ROLE OF POLYMORPHONUCLEAR LEUKOCYTES IN THE TUMORICIDAL ACTIVITY OF *Propionibacterium acnes*

by

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(ABSTRACT)

The mechanism responsible for the killing of tumor cells after injection of mice with a mixture of tumor cells and *Propionibacterium acnes* was investigated. Tumor cells were injected intramuscularly into Balb/c mice either alone or together with *P. acnes* vaccine. The tumor cells were then removed from the injection site 12 hours after injection, and transferred into fresh mice. Tumor cells from control animals given tumor cells only caused tumors when transferred into fresh mice 12 hours after injection whereas tumor cells from animals given both tumor cells and vaccine did not develop tumors in the fresh mice.

ELISA tests were done to estimate the number of tumor cells in the lesions. In control animals given 10^5 tumor cells the estimated numbers dropped to 10^3 cells at 24 hours, but thereafter rose steadily. Palpable tumors were present 7-10 days later. In animals given 10^5 tumor cells

+ 500 ug of P. acnes vaccine, estimated tumor cell numbers fell steadily, and could not be detected after 2 days. Palpable tumors never developed in these animals. These results indicate that tumor cells are killed, or rendered nontumorigenic, during the first 12 hours after co-injection into mice with P. acnes.

Histological studies showed that injection of P. acnes vaccine, with or without tumor cells, induced large numbers of polymorphonuclear leukocytes (PMNs) at 12 hours. To determine the role of PMNs in the killing of tumor cells, tumor cells were incubated with supernatant obtained from the phagocytosis mixture of PMNs and P. acnes. After a 2-hr. incubation, the tumor cells were washed and injected into fresh mice. No tumors developed, indicating that a product of the phagocytosis of P. acnes by PMNs played a role in the killing of tumor cells. Bacterial vaccines such as P. freudenreichii, which are poorly protective against tumor cells, produced phagocytosis supernatants which were unable to kill tumor cells.

Various oxygen radical scavengers/inhibitors were used to test their effect on the toxicity of the supernatant on tumor cells and chinese hamster ovary cells. Both azide and catalase rendered the supernatant nontoxigenic, suggesting that H_2O_2, produced by PMNs during phagocytosis of P. acnes, is responsible for the killing of tumor cells.

However, addition of catalase 30 minutes after the start of phagocytosis had no effect on the toxicity of the supernatant, suggesting that H_2O_2 is converted to other toxic radicals during the course of phagocytosis of P. acnes by PMNs.

The oxygen consumption levels of PMNs during phagocytosis of P. acnes or other bacterial vaccines was measured and found to be similar regardless of the antitumor ability of the vaccine used. This suggests that the difference in the ability of various vaccines to protect mice against tumors may be in the production of a particular oxygen radical by PMNs during phagocytosis, and not in the production of different quantities of the same radicals.

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INTRODUCTION

The treatment of cancer by injections of bacterial products is based on the fact that, for over two hundred years, neoplasms have been sometimes observed to regress in patients suffering from acute infections. If these cases were not too far advanced, and the infections were of sufficient severity or duration, the tumors sometimes completely disappeared and the patients remained free from recurrence.

Dr. William B. Coley developed the first "mixed toxins" preparations that were used in the treatment of cancer from 1891 until 1936 (32). This consisted of a mixture of killed cultures of Streptococcus pyogenes and Serratia marcesens (then known as Bacillus prodigiosus) (108). In spite of reports of great success in producing the recession of tumors, this type of treatment never developed into the cure for cancer that everyone had anticipated. This was mainly due to the fact that Dr. Coley's results were not reproducible. The details of toxin preparation, such as the type of growth media used, and the exact composition of the bacterial mixture were never standardized. Other physicians tried Dr. Coley's methods, but more often than not, were unable to achieve the same degree of success.

It wasn't until the 1960's that interest in the treatment of tumors by injection with bacterial preparations was renewed. Bernard Halpern, using a killed vaccine made from a strain of Corynebacterium parvum that had been isolated by Prevot, treated mice that had been injected with Ehrlich's ascites tumor cells, and was able to report a regression in the development of the tumor (60). Similar results were reported in the same year by Woodruff and Boak (180).

The organism C. parvum had been originally isolated in 1926 by Mayer from a blood culture of a patient suffering from an unidentified chronic illness acquired at childbirth (91). The bacterium was described as being coryneform in shape, as it was wider at one end than the other, and was named Corynebacterium parvum infectiosum. In 1940, Prevot referred to it using the name Corynebacterium parvum (116). He then proceeded to use it in his studies on the stimulation of the reticuloendothelial system of rabbits (117) which prompted Halpern and others to begin their investigations into the ability of the organism to inhibit the growth of transplantable tumors in mice.

By the beginning of the 1970's, research on the antitumor activity of Corynebacterium parvum was well under way. However, in the latter part of the decade, as a

result of detailed taxonomical studies by various laboratories, it was determined that Corynebacterium parvum was in fact a member of the anaerobic propionibacteria. Since that time, it has been officially classified as Propionibacterium acnes, a member of the normal flora of the human skin. In spite of this, the name C. parvum has continued to be used in scientific papers by many investigators. In the interest of accuracy and to avoid confusion, the organism will be referred to as Propionibacterium acnes throughout this thesis, even when referring to literature citations where the older name was used by the authors.

The following literature review will first discuss the classification and general characteristics of P. acnes. A discussion on its immunomodulating activity will then follow. Next, a review of the various antitumor mechanisms known to be induced in the host after injection with P. acnes will be included. Finally, the general characteristics of the polymorphonuclear leukocyte and its ability to produce various toxic oxygen radicals will be discussed in order to better understand the role of this phagocyte in the ability of the vaccine to inhibit the growth of tumors. A brief rationale for the experimental approach used in this thesis will also follow.

LITERATURE REVIEW

I. THE ORGANISM *Propionibacterium acnes*

A. Identification and Classification

Propionibacterium acnes was originally classified in the early 1900's as a member of the cutaneous corynebacteria. Like the corynebacteria, it exhibited bipolar staining as well as the classical club shape. In 1923 it was placed in the genus *Corynebacterium* by Bergey *et al* (15).

In the early part of the century it had been noted by Gilchrist that this organism, unlike the corynebacteria, grew best anaerobically (52). Studies carried out in the 1940's by Douglas and Gunter on the nature of its fermentation products led them to conclude that it should be classified in the genus *Propionibacterium* due to its production of propionic acid during fermentation of glucose (40).

In 1963, with the advent of better methods for the growth of anaerobic microorganisms, Moore and Cato were able to study the products of fermentation of this organism under *strict* anaerobic conditions (103). The ability to ferment lactate as well as glucose for the production of propionic acid led them to support the organism's

classification as Propionibacterium acnes.

In 1972 Johnson and Cummins performed a series of serological tests, cell wall composition analyses, and DNA homology comparisons using 80 strains of anaerobic coryneforms. They noticed that three of four strains labeled C. parvum could be identified as P. acnes (68). In 1974 they decided to investigate 59 strains labeled C. parvum, and found that 52 of those strains showed a high degree of homology with P. acnes DNA (36). In addition, they found that antigens from these Corynebacterium parvum strains reacted with serum against P. acnes, suggesting the sharing of cell wall antigens by the two species. The strains studied were seen also to lack mycolic acids, an indication that these organisms had little in common with the classical corynebacteria.

From these studies it was realized that this organism should indeed be classified as Propionibacterium acnes and the name C. parvum be eliminated from use. Finally, in the Approved Lists of Bacterial Names published by the International Journal of Systematic Bacteriology in 1980, such action was taken (147). So many years had passed, however, since the naming of this organism as C. parvum that the name has remained in much of the literature and only now is the correct name of P. acnes beginning to appear in scientific publications.

B. General Characteristics of Propionibacterium acnes

P. acnes is a gram-positive rod-shaped bacterium, most often isolated from human skin (35). It is indeed part of the normal flora of the skin which makes it understandable that it is one of the most common contaminants of anaerobic cultures (94, 64). It also has been found in the perianal gland of guinea pigs, but not in any other laboratory rodent (93).

As previously mentioned, this organism produces large amounts of propionic acid during growth, and is able to ferment lactate. P. acnes does not exhibit motility and is unable to form spores (35). It grows best under strictly anaerobic conditions, although it has been known to grow microaerophilically (34). Colonies grown on Brain Heart Infusion agar appear small (< 1mm), semi-opaque, and grayish after 4 days of anaerobic incubation at 37°C. P. acnes is able to liquefy gelatin, produce indole, and reduce nitrate to nitrite (35).

P. acnes strains can be divided into two types depending on cell wall sugar patterns as determined by composition analyses and serological studies. Type I strains contain galactose, glucose, and mannose as principal cell wall sugars. Type II P. acnes strains lack galactose as a cell wall component. In addition, some type

I strains are able to ferment sorbitol while type II strains are not (158).

II. IMMUNOMODULATING ACTIVITY OF P. acnes

One property of P. acnes which has been studied extensively is its ability to augment the specific or nonspecific immune response of animals. Primarily, its ability to stimulate the reticuloendothelial system (RES) of mice and its anti-tumor activity have been of great interest to researchers involved in the development of reliable methods for cancer therapy (60, 61, 180). Discussion of these effects will be herewith presented, along with a brief summary of results obtained in the study of the nature of the P. acnes cell component involved in the immunomodulation. It must be noted that much of the literature reviewed in this section refers to P. acnes by the antiquated name of Corynebacterium parvum.

A. Effects of P. acnes on the Reticuloendothelial System

Splenomegaly has been used since the early years of immunology as a measure of the stimulation of the reticuloendothelial system. After systemic administration of P. acnes, the liver and spleen increase in weight and size for a period of two weeks after which they gradually return to their original size (17, 24). This occurs as a

result of the influx of macrophages, histiocytes, and other immune system cells after intravenous injection of immunostimulants such as P. acnes (33, 43).

Splenomegaly has often been used as a measure of the ability of vaccines to stimulate the reticuloendothelial system, and this has been linked to the prevention of tumor growth in animals. In experiments performed by Bomford and others, splenomegaly was detected after intravenous injection with P. acnes and tumor cells (17, 80). In this system it was noted that, in addition to an increase in the size of the spleen, the animals did not develop tumors. This led many investigators to believe that the mechanism involved in the stimulation of the reticuloendothelial system was the same one responsible for the abrogation of the tumor's growth. However, Stimpson has found that various treatments such as extraction with trichloroacetic acid or formamide, oxidation with periodate, or acetylation, which abolish the ability of the P. acnes vaccine to induce splenomegaly, did not affect its ability to prevent the growth of a fibrosarcoma tumor (156). It seems then, that since splenomegaly does not correlate with ability to kill tumor cells, it is not necessarily a reliable way to detect antitumor activity.

B. Antitumor Activity of P. acnes Vaccines

In 1966 Bernard Halpern and co-workers were the first to describe the ability of P. acnes (then called C. parvum) to inhibit the growth of transplantable tumors in mice (60). Halpern and his co-workers gave mice intravenous injections of P. acnes at the same time, or at various times before or after intraperitoneal injection with tumor cells. They found that systemic injection of P. acnes either 2 days before, at the same time, or 2 days after intraperitoneal injection with tumor cells resulted in no tumor development. Systemic administration of the vaccine more than two days before or after injection with tumor cells resulted in the formation of tumors. Bartlett et al also used this experimental design with many tumor cell models, and were always able to demonstrate inhibition of tumor growth after systemic injection of P. acnes vaccine (11). The major difficulty encountered by this systemic injection of vaccine is that succesful therapy is dependent on the dose of P. acnes used and the time of administration relative to the time at which the tumor is inoculated, as well as the number of tumor cells present (180, 96, 141).

Many investigators have found that injections of P. acnes into the tumor site are more effective than systemic injections in abrogating the growth of the tumor, depending on the tumor system employed (12, 71, 133). In such

experiments, vaccine and tumor cells are usually mixed and are injected at the same time and at the same site (41, 63). Succesful abrogation of tumor growth has also been achieved by injection of P. acnes approximately two days before injection of tumor cells, as long as both are localized in the same site (58). The antitumor effect that results does not seem to be due to the fact that the vaccine comes into direct contact with the tumor cells, as determined by Matthews (89). In his in vitro experiments, he found that the viability of tumor cells, as measured by trypan blue exclusion, did not decrease when they were incubated with P. acnes vaccine over a period of 2 hours. These results have been succesfully repeated in our laboratory.

Local administration of P. acnes suffers from the same drawbacks as systemic administration in that succesful destruction of tumor cells is dependent on the time of vaccine administration, size of tumor, and dose of P. acnes vaccine given (82). More importantly, it must be given either at, or close to, the site of tumor injection. It seems that both systemic and local administration of vaccine can be equally succesful in inhibiting the growth of tumors in mice, depending on the tumor model used. The question remains, however, as to the nature of the mechanism triggered by injection of P. acnes when either of

the two modes of administration is employed. This indeed is the question that the series of experiments described in this thesis will attempt to answer.

C. Nature of Cell Component(s) Responsible for Immunostimulatory Activity

It has been suggested by Pringle and Cummins, as well as by other investigators, that the P. acnes cell component responsible for the stimulation of the immune system of animals is associated with the cell wall of the organism, and that it is carbohydrate (119, 118). To determine the role of the cell wall in this activity, Pringle and Cummins treated P. acnes cells in the early exponential phase of growth (12 hours) with chloramphenicol. Cells are not normally able to induce splenomegaly until they reach stationary phase at 36 hours or after. However, treatment with chloramphenicol of these young 12-hr. cultures caused a steady rise in the ability of cells to induce splenomegaly. Since this antibiotic is known to inhibit protein synthesis while not affecting cell wall synthesis, the results suggest that the material necessary for the stimulation of the reticuloendothelial system is a component of the cell wall. To confirm this, penicillin was added to cultures at various times after addition of chloramphenicol. Penicillin is known to inhibit cell wall synthesis. When added 2 hours after chloramphenicol, it

prevented the development of the splenomegaly-inducing ability of the cells, again suggesting that cell wall synthesis is essential for the immunostimulatory activity of the vaccine (119).

In addition, extensive washing of the cells, which is known not to affect the very rigid and insoluble cell wall of bacteria results in no change in the splenomegaly-inducing activity of P. acnes cells. This suggests that the material responsible for the immunostimulatory activity of the vaccine is firmly bound to an insoluble part of the cell, such as the cell wall, and thus confirms the above findings.

As evidence for the role of peptidoglycan, a component of the cell wall, in the ability of the vaccine to stimulate the reticuloendothelial system of mice, Stimpson performed studies in which he treated the vaccine with Patella vulgata lytic enzyme (PVL), which is known to hydrolyze peptidoglycan. He found that this treatment abolished the vaccine's splenomegaly-inducing activity. Although the ability to induce splenomegaly in mice cannot be correlated with antitumor activity, Stimpson found that treatment with this enzyme also abolished the antitumor activity of the P. acnes vaccine. Thus, it was concluded that intact peptidoglycan is essential to both activities of the vaccine (157).

To determine the nature of the cell wall component responsible for the stimulation of the immune system in mice, studies of the reticuloendothelial stimulatory properties of P. acnes were carried out by Cummins et al (37). They treated suspensions of P. acnes with various chemical reagents, glycosidic enzymes, or lectins. Heating at 100 °C in 0.1 N HCl as well as oxidation by periodate at 4°C rapidly destroyed the ability of the vaccine to induce splenomegaly in mice. Acylation of cells with acetic, butyric or propionic anhydride also completely abolished its activity. However, treatment with various enzymes such as glucose oxidase, β-glucosidase, β-glucuronidase, hyaluronidase, alkaline phosphatase, as well as with various proteolytic enzymes did not significantly reduce the activity of the vaccine. These data suggest that the cell component(s) responsible for the stimulation of the reticuloendothelial system is (are) not proteinaceous but rather polysaccharide in nature.

