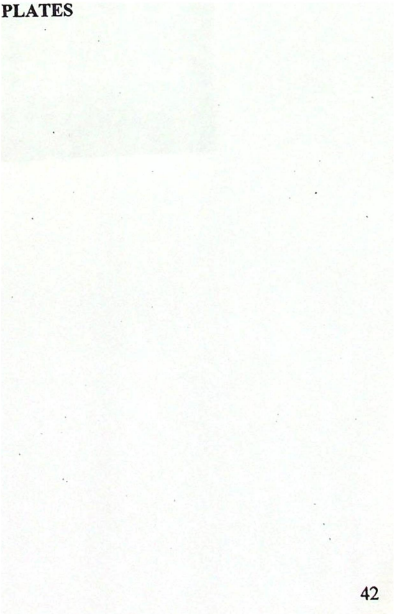

Plate 1. Sandimmun (Ciclosporine) ampoules.

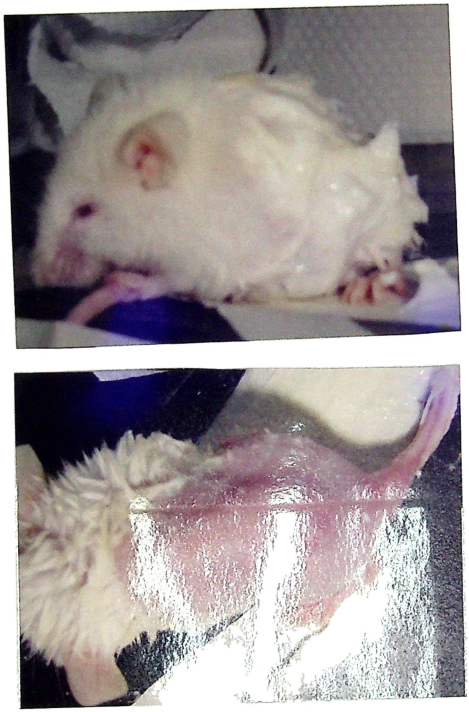

Plate 2. Mouse in the process of depilation (above) and after depilation (below).

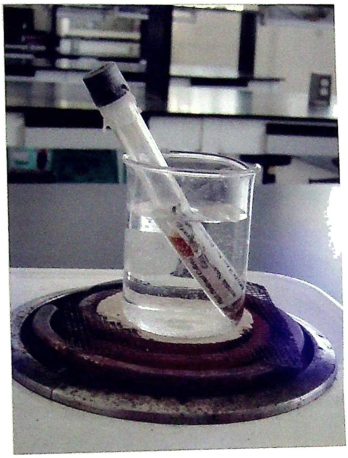

Plate 3. Sporolation of *Clostridium sporogenes*.

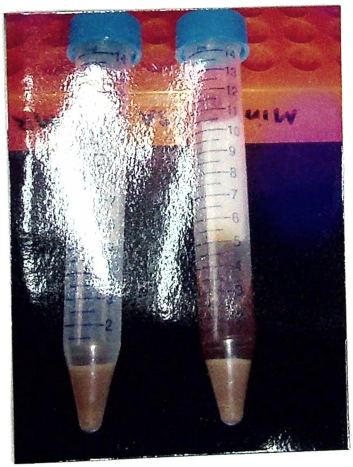

Plate 4. *Clostridium sporogenes* culture after centrifugation. Pellet contains the spores.

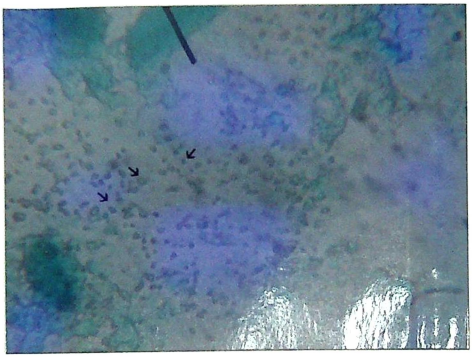

Plate 5. View of *C. sporogenes* spores after Schaeffer-Fulton staining (1000X).

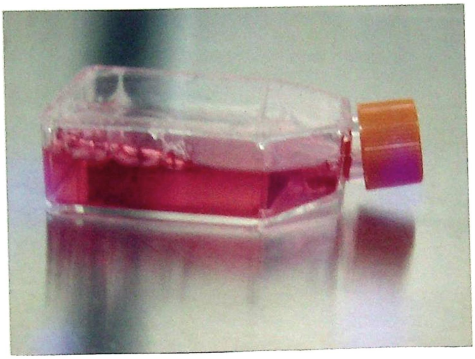

Plate 6. Culture flask containing cancer cells in fresh media.