Even though the data presented here strongly suggests that the polysaccharide and peptidoglycan components of the cell wall are both needed for the splenomegaly-inducing and antitumor activities of the vaccine, Cummins et al found that cell wall fractions were completely unable to induce splenomegaly (37). In addition, in experiments presented

in this thesis in which preparation of cell wall fractions of P. acnes were injected together with tumor cells into Balb/c mice, no antitumor activity resulted. This suggests that, even though the peptidoglycan and polysaccharide components of the cell wall are essential for the vaccine to be active, injection with cell walls alone which contain both of these components does not protect the animals against the formation of tumors. It seems that for some as yet unknown reason, the entire P. acnes cell must be injected for tumor destruction to occur.

III. KNOWN MECHANISMS OF ANTITUMOR ACTIVITY INDUCED BY P. acnes VACCINE

A. T-cell-Mediated Activity

Locally injected vaccines of P. acnes have been found to inhibit the growth of tumors in normal mice but not in athymic (nude) mice (143). Woodruff and others have indeed suggested that T- cells are involved in the supression of tumor cells (181, 182). T-cells have been found to inhibit tumor cell proliferation by a nonspecific, delayed-hypersensitivity-related response as well as through a T-cell specific response. Both will be discussed briefly within this section.

(i) Nonspecific T-cell Response

When tumor cells are injected into the footpad of a mouse previously sensitized with P. acnes the tumor is seen to grow very fast. However, if the mouse is sensitized and this treatment is then followed 6 days later by injection with a tumor cell-P. acnes mixture, a tumor never develops (166). Such results have led investigators to conclude that tumor cells are killed as "innocent bystanders", caught in the midst of a delayed-type hypersensitivity reaction prompted by P. acnes antigens present in the area. The actual cells responsible for the death of the tumor cells are still not known, but it is clear that this response, although nonspecific, is T-cell mediated. In addition it is apparent that, for the delayed-type hypersensitivity seen here, both tumor cells and vaccine must be localized in the same area. When P. acnes is instead administered systemically, a decrease in T-cell-mediated immune responses results (73). In his studies, Scott found that systemic injection with P. acnes results in the appearance of suppressor macrophages and suppressor T-cells, both of which inhibit T-cell mediated immune responses (144).

(ii) Specific T-cell Response

Specific T-cell mediated responses are thought to involve a tumoricidal T-cell. Tuttle and North found evidence for the existence of a short-lived cytotoxic T-cell (CTL) which destroys tumor cells after local injection with P. acnes (165). These CTLs are thought to arise as a result of a sequence of events involving the secretion of various lymphokines (soluble leukocyte growth factors), best known as the Interleukin Cascade (45, 97).

In simple terms, formation of CTLs first requires the presentation of foreign antigens to T-cells by macrophages. Such an act is done through interaction of specific markers on the macrophage and its corresponding receptor on the T-cell (140). The first lymphokine, Interleukin I (IL-1) has a molecular weight of 15,000 to 30,000, and is produced and released by macrophages (86, 151). Although work is still being done to determine the exact nature of the T-cell population thought to respond to this stimulus, it is thought to be the T-helper cell (115, 167). Release of Interleukin II (IL-2) by the T-helper cells follows. T-cells destined to become CTLs are thought to develop receptors for IL-2. This occurs as a result of stimulation of these pre-CTLs by the same trigger that elicited the release of IL-2 from the T-helper cells (150). IL-2 is bound by the pre-CTLs via receptors. This causes the cells

to proliferate further (134, 170). Final maturation into CTLs is accomplished with the help of gamma interferon, thought to be produced by T-helper and T-suppressor cells alike (56, 55).

Tumor growth has been associated with the suppression of various immune mechanisms and the stimulation of others. A decline in the activity of T-cells in tumor burdened hosts has been well documented (72). Host cells such as suppressor T-cells and macrophages have been found to inhibit immune mechanisms that might otherwise be cytotoxic to tumor cells (160, 10). Relative to the Interleukin Cascade discussed above, tumor cells have been reported to release factors that inhibit IL-1 production (79). In addition, suppressor T-cells have been investigated for their ability to inhibit IL-2 production (22). Gullberg and Larsson have indicated that the absence or reduction of IL-2 levels and of CTLs in a tumor burdened host is what permits the tumor to develop (57).

Where P. acnes fits in to the puzzle has been the subject of study by various laboratories. It has been shown to be capable of stimulating the production of IL-1 in vivo (179). More recently, it has been suggested that IL-2 levels are maintained in P. acnes-treated mice and that this, in turn, allows for the succesful maturation of CTLs which are thought to be instrumental in the

destruction of the tumor cells (126). However, maintenance of IL-2 levels in such experiments may simply be due to the destruction of tumor cells at an early stage so that IL-2 levels remain unaffected.

B. Activation of Macrophages

Macrophages are long-lived phagocytic cells that are localized in various organs where they provide a relatively stationary defense against organisms entering the body via the respiratory tract (alveolar macrophages), gastrointestinal tract (Kupffer cells of the liver and peritoneal macrophages), bloodstream (splenic macrophages), and lymphatics (lymph node macrophages). These cells arise from monocytes, which are formed in the bone marrow.

Injection of P. acnes into the peritoneal cavity of mice results in the mass migration of macrophages to the area: the concentration of macrophages peaks approximately 4 days later (181). These "activated" macrophages are much larger and phagocytize particles much more readily than macrophages that are normally found in the peritoneal cavity of mice (21, 107, 51). Olivetto and Bomford found that macrophages harvested from the lungs after injection with P. acnes were cytotoxic to tumor cells in vivo (112). Several investigators have suggested that activated macrophages are the effector cells responsible for anti-

tumor activity when administration of P. acnes is done systemically (142).

The way in which these macrophages are activated by injection with the vaccine is not known. Pringle et al performed studies on the degradation of P. acnes cells inside activated macrophages (120). They found that P. acnes resists degradation by macrophages, and is actually able to persist apparently untouched inside the phagocytes for considerable periods of time. Webster et al have reported the inability of monocytic lysates to degrade live P. acnes cells whereas other bacteria such as Staphylococcus aureus were degraded under the same conditions (171). This persistance has been correlated with the vaccine's ability to inhibit the growth of tumors in mice (145).

Studies conducted on the activation of macrophages by P. acnes have shown this to be a complicated mechanism. In studies using T-cell-deprived mice it has been shown that activation of macrophages is T-cell independent (16). In more recent studies, it has been suggested that processing of the bacteria by polymorphonuclear leukocytes may be an obligatory step in macrophage activation (26).

Killing of tumor cells by activated macrophages is not well understood. Direct contact between these two types of cells is necessary, but the mechanism that results in the

death of the tumor cells is not known (98). Nathan and Cohn have studied the ability of macrophages to form oxygen radicals which are cytotoxic to tumor cells (107). Although no conclusive evidence was found, these investigators were able to detect an increase in the production of hydrogen peroxide after stimulation with various phagocytosis stimulants, including P. acnes. In addition, P. acnes-activated macrophages have been reported to prevent suppressor T-cell formation, which is known to inhibit the immune system of tumor burdened animals (85). These macrophages, as mentioned earlier, also play an important role in the production of CTLs by virtue of their active participation in the release of IL-1.

C. Activation of Natural Killer Cells

In addition to cytotoxic T-cells, cells referred to as natural killer cells (NK) have been linked to the killing of tumor cells in vitro (39, 162). These cells are members of what are known as Null Cells, which are in fact a heterogenous mixture of various immature non-T-cell and non-B-cell hematopoietic cells. Specifically, NK cells are found in the spleen and peritoneal cavity and can destroy various tumor cells, particularly those that are infected with certain enveloped viruses such as the murine leukemia viruses. These cells lack the properties of B or T

lymphocytes, or of macrophages. Although it is known that NK activity is enhanced by interferon, their specific mechanism of action is unknown (42).

Lichtenstein and other investigators have shown that natural killer cells may play an important role in the killing of tumor cells after injection with P. acnes (83). Using tumor cells models that are highly sensitive to the killing effects of NK cells, Ojo et al found that P. acnes-induced peritoneal cavity cells were more destructive to the tumor cells than non-induced cells. These peritoneal cells were identified as NK cells by their ability to resist inactivation when treated with anti-macrophage antiserum or with anti-T-cell antiserum (specifically, anti-Thy 1) (111).

In vivo, it has been shown that P. acnes is able to stimulate an increase in the number of NK cells that bind to tumor cells, although no increase has been detected in the number of NK cells that actually kill tumor cells. The latter effect has been seen only when the experiment is carried out in vitro. As has been mentioned previously, gamma-interferon stimulates the maturation of CTLs. In addition, it stimulates the production of NK cells. It is known that interferon can be produced as a result of injection with P. acnes. It thus seems possible that it is through the indirect production of this hormone that the

vaccine is able to stimulate NK-cytotoxic activity (125).

The actual mechanism by which NK cells kill certain tumor cells is not known although it has been suggested by several investigators that it might be through the production of toxic oxygen radicals (127).

D. Time At Which Tumor Cells Are Killed

The mechanism involved in the killing of tumor cells by CTLs, NK cells, and macrophages must occur several days after injection with P. acnes. Nasrallah and others have determined that the activity of NK cells is first detected three days after injection (105). By comparison, macrophages are known to migrate to the site of injection in approximately four days, and CTLs even later (181, 55). Thus, it seems logical to assume that if either of these cell types are responsible for the killing of tumor cells, no killing should be expected until three or four days after injection with the vaccine.

My research has been primarily concerned with determining the mechanism that causes the destruction of tumor cells. To do so, I used a methylcholanthrene-induced tumor model in Balb/c mice and found that killing of tumor cells occurred as early as 12 hours after localized injection with P. acnes vaccine and tumor cells. The cell population present at this time is composed almost entirely

of polymorphonuclear leukocytes (PMN). In order to better understand the possible role that PMNs may play in the destruction of tumor cells, the following sections of this review will discuss the general characteristics of this cell population and their ability to produce toxic substances capable of killing tumor cells.

IV. POLYMORPHONUCLEAR LEUKOCYTES (PMNs)

Polymorphonuclear leukocytes (PMNs) are the most important phagocytic cells active against acute bacterial infection. They are the first wave of defense the immune system sends to engulf, kill, and digest foreign microorganisms. PMNs are produced in the bone marrow, and are released into the circulation in a mature state. Most of them remain adjacent to walls of small blood vessels, ready to migrate into the tissues upon stimulation (14). The following section will present the general characteristics of these dedicated phagocytes.

A. General Characteristics

PMNs are derived from pluripotent stem cells in the marrow. Myeloblasts differentiate into myelocytes through various cell divisions over a period of seven days. After

this time, the cells undergo changes in their cytoplasm and nucleus. They first develop into juveniles, then band cells, and finally become mature polymorphonuclear leukocytes after six days (121, 122, 123).

PMNs are most easily identified by the segmented appearance of their nucleus, and also by the granules contained within their cytoplasm. It is by the color of the granules when stained with Wright's Stain that PMNs have been subdivided into neutrophils (grey), basophils (blue-black), and eosinophils (red). Granules contain various enzymes and other substances, important in both the killing and digestion of microorganisms. The neutrophil contains two types of granules: azurophilic (or primary) granules and the specific (or secondary) granules. The primary granules contain the enzymes elastase, acid hydrolases, and myeloperoxidase. The latter enzyme is important in the formation of certain toxic oxygen radicals. The secondary granules contain lysozyme, collagenase, lactoferrin, and vitamin B12-binding protein (20, 174).

As mentioned above, the mature PMN has a characteristic multilobed nucleus which helps in its identification. This may actually allow the PMN to squeeze easily through small spaces, such as in between endothelial cells on its way out from a capillary into tissue. PMNs

are deficient in the amount of rough endoplasmic reticulum that they posses, indicating that synthesis of new protein is done sparingly. These cells contain many glycogen particles which serve as energy stores to be rapidly used up during phagocytosis. PMNs have certain receptors on the surface of the cell membrane. Some, like the receptors for formyl peptides, are useful in the stimulation of the phagocyte to migrate towards a particular site. Others, like the F_C receptor, help the PMNs to properly identify foreign particles by binding to the F_C portion of antibody molecules that have opsonised, or attached to, the invading antigen (168). In addition, PMNs have an efficient microtubule and microfilament apparatus that is essential in their locomotion.

About 50% of the mature PMNs are found lining the endothelial surface of blood vessels. They are short-lived cells which last about 6 hours in the bloodstream, after which time they are removed by macrophages. Inside tissues PMNs last only about two days (90, 5). The number of PMNs released from reserves in the bone marrow increases drastically at the onset of infection, sometimes as a result of the production of endotoxin by the invading bacteria. Conversely, the numbers are decreased by factors that suppress the bone marrow.

B. Mechanisms That Lead To Phagocytosis

Two to four hours after stimulation by a signal from humoral factors in the infected tissue, PMNs deform in such a way as to enable them to squeeze in between endothelial cells lining blood capillaries and thus begin their trek to the infected area (99). The motive force in this locomotion involves actin and myosin fibrils within the cytoplasm (172, 159).

Once in the tissue, PMNs readily respond to a series of substances known as chemotactic factors which guide them to the infecting organisms (176). These factors can be complement proteins such as C5a, or products of microbial metabolism such as formyl peptides (139, 153). Attachment of chemotactic factors to PMN receptors results in a change in the membrane's permeability which allows Ca^{++} into the cytoplasm from the extracellular environment, as well as from internal Ca^{++} stores (113).

There are various methods of assessing the chemotactic ability of PMNs. One *in vitro* method requires that the PMNs be placed in wells cut in an agarose semisolid medium and allowed to migrate toward the chemotactic agent in an adjacent well. The extent of migration can be measured by microscopic evaluation of the stained plate (109).

Once near the invading microorganisms, PMNs bind to molecules that have been fixed to the bacterial surface.

This enables the PMNs to recognize them as foreign, which causes several changes in the membrane that concludes with the phagocytosis of the bacteria (65). One such molecule is the immunoglobulin IgG, produced by plasma cells, which binds specifically to the surface antigens of the microorganism. PMNs recognize the Fc portion of the IgG molecule that is bound to the bacteria, and bind to the IgG by the Fc receptor on their surface. Capsulated organisms such as streptococci and Klebsiella are able to elude being phagocytized unless antibodies are present. However, most other bacteria are phagocytized even in the absence of IgG.

Another molecule which aids PMNs in the recognition of foreign particles and subsequent attachment to their surface is the complement factor C3b. This factor is produced by both the classical and alternative pathways of complement fixation. In addition, certain cell wall components of gram-positive bacteria and outer membrane components of gram-negative bacteria can combine with serum factors of the complement fixation pathways and form the necessary enzyme that will release C3b (175, 177, 104). C3b attaches to the surface of the bacteria and is then recognized by the receptor on PMNs.

Once initial attachment of receptor and ligand has occurred, the phagocyte proceeds to engulf the bacteria with its pseudopod. Actin and myosin fibrils contract as

the pseudopod forms a wall around the organism, completely surrounds it, and forms what is known as a phagosome (19). Throughout this process sequential receptor-ligand binding occurs along the pseudopod (54).

The membrane surrounding the secondary granules (those containing lysozyme and other lytic enzymes) fuses with the membrane surrounding the phagosome and forms a phagolysosome. The contents of the granules are released and, as a result, the pH inside the phagolysosome eventually drops (87). Subsequently, primary granules (containing myeloperoxidase) proceed to fuse with the vacuole and release their contents into it as well.

C. The Respiratory Burst And The Production of Oxygen Radicals

When PMNs ingest bacteria or other particles there is a marked increase in oxygen consumption by the cells, known as the respiratory burst (138). This burst is triggered when the permeability of the cell membrane is altered by binding of PMN surface receptors to their respective ligands during phagocytosis (163). Oxygen is rapidly taken up from the surrounding media and quickly converted to several active oxygen species such as superoxide anion ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$). Most of the oxygen consumed during the respiratory burst is converted to hydrogen peroxide

through the dismutation of two moles of superoxide anion per mole of hydrogen peroxide produced (66, 7). Hydroxyl radicals as well as other toxic oxygen derivatives are formed as a result of various reactions which will be discussed in the next section.

The enzyme system responsible for the initiation of the oxygen burst has been identified as a reduced nicotinamide adenine dinucleotide oxidase (6, 9). This enzyme is thought to be located on the outer surface of the plasma membrane and when activated, it acts on oxygen in the extracellular fluid. When the plasma membrane is invaginated to form the phagocytic vacuole, the enzyme now faces inward, acting on oxygen within the vacuole (74). Segal et al have suggested that a cytochrome of the b type may also be involved in this reaction (146). Since reduced pyridine nucleotides are essential for this system to work, the hexose monophosphate shunt is also activated during the respiratory burst in order to regenerate NADPH.

There are several methods used in the evaluation of the degree of activation of the respiratory burst of PMNs. One of them involves the determination of the oxidative capacity of PMNs through their ability to reduce nitroblue tetrazolium dye, which is yellow in color, to a blue-black formazan precipitate during the respiratory burst (110). Cells are examined microscopically and the number of PMNs

with formazan granules within their cytoplasm are counted. This method is somewhat unreliable in that it is largely qualitative and varies with temperature.

Another method which is widely used is measurement of the amount of light produced during the respiratory burst. PMNs are stimulated by ingestion of bacteria or by membrane perturbation by a soluble compound such as phorbol myristate acetate. Chemiluminescence results when electronically excited molecules of oxygen relax to ground state, emitting light (3). Light output is measured by a photomultiplier tube such as in a scintillation counter. Luminol enhances this light emission and serves to increase the sensitivity of the system (154).

This phenomenon has been correlated with the oxidation of glucose via the hexose monophosphate shunt and the oxidation of the particle being phagocytized. Several investigators currently believe that this oxidation may be due to the production of $^1O_2$ within the PMN (2) and/or O^{2-}, H_2O_2, or $OH\cdot$ (27, 69). Wasserman, however, contends that it is only singlet oxygen ($^1O_2$) which is responsible for chemiluminescence (169). Although the precise nature of the oxidated species is not known, chemiluminescence is definitely a product of the complex metabolic activity of PMNs during phagocytosis. It can thus be used as a measure of the generation of species which are ultimately involved

in the microbicidal activity of PMNs.

V. REACTIONS INVOLVED IN THE FORMATION OF PMN-DERIVED OXYGEN RADICALS

Complete reduction of molecular oxygen occurs by its acceptance of four electrons in order to form water. Oxygen, however, can also be partially reduced, resulting in the formation of several highly reactive products. The following is a review of the mechanisms of formation of each of the toxic species derived from the partial reduction of oxygen during the respiratory burst, as well as methods to detect each one.

A. Singlet Oxygen

This radical is formed when an absorption of energy shifts one of the two unpaired electrons on molecular oxygen to an orbital of higher energy, causing an inversion in the electrons' spin. This excess energy is then dissipated by thermal decay, or as discussed previously, by light emission. There are two forms of singlet oxygen: delta singlet oxygen ($\Delta\,^1O_2$) in which the newly paired electrons occupy the same orbital, and sigma singlet oxygen (Σ^1O_2) in which the electrons of opposite spin occupy different orbitals (169).

The chemical reactivity of singlet oxygen is due mainly to the delta form because of its electronic

configuration, and because of its relatively longer lifespan in solution. It reacts strongly with compounds at areas of high electron density such as at unsaturated carbon-carbon bonds (48). Various agents can quench singlet oxygen, inhibiting the action of this radical. Some of the more commonly used are histidine, β-carotene, azide, and α-tocopherol. However, these compounds are also able to quench other radicals so their inhibition of a reaction cannot be used as conclusive evidence of the involvement of singlet oxygen.

B. Superoxide Anion

When oxygen accepts a single electron it is converted to the superoxide anion ($O_2^{\cdot-}$), or to its protonated form, the perhydroxy radical ($HO_2^{\cdot}$) (47). The superoxide anion can react as a reductant or an oxidant, resulting in the formation of oxygen in the former, and hydrogen peroxide in the latter case. Some of the most common sources of $O_2^{\cdot-}$ are autooxidations or enzymic oxidations.

Reactions involving hemoglobin, reduced cytochrome c, reduced ferredoxins, and catecholamines are those involving autooxidations (101, 23, 102, 100). Organelles such as mitochondria and chloroplasts have been shown to generate $O_2^{\cdot-}$ through the autooxidation of reduced components of electron transport systems (84, 18, 164, 4). A number of

enzymes such as xanthine oxidase, flavin dehydrogenases, and in the case of the PMNs, NADPH oxidase produce superoxide anion. This latter enzyme is thought to be dormant in the plasma membrane of neutrophils. After suitable activation during the oxygen burst, it reduces oxygen exclusively to $O_2^{\cdot}$ (8).

Superoxide anion is thought to exert its toxic effects on cellular components by its involvement in the formation of hydrogen peroxide, and not on its own. Some investigators have proposed that the superoxide radical is actually innocuous because, in some cases, the use of superoxide dismutase has failed to alter toxicity. Further, the superoxide radical has been deemed a relatively non-reactive substance by investigators who have tested its rate of reaction with various amino acids and citric acid cycle intermediates (46, 137). On the other hand, Gebicki has suggested that the conjugate base of O_2^- could contribute importantly to the reactivity of superoxide since it has been shown to react with various compounds at a fast rate, approximately 300 M^{-1} sec^{-1} (50, 132).

Superoxide radical spontaneously dismutates to form hydrogen peroxide, a species which is a definite threat to the chemical integrity of cells. This reaction can also be catalyzed by the enzyme superoxide dismutase to produce the

same results. Fridovich and others have calculated the rate of enzymic dismutation of superoxide radical and have found it to be 10^6-times greater than that of spontaneous dismutation (49). In addition to production of hydrogen peroxide, $O_2^{\cdot}$ is involved in the generation of the most reactive of all radicals, $OH^{\bullet}$. Formation of these species will be discussed later in this review. Evidence has been presented for the direct toxicity of O_2^-. Experiments done with various scavengers of hydroxyl radicals and hydrogen peroxide have shown that, although the superoxide radical can generate $OH^{\bullet}$ from H_2O_2, it need not do so in order to exert a toxic effect (38).

Measurement of superoxide production by neutrophils is often done by the superoxide dismutase-inhibitable reduction of cytochrome c (131). Ferricytochrome C is reduced by superoxide to ferrocytochrome C. The latter compound can be measured by its absorbance at 550 nm. Superoxide dismutase inhibits this reaction by catalyzing the formation of hydrogen peroxide and thus using up all the superoxide radicals available.

C. Hydrogen Peroxide

As mentioned before, the dismutation of $O_2^{\cdot}$ either spontaneously or through the enzyme superoxide dismutase results in the formation of hydrogen peroxide (H_2O_2). This

is formed by the neutrophil during the phagocytosis-induced respiratory burst and can be detected in the phagosome. In addition, some microorganisms such as lactobacilli, streptococci, and pneumococci can generate H_2O_2. Since these organisms lack a cytochrome system, terminal oxidations in electron transport chains are catalyzed by flavoproteins, which reduce oxygen to hydrogen peroxide (183). Hydrogen peroxide is readily broken down to O_2 and H_2O by the enzyme catalase, a mechanism which many microorganisms use in order to render H_2O_2 harmless.

When combined with the enzyme myeloperoxidase and a halide, hydrogen peroxide becomes a potent antimicrobial agent. The myeloperoxidase-hydrogen peroxide-halide system is effective against a variety of bacteria, fungi, viruses, and mycoplasma, as well as multicellular organisms and mammalian cells (75). Myeloperoxidase is present in very high concentrations in the azurophil granules of PMNs. This enzyme combines with its substrate, hydrogen peroxide. to form an enzyme-substrate complex that can oxidize a variety of compounds. Among these are the halides, and it is thought that their oxidation results in the formation of a toxic agent or agents that can attack the microorganisms in a variety of ways.

One of the modes of attack is a process known as halogenation. Iodination, bromination, and chlorination

occur when either of these bulky halides (iodide, bromide, chloride) is substituted for hydrogen at crucial sites in the cell. The other way in which this system is toxic is through oxidation of sensitive groups on the cell surface. Of the oxidants formed by this peroxidase system, hypochlorous acid has been the most studied (1, 62). It is thought that this compound may further react with hydrogen peroxide to form singlet oxygen, which in turn may be contributing to the toxic effects of this system (130, 114).

Evidence for the participation of myeloperoxidase in the toxic properties of PMNs can be seen in the availability of the various halides for the formation of oxidants. Chloride, for instance, is present in PMNs at a concentration greater than that required (95). In addition, the optimum pH of the myeloperoxidase system is about 4.5 to 5.0. Since it is known that, after phagocytosis, the pH in the phagosome falls to approximately that level this enzyme system should be functional (67).

Finally, it has been found that PMNs from patients with chronic granulomatous disease are able to kill certain microorganisms. These patients lack the oxidase enzyme responsible for the production of hydrogen peroxide and are usually susceptible to many infections. When invaded by

pneumococci or streptococci, however, the PMNs are able to kill the bacteria by using the microorganisms' own hydrogen peroxide to activate the phagocyte's myeloperoxidase-hydrogen peroxide-halide system (78, 88).

Several techniques have been developed to measure hydrogen peroxide generation by PMNs. These include the oxidation of formate-^{14}C by catalase (66), the oxidation of scopoletin by horseradish peroxidase (128), the formation of a peroxidase-hydrogen peroxide enzyme-substrate complex with a measureable shift in absorption spectrum (70), and the release of oxygen by catalase (183).

D. Hydroxyl Radical

Hydrogen peroxide can itself be further reduced to form the highly reactive hydroxyl radical (OH•). This occurs as depicted in the classical Haber-Weiss reaction in which a trace metal acts as an oxidation-reduction catalyst. The metal is alternatively reduced by superoxide anion and oxidized by hydrogen peroxide with the overall reaction resulting in the generation of hydroxyl radical by the interaction of superoxide and hydrogen peroxide (92, 59). Some metal chelators such as ethylenediamine tetraacetic acid (EDTA) stimulate the formation of hydroxyl radicals by the Haber-Weiss reaction while others, such as

diethylenetriamine pentaacetic acid (DTPA) are inhibitory (59).

It has often been debated whether this mechanism for the production of hydroxyl radicals actually occurs within living systems. Elstner found that, upon illumination, chloroplast lamellae produce OH• from $O_2^{\cdot-}$ plus H_2O_2 (44). In addition, liver microsomes have been found to exhibit a nonspecific alcohol-oxidizing activity that appears to be due to OH•, also generated from $O_2^{\cdot-}$ plus H_2O_2 (25). Most importantly for this review, suspensions of neutrophils, which produce large amounts of $O_2^{\cdot-}$ when activated, have also been shown to generate hydroxyl radicals (124, 135).

Hydroxyl radicals can be indirectly detected by the use of mannitol and other sugar alcohols as scavengers. However, several methods have been proposed for the direct measurement of hydroxyl radical formation by phagocytes. Certain thioethers like keto-4-methylthiobutyric acid (KMB) are oxidized with the release of ethylene. Ethylene formation is initiated by the removal of an electron from the sulfur of the thioether. Beauchamp and Fridovich have shown that the hydroxyl radical is indeed capable of this reaction (13). When stimulated by phagocytosis, neutrophils convert KMB to ethylene, which can then be measured by gas chromatography (77).

The role of OH• in ethylene formation by polymorphonuclear leukocytes has been questioned on several grounds. It can be initiated by oxidants other than OH , and ethylene formation by phagocytes is dependent largely on myeloperoxidase. This suggests that either this enzyme is required for OH• formation, or that ethylene formation occurs largely by an OH•-independent mechanism (77).

Electron spin resonance spectroscopy using the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) has recently been applied to the measure of hydroxyl radical formation by neutrophils (129). Spin traps react readily with free radicals to produce a relatively long-lived free radical adduct with an identifiable electron spin resonance spectrum. The same characteristic spectrum that is formed when DMPO reacts with OH is seen when DMPO is incubated with neutrophils, thus offering a reliable method of measuring this particular radical.

D. Tumoricidal Activity of Oxygen Radicals

Although the primary function of PMNs is the phagocytosis and destruction of microorganisms, leakage or secretion of toxic agents can occur with the potential for damage to adjacent cells. The cell-free myeloperoxidase-hydrogen peroxide-halide system has been found to be toxic

to spermatozoa (149), erythrocytes (76), leukocytes (29), and platelets (28). In 1975, Clark and Klebanoff reported the cytotoxic effect of PMNs on mammalian tumor cells (30). Since then, a number of publications have appeared on the subject.

Nathan and co-workers have found that production of hydrogen peroxide occurs upon activation of PMNs by phorbol myristate acetate, which causes target cells to lyse in about three hours (106). Glaves has used an arrested Lewis lung carcinoma cell system to determine whether oxygen radicals are responsible for the death of these cells during metastases. She has been able to correlate the intravascular death of the cancer cells with the production of superoxide anion by PMNs (53).

Weiss et al, working with a T-lymphoblast cell line, have suggested that cytolysis is actually caused by hypochlorous acid. In their experiments, cytolysis was inhibited by catalase and by myeloperoxidase inhibitors such as azide, but stimulated by superoxide dismutase. This indicates a role for both H_2O_2 and myeloperoxidase in the cytolytic event. Compounds known to scavenge hypochlorous acid (tryptophan, methionine) prevented cell lysis in both a cell-free hypochlorous acid system as well as in one where PMNs were used (173).

Almost all the investigations being carried out on this subject have used either cell-free systems, or PMNs obtained from the blood or peritoneal cavity which have then been stimulated with phorbol myristate acetate to produce oxygen radicals. As mentioned previously, this substance is known for its ability to strongly stimulate the respiratory burst of PMNs to produce radicals capable of killing tumor cells in vitro. It is possible, then, that other substances known for their ability to inhibit the growth of tumors may also be able to stimulate PMNs into producing toxic oxygen radicals, and that this mechanism is what kills the tumor cells. One of these, OK-432, is a streptococcal preparation that has been widely used in Japan as a biological response modifier. Recently, this preparation has been investigated for its ability to kill ascites tumors by inducing PMNs to accumulate in the ascitic fluid (81). Although it has not been demonstrated in this system, killing of tumor cells may be due to oxygen radicals produced by these PMNs.

VI. SUMMARY AND RATIONALE FOR THE EXPERIMENTAL APPROACH USED IN THIS THESIS

The ability of the organism Propionibacterium acnes to inhibit the growth of certain tumors in animals is

indisputable. Although many experiments have been performed in the last decade on the nature of its antitumor activity, no definitive results have been obtained as to the actual mechanism which prevents the growth of tumor cells. In addition, there has been no study on the minimum time necessary for the effects of the vaccine to cause the death of tumor cells. It seems logical that one way to determine what immunological mechanism is responsible for preventing tumor growth after injection with P. acnes is to determine the time at which the tumor cells are killed.

As part of the results presented in this thesis, I have determined that destruction of tumor cells occurs as early as 12 hours after injection of mice with tumor cells and P. acnes vaccine. Through histological studies of the injection site 12 hours after injection, I have determined that PMNs are the primary host cell population present. Since it is possible for PMNs to produce substances toxic to tumor cells, it seemed logical that studies examining the role of PMNs in the killing of tumor cells after stimulation by P. acnes should be carried out. In doing so, the actual mechanism responsible for the killing of tumor cells in the methylcholanthrene tumor model used may be elucidated.

MATERIALS AND METHODS

Bacterial Strains

The following bacterial strains were obtained from the VPI Anaerobe Laboratory Culture Collection:

Propionibacterium acnes type I strain 0009

Propionibacterium freudenreichii strain 0407

Propionibacterium granulosum strain 5888

Propionibacterium avidum type II strain 0589

P. acnes was used in histological studies and Enzyme-linked Immunosorbent Assay (ELISA), and to prepare cell walls. *P. acnes* and *P. freudenreichii* were used in the preparation of vaccines for the anti-tumor assay, lesion transfer experiments, chemiluminescence studies, phagocytosis assay, and in determinations of O_2 consumption by phagocytes.

Micrococcus lysodeikticus ATCC 4698 was obtained as a spray-dried powder from Miles Laboratories, Elkhart, IN., and, together with *P. granulosum* and *P. avidum*, it was used in the O_2 consumption assay.