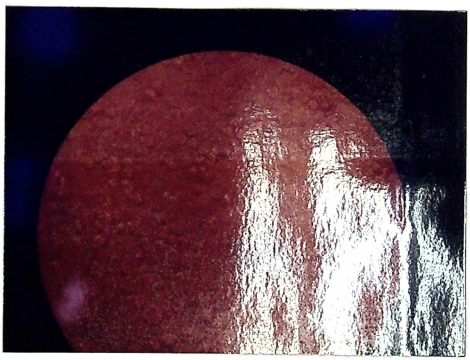

Plate 7. Appearance under an inverted light microscope of cancer cells contained in a culture flask with media (40X).

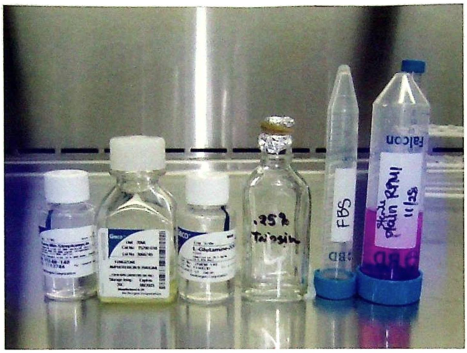

Plate 8 Reagents used in subculturing and harvesting cancer cells. From left to right: penicillin-streptomycin, amphotericin, L-glutamine, trypsin, fetal bovine serum, Roswell Park Memorial Institute medium.

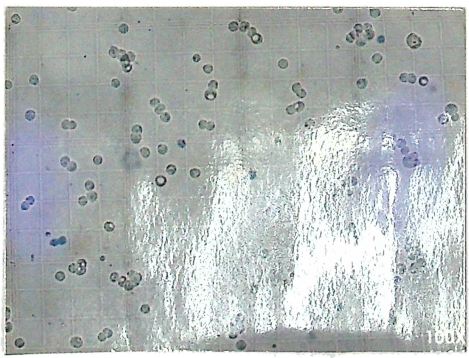

Plate 9. View of trypanized cancer cells (400X). Viable cells remain unstained; dead cells are stained blue.

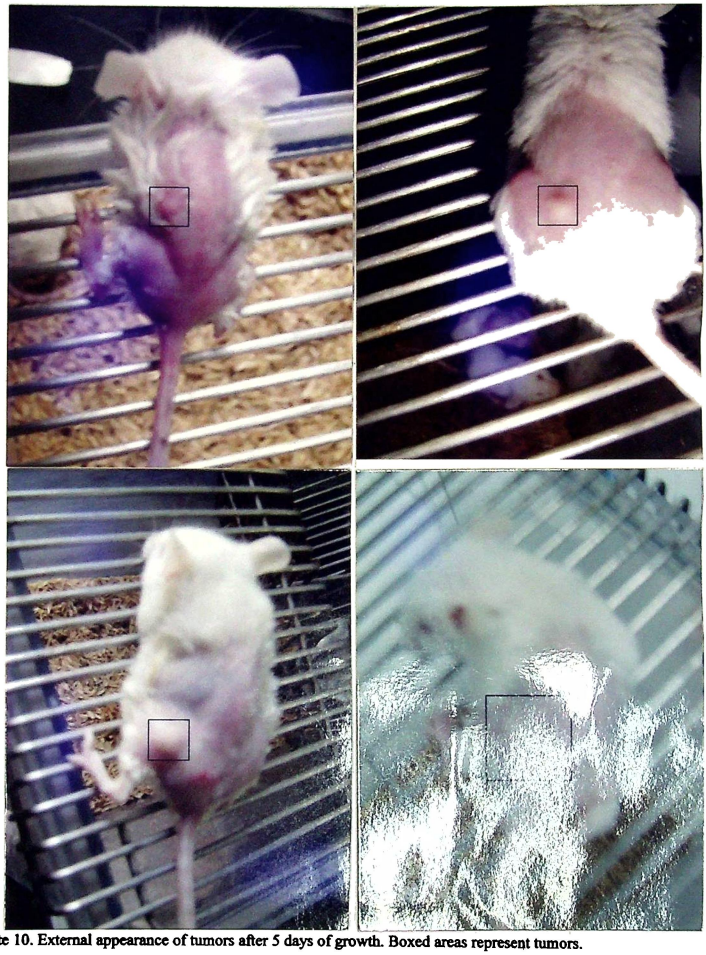

Plate 10. External appearance of tumors after 5 days of growth. Boxed areas represent tumors.