Animals

Male Balb/c mice, approximately 6 weeks old weighing 16-18 g were obtained from Dominion Laboratories, Dublin, VA. They were fed Purina Mouse Chow (Dominion Labs) and supplied with tap water *ad libidum*. These mice were used

to maintain the fibrosarcoma tumor line. In addition, this strain of mice was used in the antitumor assay, histological studies, lesion transfer experiments, and ELISA. Balb/c mice were also used to obtain polymorphonuclear leukocytes and fresh serum to be used in the chemiluminescence assay, phagocytosis assay, Chinese Hamster Ovary cell assay, and O_2 consumption experiments.

Rabbits were used to develop polyclonal serum reactive against tumor cells from the methylcholanthrene-induced fibrosarcoma line, and to develop serum against P. acnes 0009 strain. These animals were obtained from the Animal Research facility at VPI & SU, Blacksburg, VA., where they were housed and fed according to specifications, by personnel from the Virginia-Maryland School of Veterinary Medicine Animal Research Division.

Chinese Hamster Ovary Cells

Cultured Chinese Hamster Ovary cells (CHO) were obtained from Julianna Toth, in the laboratory of Dr. Tracy Wilkins, VPI & SU, Blacksburg, VA. The cells were grown in Modified Eagle's Medium supplemented with 5% fetal calf serum (Difco Laboratories) in 96-well microculture plates at 37 °C under a CO_2 atmosphere. All experiments were carried out on wells containing CHO cells grown to 50% confluency. Healthy CHO cells were identified as spindle-

shaped cells, firmly attached to the bottom of the plate, while dead cells were identified as those having assumed a rounded appearance after 24 hours incubation.

Chemicals

All chemicals and antisera were obtained from Sigma Chemical Company, unless specified otherwise.

Vaccine Preparation

All *Propionibacterium* strains used were maintained in chopped meat medium under strict anaerobic conditions and subcultured into tubes containing 50-ml of sterile Peptone Yeast Glucose (PYG) medium with Tween, using the techniques described in the VPI & SU Anaerobe Laboratory Manual. The cells were incubated at 37°C and transferred anaerobically to fresh medium daily for at least two days. A 50-ml culture of each strain was used to inoculate 2.5 L of pre-warmed Trypticase Yeast Glucose (TYG) medium containing 0.05% Tween. This was subsequently incubated at 37°C with constant stirring. *P. acnes* cells were allowed to grow under anaerobic conditions for either 12 hours (logarithmic growth phase) or 48 hours (early stationary growth phase) before harvesting. The cultures of *P. acnes* grown for 12 hours are referred to as "12-hr. 0009" cells and those grown for 48 hours are referred to as "48-hr. 0009" cells.

All other Propionibacterium strains were grown for 48 hours only.

After either 12 hours or 48 hours of incubation, all cultures were heat killed by placing the culture flasks in a 56 °C water bath for thirty minutes. Each bacterial strain was harvested separately by centrifugation at 10,000 x g for 20 minutes, followed by washing three times with distilled water. The washed bacteria were finally resuspended in distilled water and lyophilized. The yield was approximately 2 g dry weight of bacterial cells per 2.5 L culture.

Cell walls were prepared from 48-hr. P. acnes cells as described by Johnson and Cummins (68). Briefly, cells (500 mg) were disrupted by shaking in a Braun mechanical cell homogenizer for 5 minutes at 4,000 cycles/min. with 0.1 mm diameter glass beads (20 ml). Disrupted 48-hr. P. acnes cells were then separated from the glass beads by filtration through a sintered glass filter, and treated with pronase (50 ug/ml, Calbiochem, LaJolla, CA) at 56°C for 1 hour. After further centrifugation at 20,000 x g for 30 minutes, the cell wall layer (upper) was gently washed away from the whole cell pellet (lower) with distilled water. Cell walls were subsequently centrifuged at 5,000 x g for 10 min., washed three times with distilled water and lyophilized.

Stock suspensions were prepared of all lyophilized, killed cultures using sterile phosphate buffered saline to achieve a concentration of 10 mg/ml. These were kept at 4 ^{o}C until use.

Tumor Cell Preparation

Tumor cells were obtained from a transplantable fibrosarcoma tumor maintained in Balb/c mice. The tumor originates from the laboratory of Dr. Klaus Elgert, VPI & SU, Blacksburg, VA. It was induced 10 years ago by W.L. Farrar (Dr. Elgert's laboratory) by injection of Balb/c mice with methylcholanthrene by the method of Smith and Konda (152). The tumor is transplanted every two weeks into the right hind leg of normal Balb/c mice via intramuscular injection of a single-cell suspension of 1 X 10^5 viable cells prepared from 2-week old excised tumors.

Tumor cells to be used in the preparation of cell suspensions for routine *in vivo* tumor passage, or to be injected with bacterial vaccines for the purpose of conducting experiments, were prepared as follows. A mouse having a tumor measuring approximately 10 mm in diameter was killed by cervical dislocation. The tumor was then aseptically removed and placed on a 50-gauge wire mesh inside a stainless steel cup, containing 7-10 ml of ice cold Hank's Balanced Salt Solution (Grand Island Biological

Co.). Using a glass syringe plunger, the tumor material was pushed through the wire mesh, collected in the cup, and transferred to a 15-ml conical centrifuge tube. The cells were then centrifuged at 400 x g for 5 min, and washed twice with 10 ml cold Hank's Balanced Salt Solution (HBSS) before finally being resuspended in about 1.5 ml HBSS for counting.

A 0.1 ml portion of cells was suspended in 0.1 ml 0.4% trypan blue dye (Gibco) and 0.3 ml phosphate buffered saline and mixed. A pasteur pipette was used to fill the counting chamber of a hemacytometer (American Optical, Buffalo, NY) and viable cells (those excluding trypan blue dye) were counted. For routine transfer of tumors, the concentration of tumor cells was adjusted to 10^6 cells/ml, and 0.1 ml/mouse were injected intramuscularly into the right hind leg, using a 26-gauge needle and a 1.0-ml tuberculin syringe (Beckton-Dickinson, Rochelle Park, NJ).

For use in testing the anti-tumor ability of the various bacterial vaccines, in histological studies, lesion transfer experiments, and ELISA determinations the concentration of tumor cells was adjusted to 5 X 10^6 cells/ml. Equal volumes of tumor cells and of 10 mg/ml bacterial stock solution were then mixed, resulting in a suspension containing 1 X 10^6/ml tumor cells and 5 mg/ml vaccine. Mice to be tested received an injection of 0.1 ml

of this mixture, corresponding to 10^5 tumor cells and 0.5 mg vaccine. Development of tumors with time was determined by periodic measurement of the thickness of the right hind leg of each mouse to the nearest 0.1 mm using a Manostat caliper.

Anti-P. acnes Antiserum

Antiserum specific against P. acnes 0009 strain was obtained from rabbits by the following protocol: intravenous injection of 1 ml of a partially disintegrated suspension of 48-hour 0009 heat-killed whole cells (HKWC) was performed twice a week for three weeks. The serum was tested by agglutination of the material used to immunize the animals one week after the last injection. After titers of at least 1/320 were detected, the animals were bled out from the heart and the blood collected in a sterile centrifuge tube. The blood was allowed to clot and the serum recovered and stored at 4°C with 0.02% sodium azide added as a preservative.

This antiserum was used in the Fluorescent Antibody Assay to detect the presence of P. acnes cells after separation of various cell types by density centrifugation.

Anti-Tumor Antiserum

Rabbits were injected subcutaneously at the back of the neck with 1 ml of an emulsified suspension containing 1 X 10^5 tumor cells in 0.5 ml of HBSS buffer and 0.5 ml Freund's Complete Adjuvant. At one month, the rabbits were injected once subcutaneously with a suspension of 1 X ^{5} tumor cells in HBSS buffer. The titer was tested after one week by the ELISA method described in a later section, using various concentrations of tumor cells and antitumor antiserum. After a titer of at least 1/1,280 was detected, the rabbits were bled out completely and the blood was collected in a sterile centrifuge tube and kept overnight at 4°C. The serum was then obtained by centrifugation.

The ELISA procedure was also used to determine whether the antitumor antiserum was specific for tumor cells only or whether it would react with other cell types. The serum was added to mouse spleen cells, and, according to the ELISA procedure, it was determined that it cross-reacted with spleen cells. The serum was therefore adsorbed with a suspension of splenocytes obtained from fresh Balb/c mice in order to remove antibodies from the serum that would react with antigenic determinants on cells other than tumor cells. The procedure used was as follows: spleens were obtained from normal mice and a suspension of single cells was obtained after treatment of the spleens with a glass

homogenizer in PBS. The splenocytes were centrifuged and washed twice at low speed in PBS and 10^6 cells/ml were resuspended in 10 ml of the anti-tumor serum. The serum-splenocyte mixture was kept at 4°C overnight, after which time it was centrifuged and the splenocytes discarded.

This procedure was performed 9 times using fresh splenocytes each time. The serum was again tested for cross-reactivity with spleen cells and for reactivity with tumor cells using the ELISA method and found to be 100X more specific for tumor cells than for the other cell types. This adsorbed anti-tumor serum was used in all subsequent ELISA procedures at a 1/1000 dilution.

Fluorescent Antibody Test

This assay was used to determine whether succesful separation of tumor cells from P. acnes cells was achieved after centrifugation in a density gradient in the Lesion Transfer experiments.

The cell suspension to be tested for contamination with P. acnes cells was heat-fixed onto a clean slide and maintained in a moist chamber throughout the assay. Anti-P. acnes antiserum was added to cover the cells and the slide was incubated at room temperature for 1 hour. At this time, the slide was placed in a Coplin jar containing FTA buffer and washed for 30 minutes with constant

stirring. Goat anti-rabbit antiserum labelled with fluorescein-isothiocyanate (FITC) was added onto the fixed cells on the slide and incubated for 30 minutes at room temperature. The slide was then washed thoroughly with FTA buffer as described before, and then examined under a fluorescent microscope for the presence of apple-green fluorescence, indicative of the presence of P. acnes cells.

Histology

Groups of 5 mice were injected intramuscularly in the right hind leg with either tumor cells only, P. acnes cells only, tumor cells + P. acnes cells, or with latex beads. At various times after injection, the mice were killed by cervical dislocation and their leg tissue removed. The tissue from each sample was minced into pieces approximately 3 mm in diameter, briefly rinsed in PBS, and suspended in a buffered formalin solution for fixation overnight. The samples were submitted to the Histology Laboratory at the Virginia-Maryland Regional College of Veterinary Medicine's Research Center, VPI & SU, Blacksburg, VA. for sectioning, staining with hematoxylin and eosin, and mounting on slides.

Sections of each piece of tissue in a sample were examined under the light microscope and notes were made as to the types of cells present in the tissue at the various

times after injection with the materials mentioned above. Specifically, the types of immune cells present were noted, as well the relative numbers of cells present at each time interval tested.

Enzyme-Linked Immunosorbent Assay

This procedure was developed in order to detect changes in the level of tumor cell antigen present in the injected area with time so as to indirectly determine the time at which tumor cells are inactivated, or killed after injection into Balb/c mice in a mixture with P. acnes vaccine.

First, it was used to determine the antibody titer of the antitumor antiserum, as well as to test for its specificity to tumor cells, as described in the section "Anti-Tumor Antiserum", above. Control wells were prepared using a Falcon flexible microtiter plate in which various concentrations of tumor cells were tested against various concentrations of antitumor antiserum. The amounts of the various reagents used as well as the procedure followed are described in more detail in the next paragraph. It was found that the adsorbed serum could be diluted to at least 1/1,280 and still provide accurate absorbance readings on the lowest concentration of tumor cells tested. It was also established by coating some wells with spleen cells

that the serum was only weakly reactive with cell types other than tumor cells, producing absorbance readings of about 0.05 units (background readings were 0.03 units).

After establishing the serum dilution to be used throughout the rest of the experiments, a standard curve of absorbance vs. tumor cell number was first constructed in order to provide a means of correlating absorbance findings in the experimental assays to actual tumor cell numbers present. A 10-13 day-old tumor was obtained from a tumor-burdened Balb/c mouse and homogenized as described previously. Tumor cells were counted and appropriate dilutions in carbonate buffer (pH=9) were made to yield 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 tumor cells/0.2 ml suspensions. Three wells of a Falcon flexible polycarbonate microtiter plate were coated with 0.2 ml of each of the tumor cell dilutions and incubated overnight at 37°C. Controls consisted of wells to which no tumor cells were added (control #1), wells containing 10^5 tumor cells but to which no anti-tumor serum was to be added (control #2), and wells containing 10^5 tumor cells to which no goat anti-rabbit conjugated serum was to be added (control #3).

All wells were then washed 5X with Phosphate Buffered Saline-Tween 20 buffer (PBS-T, pH-7.4) using an ELISA automatic wash apparatus. Then, the previously adsorbed anti-tumor serum was diluted 1/1000 in PBS-T buffer and 0.2

ml were added to each well except control #2, to which PBS-T was added. After incubation for 1 hour at 37°C, all wells were washed 3X with PBS-T buffer. Goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase was diluted 1/500 in PBS-T and 0.2 ml were added to each well, except control #3 to which PBS-T was added. The plate was incubated once again for 1 hour at 37 °C. All wells were then washed 3X in PBS-T, after which 0.2 ml of a 1 mg/ml solution of alkaline phosphatase substrate in diethanolamine buffer (pH=9.6) was added to each well, and incubated at room temperature. After 30 minutes, development of color by the reaction of the enzyme with its substrate was stopped by addition of 0.05 ml of 4N NaOH to each well. Absorbances for each well were recorded using an ELISA automatic reader set at 405 nm.

The experimental assay consisted of groups of 3 mice, injected in the right hind leg with either tumor cells alone, or with tumor cells and P. acnes vaccine. After 12 hours, 24 hours, 2 days, or 4 days, the mice were killed by cervical dislocation, their leg tissue removed and digested in 2.5 ml each of a 1 mg/ml trypsin and 1 mg/ml collagenase enzyme preparation for 30 minutes at 37°C to release the tumor cells. The cells obtained from each lesion were centrifuged, washed in PBS, and finally resuspended in 0.2 ml carbonate buffer. The digested samples from each of the

three mice in each experimental group were placed in a separate well of a Falcon microtiter plate and the plate was incubated overnight at 37°C. The rest of the ELISA assay was performed as described previously in this section for the preparation of the standard ELISA curve. The absorbance readings obtained for the experimental samples were translated into actual tumor cell numbers by using the standard curve previously prepared.

Lesion Transfer Assay

This assay was performed in order to determine at what time after injection of P. acnes vaccine and tumor cells into Balb/c mice, the tumor cells are rendered nontumorigenic, or are killed. The rationale for this assay is based on the development or failure to develop tumors in fresh mice which have been injected with lesions from mice originally injected with tumor cells and vaccine. These lesions were removed after certain periods of time, digested, freed of residual P. acnes cells, and reinjected into fresh mice. Development of tumors in the recipients of these lesions would indicate that the tumor cells had not yet been destroyed in the original mice at the time that the lesions were removed and transferred to the fresh mice. On the other hand, if the fresh mice did not develop tumors after receiving the washed lesions from the original

mice, this would indicate that the tumor cells had been killed, or rendered unable to produce tumors, by the time they were removed from the original mice.

Four groups of nine Balb/c mice each received an intramuscular injection in the right hind leg consisting of 500 ug of 48-hr. 0009 cells and 10^5 tumor cells. Four additional groups of nine mice each received injections of 10^5 tumor cells only, as controls. After 12 hours (group 1), 24 hours (group 2), 48 hours (group 3), and 4 days (group 4), the lesions were removed from the legs and digested with trypsin and collagenase, as described in the ELISA experiments, to release the tumor cells.

The suspensions of cells obtained from each lesion were washed separately in HBSS and each individual lesion of each of the 9 mice in each group was resuspended in 1 ml of HBSS for a total of 9 ml of lesion cell suspension per group. Lesion cell suspensions in each group of nine mice were combined to form three batches, each containing the lesions from 3 mice. Each of the three 3-lesion batches in a group was layered on top of a Ficoll double gradient, which was prepared by layering 3 ml of a Ficoll solution of specific gravity = 1.077 over 3 ml of a Ficoll solution of specific gravity = 1.119. The double gradient with the sample was centrifuged at 200 x g for 30 minutes to separate tumor cells from other types of cells. Tumor

cells pelleted to the bottom while host cells formed a layer at the interface of the two Ficoll gradients.

The tumor cell pellet from each 3-lesion batch was carefully removed, washed in HBSS and examined for contamination with P. acnes cells by the Fluorescent Antibody technique described in this section using anti-0009 antiserum developed in rabbits. In addition, the layer of host cells was also examined for the presence of P. acnes in the same way.

The three tumor cell suspensions in each group were then injected in the right hind leg of each of three fresh mice and tumor development determined by measurement of thigh diameter with time using calibrated calipers as described before.

Chemiluminescence

This assay was performed so as to determine whether there exists a difference in the degree of activation of the respiratory burst of polymorphonuclear leukocytes when different vaccine materials are used. Specifically, this method was employed to test the difference in activation of PMNs by 48-hr. 0009, a vaccine which protects mice against the development of tumors, and 12-hr. 0009 cells, 0009 cell walls, as well as latex beads, all of which are either only partially protective or not protective at all.

Four mice were each injected intraperitoneally with 0.5 ml of a 5% glycogen solution in PBS in order to elicit the migration of PMNs to the area. Five hours later, the mice were anesthetized with Methofane for 5 minutes. Mice were decapitated and their blood was collected and stored at 37°C for 20 minutes in order to obtain the serum. Ice-cold Hank's Balanced Salt Solution lacking phenol red (HBSS-PR) was used to perform an intraperitoneal lavage of each animal in order to harvest the polymorphonuclear leukocytes. These were counted using a hemacytometer and adjusted to a concentration of 1 X 10^6 PMNs/ml HBSS-PR. Erythrocytes that may be present from the peritoneal fluids and which can interfere with chemiluminescence measurements were removed by exposing the PMN suspension to ice-cold hypotonic saline of 0.22% for 30 seconds with slight vortexing, followed by the addition of an equal volume of 1.54% ice-cold saline to restore the PMN suspension to isotonicity.

Preparations consisting of 0.2 ml of 250 ug/ml 48-hr. 0009 cells, 12-hr. 0009 cells, 0009 cell walls, or latex beads were opsonized by being resuspended in an equal volume of fresh mouse serum and incubated at 37°C for 30 minutes under constant rotation. The cells and beads were then centrifuged in a microcentrifuge at high speed and resuspended separately in 0.4 ml of HBSS-PR to yield 50 ug

of opsonized cells or beads, which corresponds to 2×10^8 organisms or beads. Luminol was used to enhance chemiluminescence readings. A 2×10^{-5} mg/ml solution in DMSO was prepared.

Readings with PMNs only were made first by transferring 1 ml of PMNs in HBSS-PR to each of twelve previously dark-adapted polyproplylene scintillation holders and chemiluminescence was measured immediately using a Beckman LS-250 liquid scintillation spectrophotometer at room temperature with a gain of 275 and a window of 0 to 500, and with its coincident circuit switched to the rear photomultiplier tube in order to provide maximum sensitivity for chemiluminescence (155). The vials were equilibrated for about 5 minutes in the counting chamber until they reached a stable background of approximately 10,000 cpm. Phagocytosis was then initiated in all vials by adding 0.4 ml of one of the four preparations of opsonized organisms or beads, and 0.4 ml of luminol to each of three vials to yield a final concentration of 1×10^{-8} M luminol and 200 organisms or beads per PMN. The vials were immediately hand-shaken for 10 seconds, placed back into the counting chamber, and counted at 1-minute intervals for at least 30 minutes.

Phagocytosis Cytotoxicity Assay

This assay was developed in order to test whether PMNs are capable of killing tumor cells after having phagocytosed P. acnes cells, and whether the destruction of tumor cells which occurs after injection of Balb/c mice with tumor cells and P. acnes cells mixture is due to the release of certain products by PMNs during phagocytosis of P. acnes cells through oxygen-burst reactions. In addition, this assay was used to determine the nature of such products by the use of scavengers and inhibitors of various oxygen radicals.

Fresh PMNs were obtained and vaccine preparations were opsonized according to the methods and concentrations used for the Chemiluminescence Assay. Opsonized cell preparations included 48-hr. P. acnes cells (group 1), 12-hr. P. acnes cells (group 2), P. acnes cell walls (group 3), P. freudenreichii cells (group 4), and latex beads (group 5). The opsonized preparations (1.5 ml each) were mixed with 3 ml of 10^6 PMNs at a ratio of 200 organisms or beads per PMN, and incubated in HBSS at 37°C for 30 minutes in order to allow for phagocytosis to take place. The phagocytosis mixture for each of the five groups was then centrifuged at 60 x g for 10 minutes and the supernatants were saved. Tumor cells were obtained from a tumor-burdened mouse as described before and their concentration adjusted to 3 X 10^5 cells/ml. The supernatant obtained

from each phagocytosis group was divided in three parts and each part was used to resuspend 1 ml of tumor cells and then was incubated at 37°C for 2 hours. A control group was included which contained only tumor cells in HBSS-PR in order to determine the effects of incubation time and temperature on these cells. In addition, tumor cells were incubated with PMNs only or with 48-hr. P. acnes cells only as additional controls.

The three tumor-cell-supernatant preparations for each group were centrifuged, washed with HBSS, and each one resuspended in 0.1 ml HBSS and injected into the right hind leg of a fresh Balb/c mouse. Thigh diameter measurements were performed on each group of mice as described previously in order to follow the development of tumors with time.

To test for the effect of various radical scavengers and inhibitors on the toxicity of the supernatant obtained from the phagocytosis mixture of PMNs and 48-hr. P. acnes cells, 3 ml of PMNs were incubated with 1.5 ml of opsonized 48-hr. 0009 and 1.5 ml of an oxygen radical scavenger solution for 30 minutes at 37 °C. As a positive control, HBSS-PR was added in place of the scavengers. The concentration of scavenger solution used in the experimental groups varied with the scavenger used. Sodium azide was used at a concentration of 1×10^{-3} M, catalase

from bovine liver and superoxide dismutase (DDI Pharmaceuticals, Los Angeles, CA) were used at a concentration of 75 ug/ml each, and D-mannitol (Difco Laboratories) and L-methionine were used at 0.02 M each. After the incubation time, the phagocytosis mixtures were centrifuged to obtain the supernatant which was then used to resuspend 10^5 tumor cells. The cells were incubated at $37^{\circ}C$ for 2 hours and then injected into fresh Balb/c mice, as described above. Thigh diameter measurements were then performed with time.

Chinese Hamster Ovary Cell Cytotoxicity Assay

This assay was developed in order to serve as a second model to test for the ability of supernatant obtained from the incubation of PMNs and various vaccine materials to kill tumor cells. Instead of resuspending fibrosarcoma tumor cells in the supernatant, incubating it for 2 hours, and then injecting the cells into fresh mice, this assay consists of adding the supernatant directly to a culture plate containing Chinese Hamster Ovary cells and looking for death of the cells after 24 hours. This assay was used to repeat the experiments carried out in the Phagocytosis Assay. In addition, it was also used in time studies in which the various oxygen radical scavengers described previously were added to the phagocytosis mixture of PMNs

and 48-hr. P. acnes cells either at the start of phagocytosis or 30 minutes after.

Supernatants obtained from the incubation of PMNs and either 48-hr. P. acnes cells, P. freudenreichii cells, or latex beads for 30 minutes were diluted 1/2, 1/5, and 1/10 in HBSS-PR. Chinese Hamster Ovary (CHO) cells were grown in Minimum Essential Medium + 10% fetal calf serum in 96-well microtiter culture plates to 50% confluency in a total volume of 200 ul. They were inoculated in triplicate with 20 ul of each supernatant undiluted, or at the dilutions specified above so that the final dilutions of phagocytosis supernatant in each well was 1/20, 1/50, and 1/100. The cells were incubated in a CO_2 incubator at 37°C overnight, after which time the cells were examined under phase contrast to determine whether cell death had occurred. This is defined as the change in cell morphology from spindle-shape to a rounded configuration of the cells due to their detachment from the surface of the well at death. As controls, some wells were inoculated with supernatant from PMNs only, vaccine only, HBSS-PR buffer only, or not inoculated at all.

In time studies performed with radical scavengers, PMNs and 48-hr. P. acnes cells were incubated with the various scavengers having been added to the mixture either at the start of the 30-minute phagocytosis period or after

30 minutes of incubation of PMNs with vaccine. In the latter case, the mixture of PMNs, 48-hr. 0009, and scavenger was incubated for 30 minutes after the addition of scavenger. The same controls were used as stated above. In addition, a positive control was used in which no scavenger was added and the phagocytosis mixture of PMNs and 48-hr. P. acnes was incubated for either 30 minutes or 60 minutes. CHO cells were then examined for change in morphology after 24 hours.

Oxygen Consumption Assay

Measurement of the amount of oxygen consumed by PMNs during phagocytosis of 48-hr. P. acnes cells was used to determine whether the killing of tumor cells by toxic oxygen radical(s) is due to a general increase in the amount of radicals produced during phagocytosis, or to the production of specific toxic radical(s).

A Clark cell apparatus equipped with an oxygen electrode was used to measure the amount of oxygen consumed by PMNs during phagocytosis of various protective and non-protective vaccine materials. The oxygen electrode was calibrated in order to determine the microliters of oxygen that dissolve per ml of HBSS-PR buffer under experimental conditions by using the formula $c = (100)(n)/(2)(X)(v)$ where v is the volume of HBSS-PR buffer used, n is the

umoles of NADH used, X is the recorder deflection, and c is the concentration of oxygen in umoles/ml. To obtain the microliters of oxygen that dissolve per ml of buffer, the value for c is multiplied by 22.4.

A solution containing 6.67 ug/ml PMS and 133 ug/ml catalase in HBSS-PR was prepared. An NADH solution was also prepared which consisted approximately of 2.0 umoles/ml beta-NADH. The exact concentration of NADH was determined spectrophotometrically by measuring the absorbance of a 1:100 diluted solution at 338 nm. The absorbance value obtained was 0.122. This figure was then used to obtain the concentration of the diluted NADH solution using Beer-Lambert's law: A = (e)(c)(l) where A = absorbance (0.122), e = molar extinction coefficient of NADH (6200), c = the concentration of NADH in moles/L, and l = width of cuvette (1 cm). The value obtained for the concentration of NADH in the diluted solution was 0.02 umoles/ml. This value was then multiplied by the dilution factor of 100 in order to obtain the concentration of the undiluted solution of NADH, which equaled 2 umoles/ml. This solution was kept in the dark throughout the calibration of the electrode, which was performed at 37°C. Of the PMS-catalase solution, 1.7 ml were added to the Clark cell under constant stirring and aeration. After stabilizing and zeroing both the Clark cell and the

recorder, 50 ul of the NADH solution were injected and the recorder deflection was used to calculate the microliters of oxygen per ml of HBSS-PR that dissolve in this buffer.

In the experimental assay, PMNs were obtained from the peritoneal cavity of mice which had been injected with 0.5 ml of a 5% glycogen solution as described previously. The following vaccine materials were opsonized by incubating 0.5 ml of a 1 mg/ml solution of each with 0.5 ml of fresh mouse serum for 30 minutes at 37°C: 48-hr. P. acnes, P. freudenreichii, P. granulosum, P. avidum, Micrococcus lysodeikticus, and latex beads. Each vaccine preparation was then washed and resuspended in 1.0 ml of ice-cold HBSS-PR to yield 500 ug/ml. The suspension of PMNs was adjusted to a concentration of 1 X 10^6 PMNs/ml, 1.3 ml of which was placed in the Clark cell, aerated, and the deflection in the recorder (slope of the line) was determined in units per minute. This corresponds to the amount of oxygen normally consumed by PMNs without stimulation (endogenous oxygen consumption). After 5 minutes, 100 ul of one of the opsonized vaccine preparations was added to the PMNs in the Clark cell to initiate phagocytosis, and the recorder deflection measured for 30 minutes. Again, the slope of the line was determined in units per minute and this figure was multiplied by the calibrated microliters of oxygen that

dissolve per ml of HBSS-PR to obtain the microliters of oxygen consumed per ml of HBSS-PR buffer per minute.

RESULTS

Antitumor Effect of Various Vaccine Materials

The studies carried out for this thesis represent an attempt to identify the nature of the host mechanism, triggered by injection with *Propionibacterium acnes*, that prevents the growth of tumors in Balb/c mice after injection with tumor cells and vaccine. In order to establish the uniqueness of this antitumor effect, various other materials were used as vaccines, and compared with *P. acnes* for their ability to protect mice from the development of tumors.

Table 1 represents the thigh diameter measurements of Balb/c mice at various times after injection with 10^5 tumor cells alone, or with 10^5 tumor cells and 500 ug of one of several vaccine materials. Injection with tumor cells only results in the formation of palpable tumors after approximately 10 days. Injection of tumor cells with a 48-hr-old culture of *P. acnes* heat-killed whole cells ("48-hr. 0009") results in no tumor development even after 90 days. When 12-hr-old *P. acnes* heat-killed whole cells ("12-hr. 0009") or *P. acnes* cell walls are used, tumors develop at the same rate as controls receiving only tumor cells. The same also occurs when heat-killed *P. freudenreichii* cells ("0407") are used. These results confirm the findings of

TABLE 1

ANTITUMOR EFFECT OF VARIOUS VACCINE MATERIALS

Thigh Diameter Measurements (mm)[a]

Day	TC[b] only	TC+48hr *P. acnes*	TC+12hr *P. acnes*	TC+*P. acnes* CW	TC+*P. freud.*	TC+latex beads
6	5.0	6.0	5.1	5.7	5.1	4.8
10	6.3	5.4	6.0	6.5	6.5	5.4
14	9.2	5.6	8.9	8.4	9.6	8.7
20	13.8	5.0[c]	13.2	11.2	14.3	13.8

[a] Average of 5 mice.

[b] TC = 10^5 tumor cells.

[c] No tumors developed even after 90 days.

many investigators on the ability of 48-hr. 0009 cells to protect mice against the development of tumors. This vaccine preparation was used in all assays throughout this research as the "protective" vaccine preparation.

Determination of Time at Which Tumor Cells Are Killed

As stated before, the ultimate goal of this research is to determine the nature of the mechanism responsible for the destruction of tumor cells after concomitant injection into Balb/c mice with P. acnes. As an initial step, experiments to determine the time at which the tumor cells are killed were carried out. Two separate methods were used: lesion transfer experiments, based on the ability of tumor cells harvested from lesions to cause the development of tumors in fresh mice, and tumor antigen assays, which are based on the detection of tumor cells in these lesions by antigen-antibody interactions. The results obtained with each method are discussed below.

(i) Lesion Transfer Experiments

To determine the time at which tumor cells are killed after injection of Balb/c mice with protective vaccine, the tumor cells' viability was tested by first removing the lesions from these mice at specific times. This was followed by separation of tumor cells from other cells in

the lesion through centrifugation in a Ficoll double gradient, and injection of the tumor cells into fresh mice. The rationale behind this assay is that tumor cells from lesions not able to develop into tumors in the fresh mice would either be dead or somehow rendered non-tumorigenic.

The lesions were removed at 4 days, 48 hours, 24 hours, or 12 hours after injection with tumor cells and vaccine, and the extracted tumor cells were injected into the fresh mice. As can be seen from Figures 1 through 4, tumors did not develop in mice injected with tumor cells from the lesions obtained from mice that had been previously treated with P. acnes. However, the transfer of tumor cells from mice injected with tumor cells alone resulted in the development of palpable tumors approximately 15 days after the transfer of the lesions into fresh mice, regardless of the time at which the lesions were removed. These results suggest that the tumor cells were killed quite rapidly, since in the presence of P. acnes, tumors did not develop in the fresh mice even if the lesions were transferred as early as 12 hours after injection.