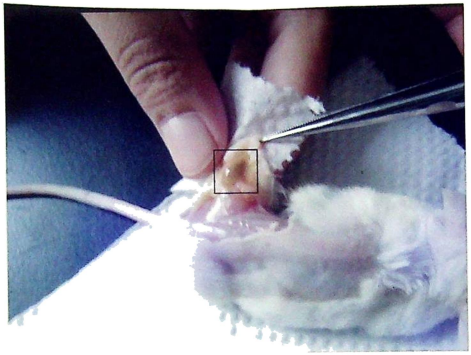

Plate 11. Tumor excision. Boxed area represents the tumor.

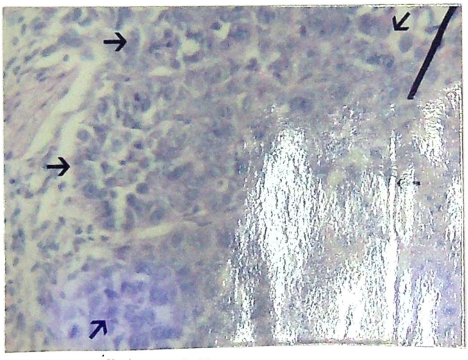

Plate 12. Numerous tumor cells (surrounded by arrows) in histological section of control (untreated) tumor. (400X).

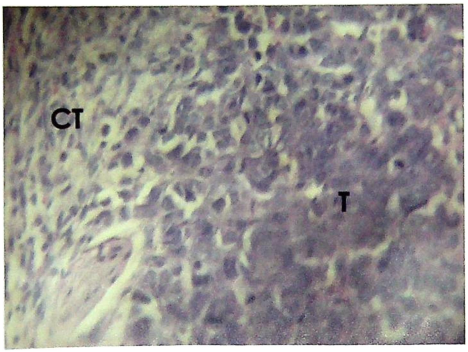

Plate 13. Tumor cell (T) infiltration of the wall of fibrous tissue (CT) in control (untreated) tumor (400X).

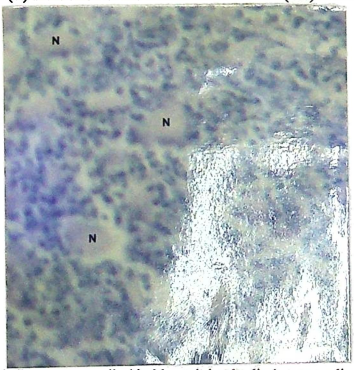

Plate 14. Numerous inflammatory cells (darkly staining bodies) surrounding necrotic areas (N) in tumor treated with *C. sporogenes* for 24 hours (400X).

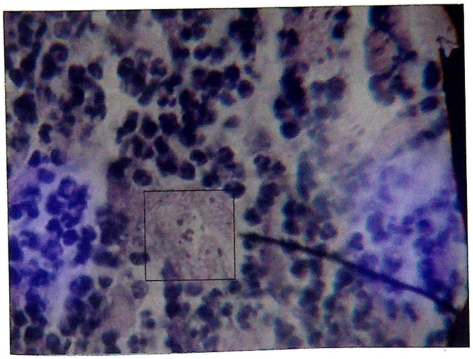

Plate 15. *C. sporogenes* endospores found in histological section of tumor treated for 24 hours. Small, circular bodies inside the boxed area represent the endospores (1000X).

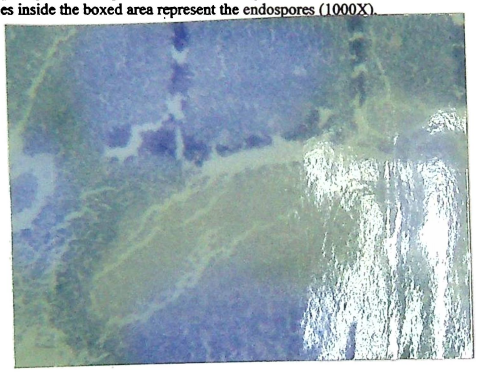

Plate 16. Inflammatory cells (darkly staining bodies) and necrotic core (pink region) in tumor treated for 24 hours (100X).