(ii) Enzyme-linked Immunosorbent Assay

In order to support the findings from the Lesion Transfer Experiments, an ELISA was performed to measure

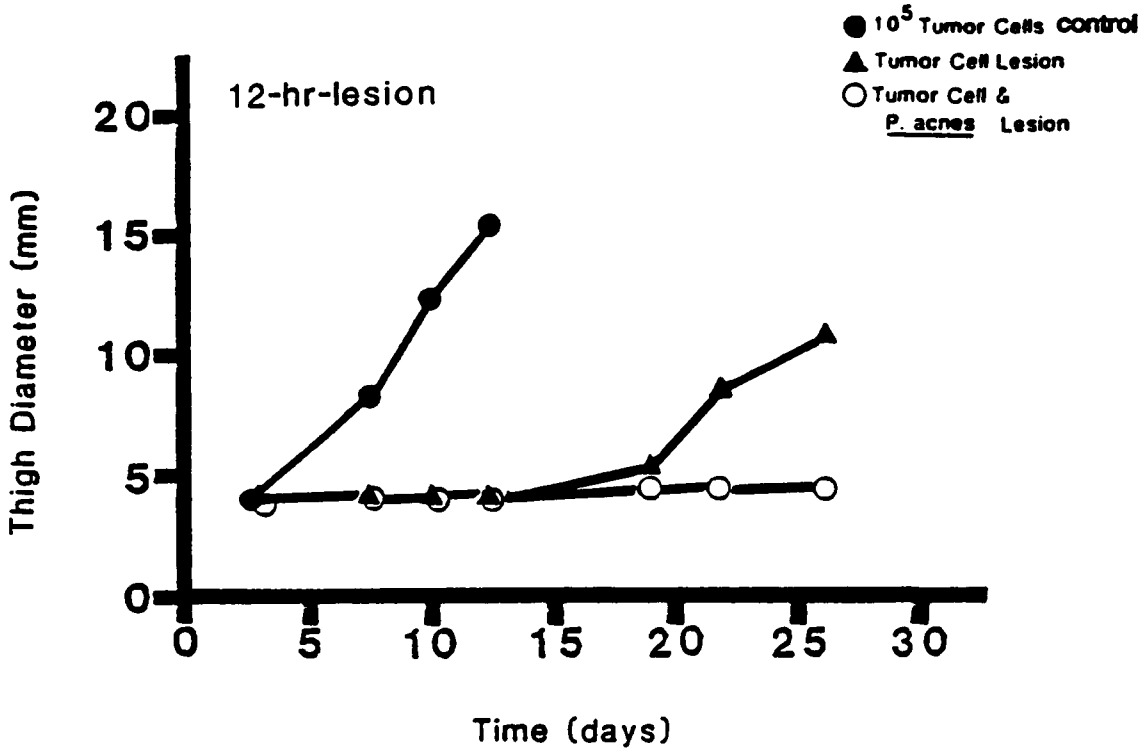

Figure 1: Tumor development in fresh Balb/c mice injected with tumor cells obtained from 12-hr-old lesions of mice previously injected with either tumor cells only or tumor cells + P. acnes. Control mice received 10^5 tumor cells obtained from a disaggregated 13-day tumor. Data points represent the average of 5 mice.

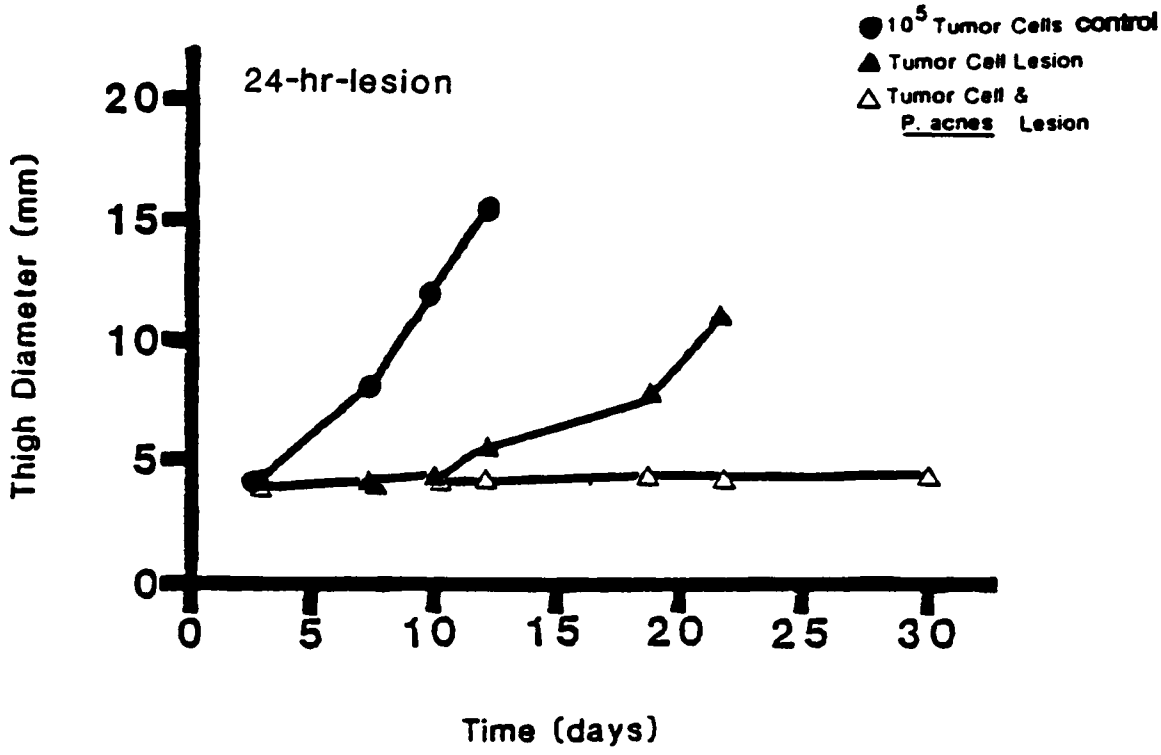

Figure 2: Tumor development in fresh Balb/c mice injected with tumor cells obtained from 24-hr-old lesions of mice previously injected with either tumor cells only or tumor cells + P. acnes. Control mice received 10^5 tumor cells obtained from a disaggregated 13-day tumor. Data points represent the average of 5 mice.

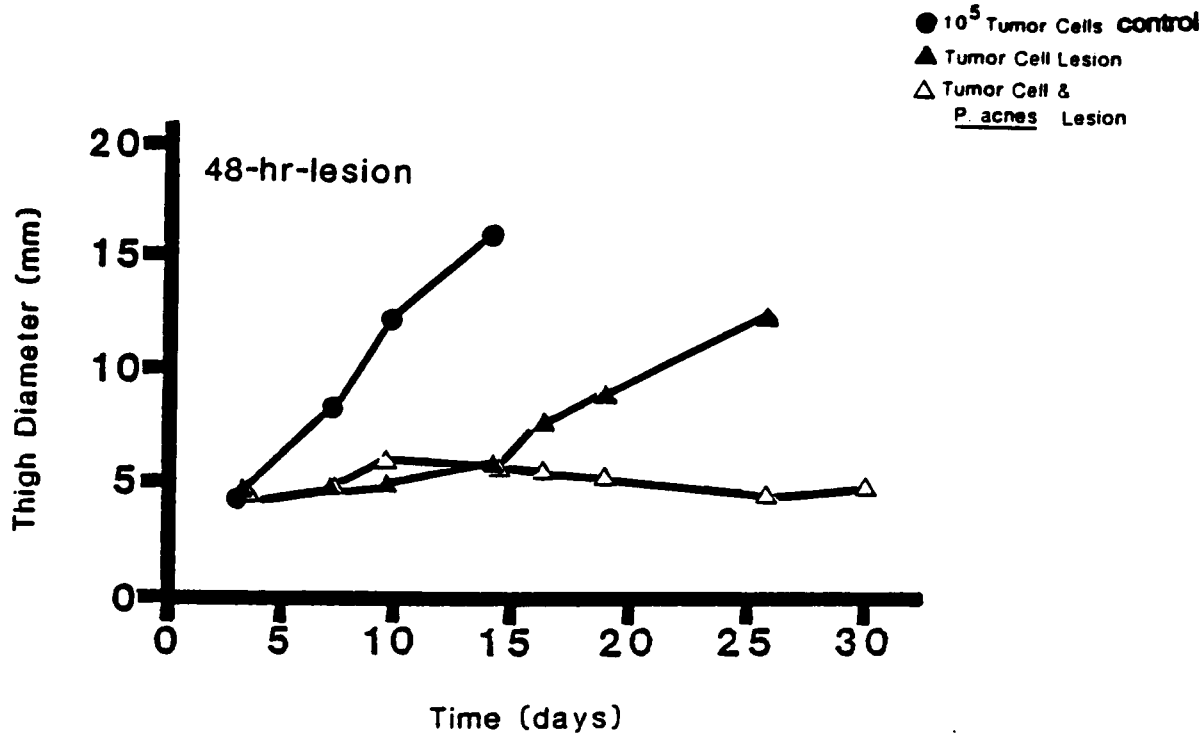

Figure 3: Tumor development in fresh Balb/c mice injected with tumor cells obtained from 48-hr-old lesions of mice previously injected with either tumor cells only or tumor cells + P. acnes. Control mice received 10^5 tumor cells obtained from a disaggregated 13-day tumor. Data points represent the average of 5 mice.

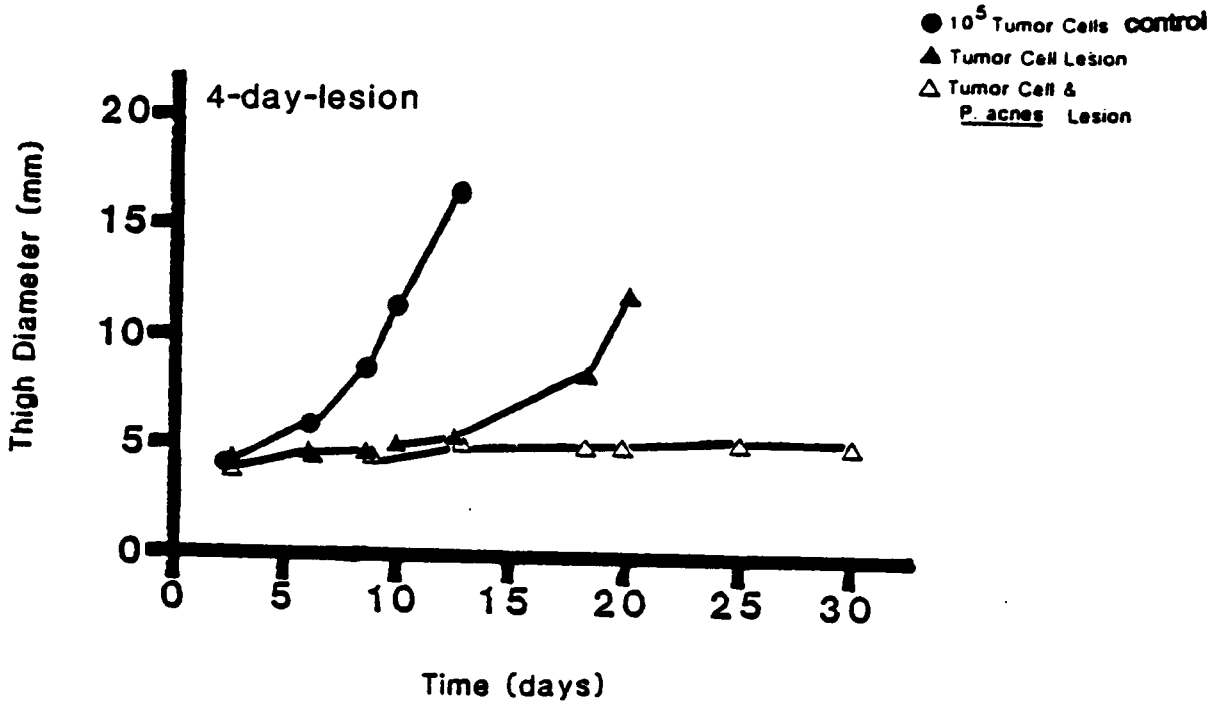

Figure 4: Tumor development in fresh Balb/c mice injected with tumor cells obtained from 4-day-old lesions of mice previously injected with either tumor cells only or tumor cells + P. acnes. Control mice received 10^5 tumor cells obtained from a disaggregated 13-day tumor. Data points represent the average of 5 mice.

levels of tumor cell antigen in each of the lesions. These values were used as an indirect measure of tumor cell numbers, and thus of viability of tumor cells at various times after injection. A standard curve was first constructed relating absorbance in the ELISA test to numbers of tumor cells (Figure 5), and this was used to determine tumor cell numbers in the various lesions examined.

At specific times, lesions from mice injected with tumor cells alone, or with tumor cells and P. acnes, were obtained, and rabbit anti-tumor antiserum was used to determine absorbance readings vs. time at which the lesion was removed. Figure 6 represents the estimated numbers of tumor cells vs. time at which the lesions were removed. If tumor cells alone were injected, there was an initial drop in the number of tumor cells from 10^5 to approximately 10^3 cells 12 hours after injection. However, after 1 day, the number of cells increased rapidly, achieving an absorbance reading corresponding to 10^5 tumor cells approximately 7 days later. This initial two-log drop in the number of tumor cells seems to be a normal effect, since it occurred in controls when only tumor cells were injected. It may represent a period of time in which the tumor cells were trying to adapt to the conditions in the mouse leg tissue. The fact that tumor cell numbers rose again after this time

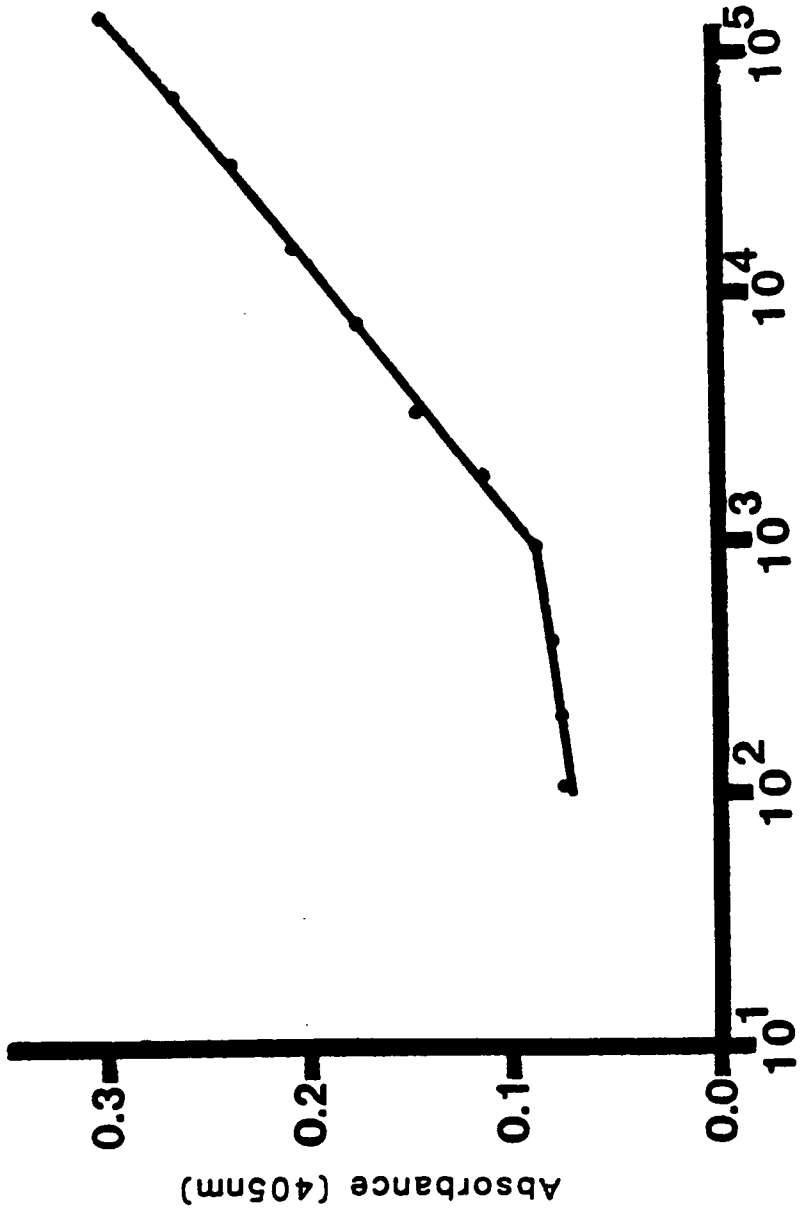

Figure 5: Standard curve of absorbance vs. tumor cell numbers obtained by Enzyme-linked Immunosorbent Assay.

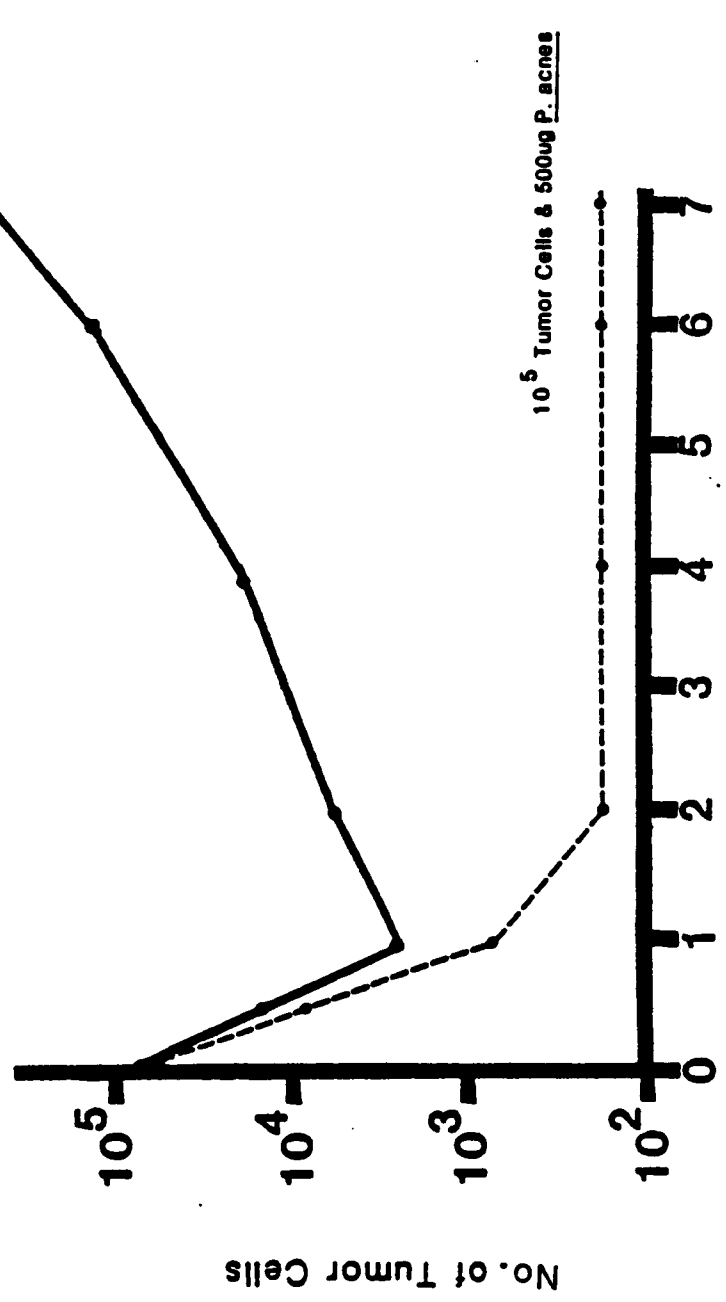

Figure 6: Tumor cell number present in lesions vs. time at which lesions were removed from Balb/c mice and subjected to analysis by ELISA. The curves represent results with mice injected with tumor cells only or with tumor cells + P. acnes. Data points represent tumor cell numbers obtained from the ELISA Standard Curve (Figure 5), using the average of 3 absorbance readings for each lesion.

is supportive evidence of this theory. It must be remembered, however, that ELISA measures levels of tumor antigen and not tumor cell numbers. Thus, assumptions that a decrease in absorbance readings is indicative of a drop in tumor cell numbers may not be valid. This subject will be dealt with in more detail in the Discussion Section of this thesis.

When P. acnes was injected together with the tumor cells the estimated number of tumor cells dropped at 1 day, even further than when only tumor cells were injected. However, the number of tumor cells failed to rise with time, suggesting that in the presence of P. acnes the tumor cells were either killed and cleared away from the lesion area by the time the lesion was removed for the assay, or that the antigenic configuration of the tumor cells changed in such a way as to be unreactive with the antitumor antiserum. Whichever is the case, no tumors developed.

The results obtained from this assay agree with those obtained in the Lesion Transfer Experiments in that it appears that tumor cells were killed or rendered non-oncogenic as early as 12 hours after injection with P. acnes. Mice injected with these early lesions, as seen in the Lesion Transfer Assay, were unable to develop tumors, and according to the determinations by ELISA, the number of tumor cells in lesions in which P. acnes was present

dropped to a low level 12 hours after injection, without any subsequent rise.

Histological Studies

Having determined that tumor cells appeared to be killed as early as 12 hours after injection, cytological studies were carried out to determine the types of host cells in the lesions at that time.

Figure 7 shows that 12 hours after injection of Balb/c mice with P. acnes cells, approximately 95% of the population of host cells present in the lesion was composed of polymorphonuclear leukocytes (PMNs). Injection of mice with mixtures of P. acnes and tumor cells also showed PMNs to be the predominant cell in the lesion 12 hours after injection (Figure 8). In contrast, in 12-hour sections obtained from mice injected with tumor cells only, PMNs were hardly detectable (Figure 9). These results suggest that injection with P. acnes elicited the migration of PMNs to the lesion in large numbers. To determine whether this phenomenon happens only when protective vaccines are used, injection with substances such as latex beads, which do not protect mice against the development of tumors, was also performed. This also resulted in the appearance of large numbers of PMNs in the lesion 12 hours after injection

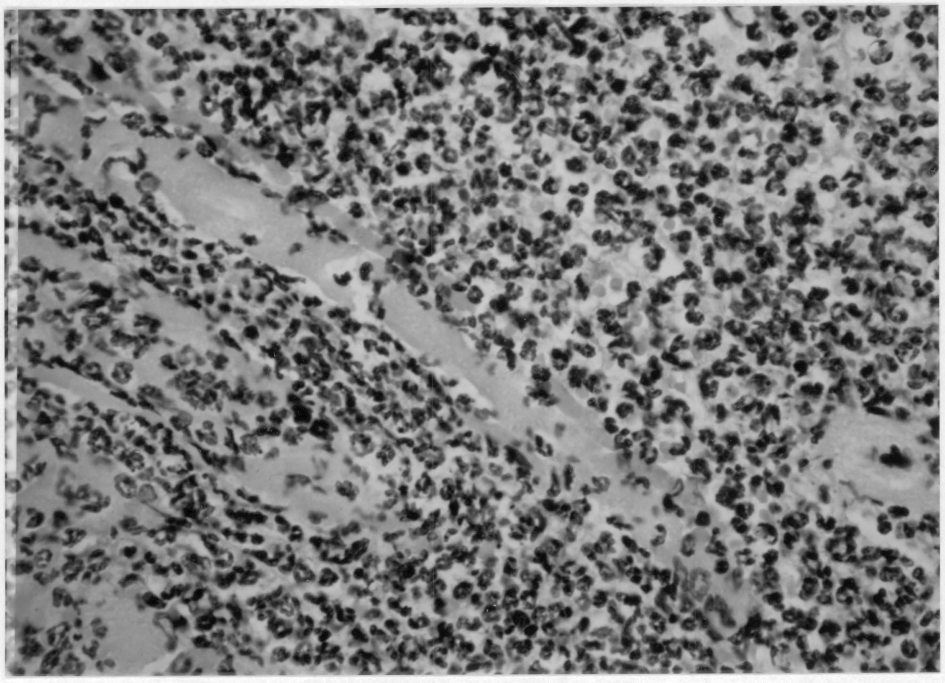

Figure 7: Hematoxylin and Eosin staining of muscle tissue section from Balb/c mouse leg, 12 hours after intramuscular injection with 48-hr. P. acnes vaccine.

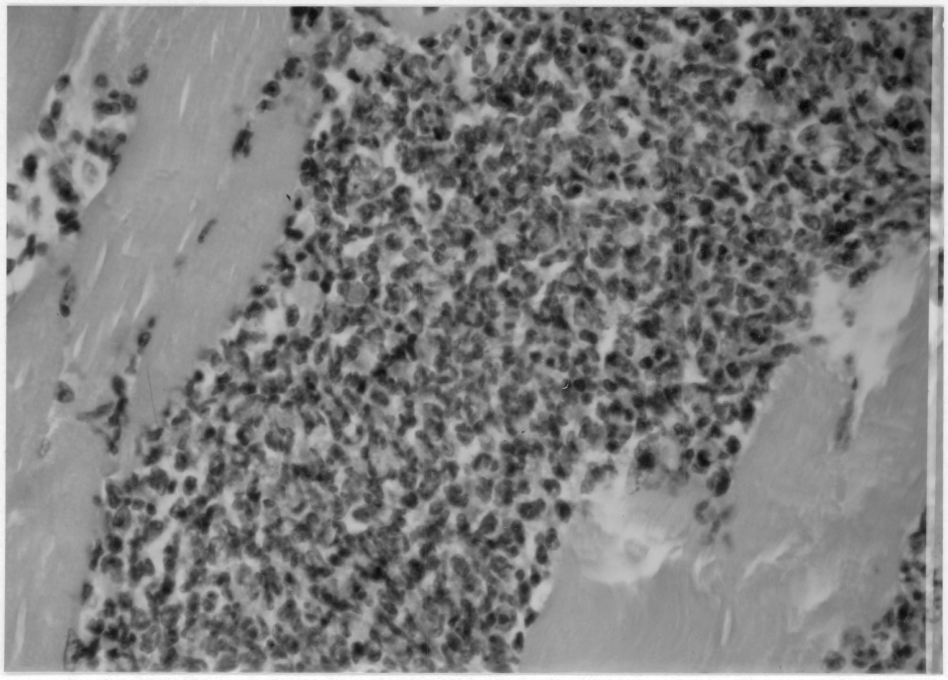

Figure 8: Hematoxylin and Eosin staining of muscle tissue section from Balb/c mouse leg, 12 hours after intramuscular injection with 48-hr. P. acnes + tumor cells.

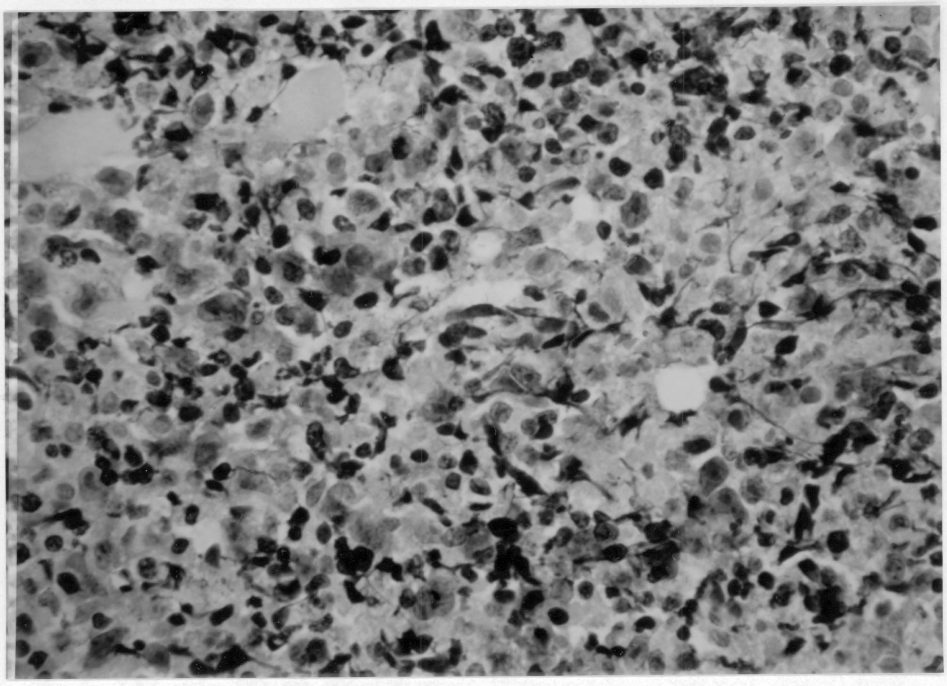

Figure 9: Hematoxylin and Eosin staining of muscle tissue section from Balb/c mouse leg, 12 hours after intramuscular injection with tumor cells.

(Figure 10). P. acnes thus is not unique in its ability to cause the migration of PMNs to the injected area. However, this does not eliminate the possibility that PMNs may be involved in the killing of tumor cells.

Chemiluminescence

As discussed in previous sections of this thesis, PMNs are able to produce toxic oxygen radicals during the act of phagocytosis in an event known as the "oxygen burst" in which the PMNs convert almost all the oxygen to superoxide. This and other radicals produced by PMNs have been studied for their effect on tumor cells in vitro. Nathan has suggested that the production of hydrogen peroxide during phagocytosis of phorbol myristate acetate by PMNs, and its subsequent excretion into the surrounding medium, is what eventually kills the tumor cells (106). Although the fact that P. acnes prevents the growth of tumors in animals has been known for many years, no studies have been done on its ability to stimulate PMNs to produce toxic oxygen radicals, capable of killing tumor cells. It has been shown that there is a difference in the protective ability of P. acnes vaccine and other vaccine materials against the formation of tumors. To determine whether that difference is in the ability of PMNs to produce different toxic oxygen radicals depending on the material being phagocytized, a

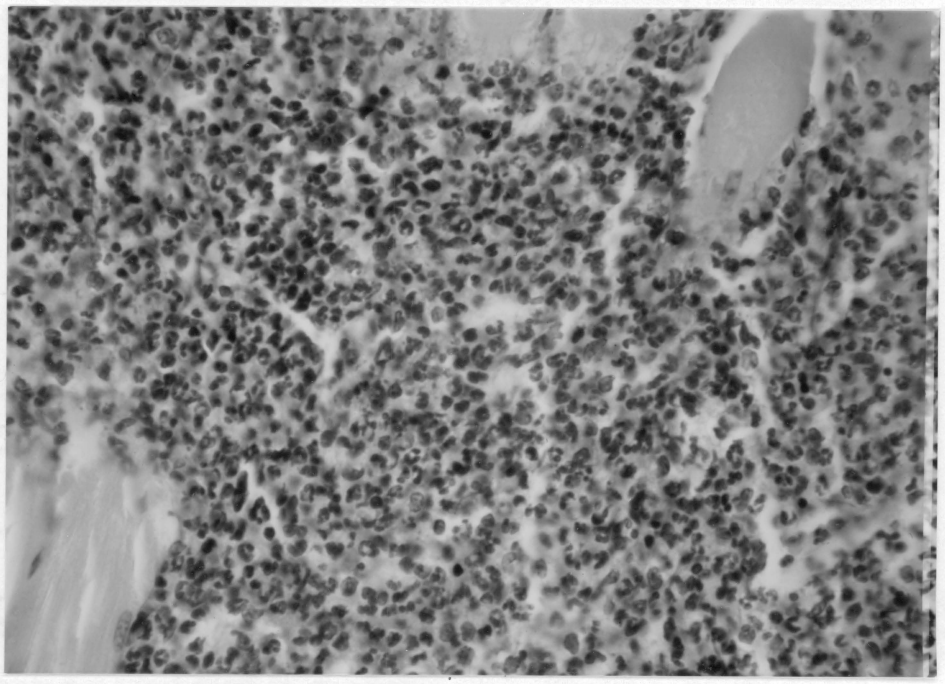

Figure 10: Hematoxylin and Eosin staining of muscle tissue section from Balb/c mouse leg, 12 hours after intramuscular injection with latex beads.

chemiluminescence assay was developed to obtain a measure of the relative levels of oxygen radical produced during phagocytosis of various materials.