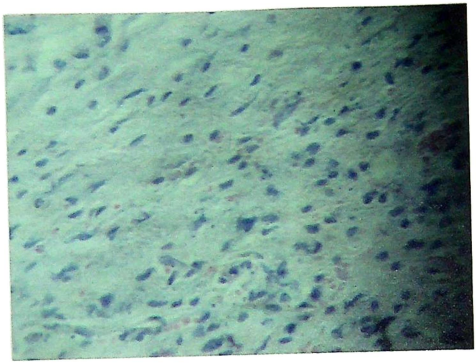

Plate 17. Fibroblasts and collagen fibers in tumor treated for 48 hours (400X).

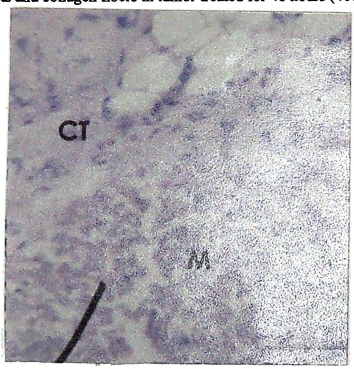

Plate 18. Damaged muscle tissue intermixed with necrotic debris (M) and surrounded by a thin fibroblast wall (CT) in tumor treated for 48 hours (400X).

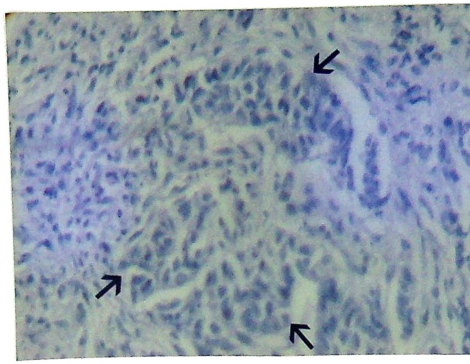

Plate 19. Single tumor nest in tumor treated with *C. sporogenes* for 72 hours (400X).

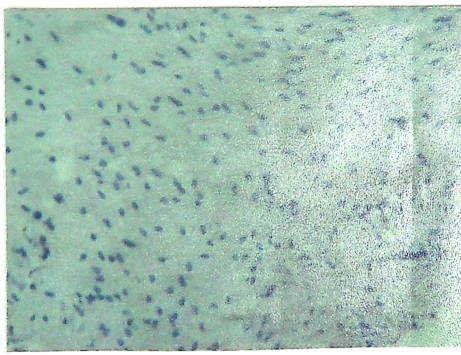

Plate 20. Dense fibroblasts and collagen fibers in tumor treated for 72 hours (400X).

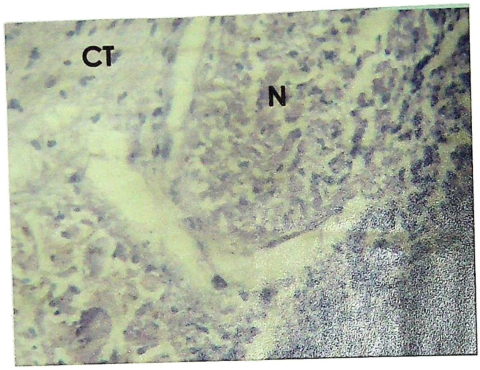

Plate 21. Area consisting of necrotic debris (N) walled off by lymphocytes (I) and fibroblasts (CT) in tumor treated for 72 hours (400X).

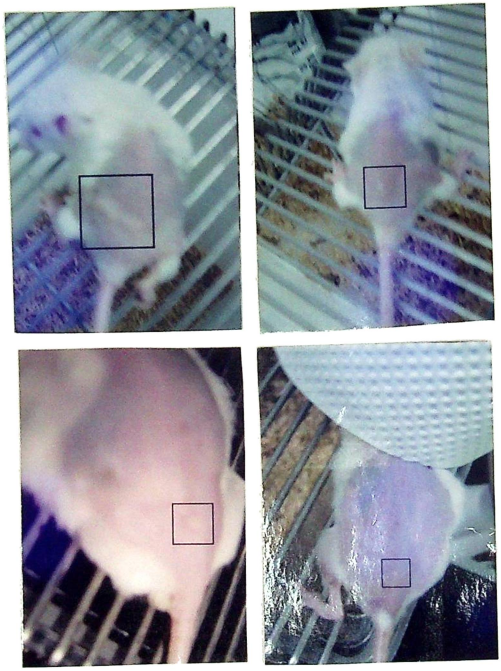

Plate 22. Tumor regression in a mouse observed 0 (upper left), 24 (upper right), 48 (lower left), and 72 (lower right) hour/s after treatment with *C. sporogenes* endospores.