As seen in Figure 11, the highest response for all materials was detected approximately from 1 to 2 minutes after the mixture of PMNs with the opsonized vaccine preparations. Both 48-hr. 0009 cells and cell walls were able to elicit a higher chemiluminescence response from PMNs than 12-hr. 0009 cells. The highest peak for 12-hr. 0009 cells detected was about 86,000 cpm. In contrast, the other two preparations produced peaks of about 140,000 cpm. 48-hr. 0009 cells elicited a second peak 15 minutes after the first peak, the height of which corresponded to 15,000 cpm from the baseline. With 12-hr. 0009 cells no second peak was detected. However, a second peak was detected when 0009 cell walls were used, although it was only about 5,000 cpm from the baseline. Latex beads did not elicit any chemiluminescence. This may be due to failure of PMNs to effectively phagocytize them.

The second peak observed when 48-hr. 0009 cells and cell walls were used may be significant in that it may represent a later production of more oxygen radicals. The first peak may represent production of singlet oxygen as well as superoxide radical whereas the second peak may represent the subsequent production of other radicals and

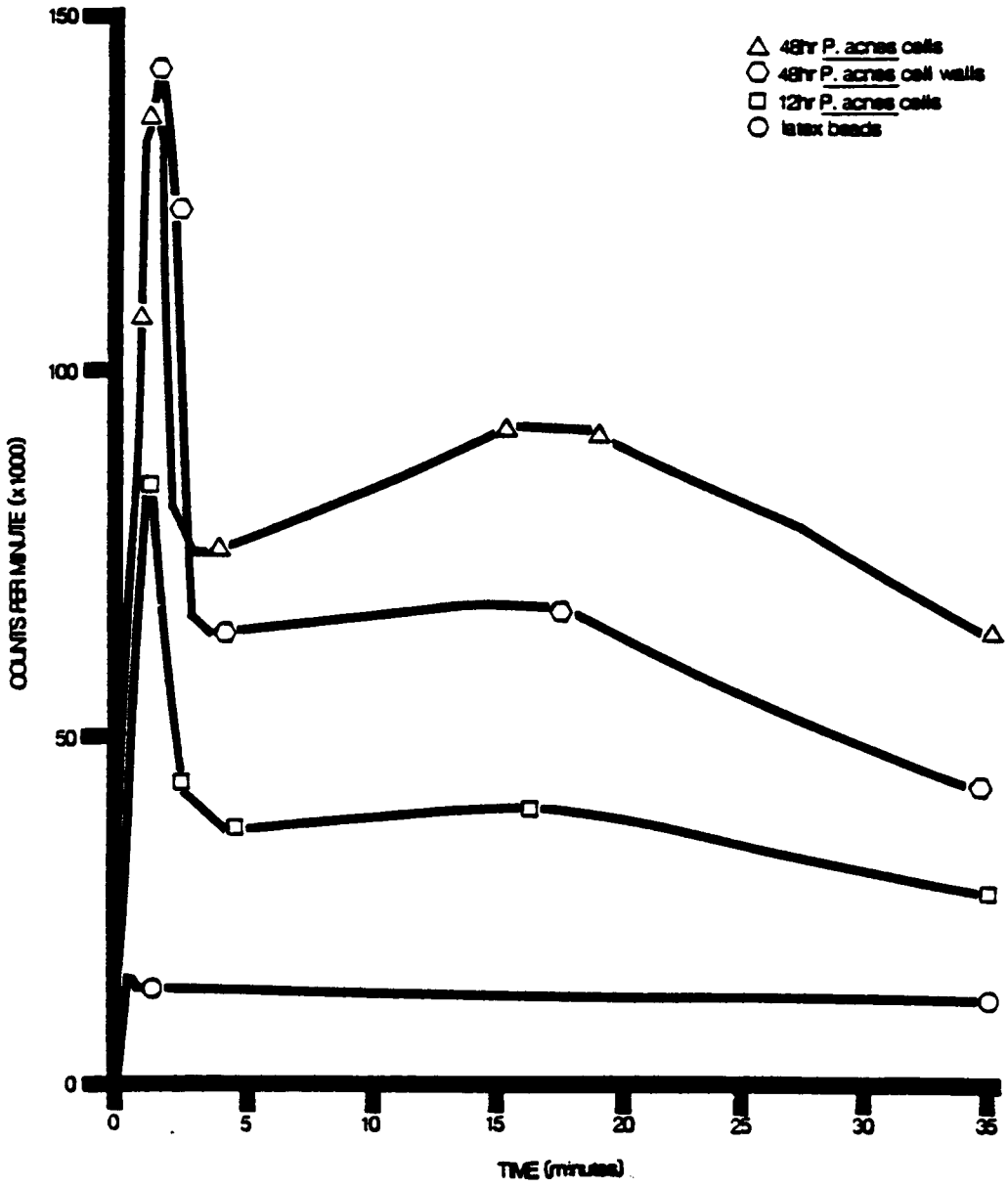

Figure 11: Effect of various P. acnes cell preparations on chemiluminescence emmission of polymorphonuclear leukocytes during phagocytosis.

their derivatives, such as H_2O_2 and OH.

These results indicate that there is a difference in the radical-producing profile of PMNs depending on whether they are involved in the phagocytosis of protective or nonprotective vaccine materials.

Phagocytosis Cytotoxicity Assay

A Phagocytosis Assay was developed to determine whether tumor cells can be killed by the types and amounts of oxygen-derived radicals produced during phagocytosis of P. acnes cells by PMNs. This assay was also used to determine whether the difference in radical production detected in the Chemiluminescence Assay could account for the difference in the antitumor activity of the various vaccine materials.

Supernatants were obtained from phagocytosis mixtures as listed in Table 2, and were incubated with tumor cells for 2 hours. Thigh diameter measurements of mice injected with the tumor cells that had been previously incubated with PMNs and 48-hr. 0009 cells did not increase as compared to controls injected with buffer only. Mice injected with tumor cells previously incubated with any of the other cell preparations, or with latex beads all developed tumors. These results indicate that whatever the toxic substance is in the supernatant that caused the

TABLE 2

TUMOR DEVELOPMENT AFTER INCUBATION OF TUMOR CELLS WITH SUPERNATANTS FROM VARIOUS PHAGOCYTOSIS MIXTURES

Material Injected	Thigh Diameter Measurements (mm) 17 days	21 days
HBSS-PR buffer	5.0	5.0 (0)[b]
10^5 tumor cells incubated with supernatants from mixture containing:		
10^6 PMNs + 48-hr. 0009	5.0	4.9 (0)
" + 12-hr. 0009	16.1	17.2 (3)
" + 0009 cell walls	14.8	16.8 (3)
" + 0407	15.1	17.0 (3)
" + latex beads	14.3	16.6 (3)
10^6 PMNs ONLY	14.5	16.9 (3)
48-hr. 0009 ONLY	14.0	16.1 (3)

[a] Average of three mice.

[b] Number in parentheses represents the actual number of mice that developed tumors.

death of tumor cells, it was produced after mixing PMNs with 48-hr. 0009 cells, and not by PMNs alone or by 48-hr. 0009 cells alone. Also, the death of tumor cells did not occur during the 2-hour incubation of the cells in buffer alone since controls in buffer regularly produced tumors.

The supernatant obtained from the PMN-48-hr.-0009-cell mixture contained a substance(s) capable of killing tumor cells after a 2-hour incubation period. This did not happen when tumor cells were incubated in supernatants obtained from mixtures of PMNs with any of the other nonprotective vaccine preparations. It thus seems that whatever was produced, or released, by PMNs during phagocytosis of 48-hr. 0009 cells was not produced when other vaccines were being phagocytized, or at the very least, not in the same quantities as when 48-hr. 0009 cells were used.

To determine whether the material in the supernatant responsible for the killing of tumor cells is in fact an oxygen radical produced during phagocytosis of 48-hr. 0009 cells by PMNs, various oxygen radical scavengers and inhibitors were used in the Phagocytosis Assay. Table 3 represents the known effects of certain substances on the production of various oxygen radicals. Table 4 contains the data on the effect of the various radical scavengers and inhibitors on PMN-mediated cytotoxicity of tumor cells.

TABLE 3

KNOWN EFFECTS OF ADDITIVES USED ON OXYGEN RADICALS[a]

Additive	Known Effect
Azide	Inhibits myeloperoxidase enzyme found in lysosomes of PMNs
Catalase	Breaks down hydrogen peroxide ($H_2O_2 \longrightarrow H_2O + O_2$)
Mannitol	Scavenges hydroxyl radicals ($OH\cdot$), a derivative of H_2O_2
Methionine	Scavenges hypochlorous acid (HOCl), formed from H_2O_2 and Cl^-
Superoxide Dismutase	Enzyme that catalyzes the reaction: $2\ O_2\cdot^- + 2H^+ \longrightarrow H_2O_2$

[a] See reference 49.

TABLE 4

EFFECT OF RADICAL SCAVENGERS AND INHIBITORS ON PMN-MEDIATED CYTOTOXICITY

Material Injected	Thigh Diameter Measurements[a] 17 d.	21 d.	Protective Index[c]
HBSS-PR buffer	5.2	5.1	
10^5 tumor cells incubated with supernatant from mixture containing:			
10^6 PMNs + 0009	5.3	5.2	99.1
10^6 PMNs + 0009 +:			
Mannitol	8.5	11.4	44.7
Methionine	5.7	6.6[b]	86.8
Catalase	12.7	14.2	20.2
Azide	14.4	16.9	-3.5
10^6 PMNs ONLY	14.1	16.5	0.0

[a] Average of three mice.

[b] Tumor developed in 1/3 mice.

[c] [(increase in thigh diameter of PMNs ONLY - increase in thigh diameter of experimental groups)/increase in thigh diameter of PMNs ONLY] X 100.

As can be seen, the use of azide, catalase, and mannitol resulted in the greatest reduction in the protective ability of 48-hr. 0009 cells. In all three cases, three out of three mice developed tumors. When methionine was used, the protective index also declined although to a lesser extent, with only one mouse out of three developing a tumor.

Chinese Hamster Ovary (CHO) Cell Cytotoxicity Assay

This assay was used to confirm the results obtained in the Phagocytosis Cytotoxicity Assay regarding the toxicity of the PMN-48-hr.-0009-cell supernatant and the nature of the radical(s) responsible for the killing of tumor cells. The assay provided results much faster due to the elimination of the waiting time required for the development of tumors in the fresh mice. In the CHO cell assay a cell culture was used and cytotoxicity was determined after overnight incubation of the cells with the supernatant in question.

As in the Phagocytosis Assay, supernatant obtained from the phagocytosis mixture of PMNs and 48-hr. 0009 cells exhibited the most toxicity, as it was able to kill CHO cells at a higher dilution than the supernatants obtained from other phagocytosis mixtures (Table 5).

To determine whether the material in the supernatant

TABLE 5

EFFECT OF SUPERNATANT FROM VARIOUS PHAGOCYTOSIS MIXTURES ON CHINESE HAMSTER OVARY (CHO) CELLS

	CHO cell death[a]			
	Supernatant Dilutions			
	Undiluted	1/2	1/5	1/10
CHO cells incubated with supernatant from mixture containing:				
CONTROL: no supernatant added	-	-	-	-
HBSS-PR buffer	-	-	-	-
PMNs ONLY	-	-	-	-
PMNs + 48-hr. 0009	+	+	+	+
PMNs + 0407	+	-	-	-
PMNs + latex beads	+	-	-	-

[a] Death is measured by morphological change of all cells from spindle- to round-shape, as they become detached from the surface of the microtiter plate when dead. This is indicated by a "+". No change in morphology, as compared to control, is indicated by a "-".

responsible for the killing of CHO cells is in fact an oxygen-derived radical produced during phagocytosis by PMNs, radical scavengers and inhibitors were used. In addition, the effect of the time at which the scavengers/inhibitors were added to the phagocytosis mixture was studied using the CHO cell model. As can be seen from Figure 12, addition of catalase at the start of phagocytosis of 48-hr. 0009 cells reduced the cytotoxic effect of the supernate, as detected by the decrease in the dilution of the supernatant that still caused rounding of CHO cells. When added 30 minutes after the start of phagocytosis, catalase had practically no effect in the reduction of toxicity of the supernatant. Addition of superoxide dismutase produced similar results. Methionine did not seem to have a significant effect on the toxicity of the supernatant when added either at the start or 30 minutes after. Similarly, addition of mannitol at the start had no effect. However, when added 30 minutes after the start of phagocytosis, it reduced the cytotoxic effect of the supernatant substantially. These results suggest that hydrogen peroxide played a major role in the killing of tumor and CHO cells since catalase did have an effect when added at the start of phagocytosis in decreasing the toxic ability of the supernatant. However, the fact that the effect of catalase decreased when added 30 minutes

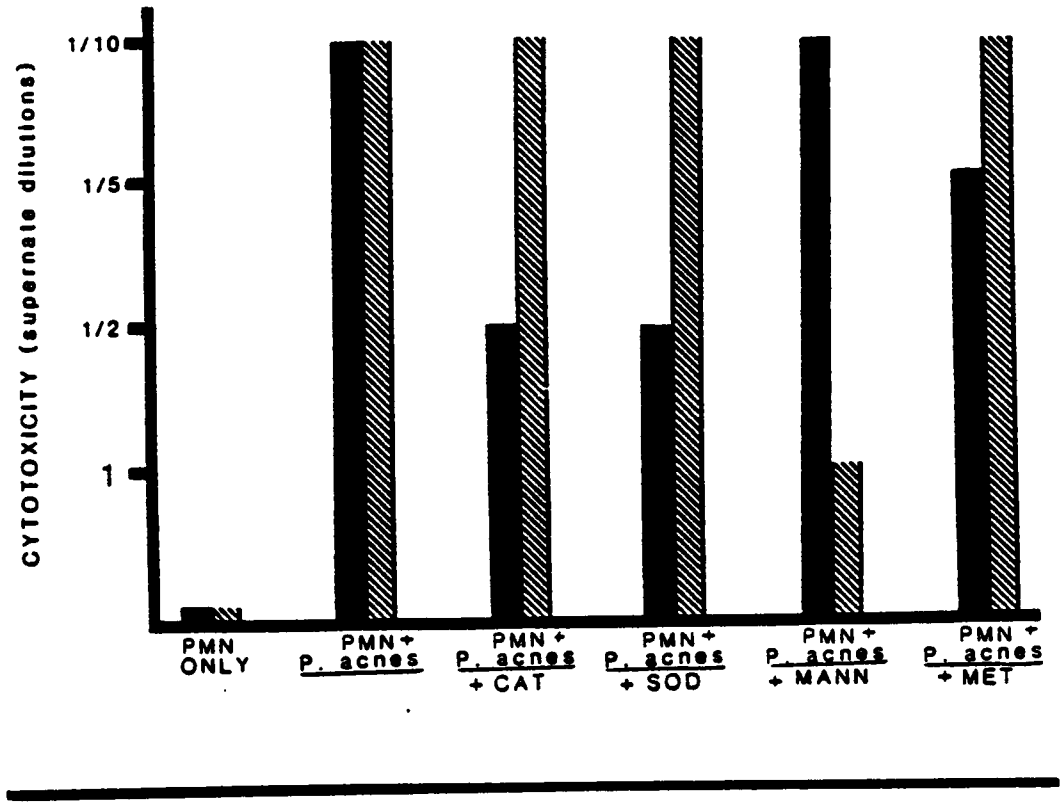

Figure 12: Effect of time of addition of various radical scavengers/ inhibitors on toxicity of supernatant from phagocytosis mixture containing PMN's and 48-hr. P. acnes cells. Solid bars indicate addition of scavenger/inhibitor at the start of phagocytosis and hatched bars indicate addition 30 minutes after the start of phagocytosis. Scavenger/ inhibitors used are catalase (CAT), superoxide dismutase (SOD), d-mannitol (MANN), and methionine (MET). Controls consist of PMN's only or PMN's + P. acnes cells to which no scavenger/inhibitor was added.

later and the effect of mannitol increased suggests that hydrogen peroxide may act only as a precursor in the production of hydroxyl radicals or some other toxic radical.

Measurement of Oxygen Consumption by PMNs

The results presented above suggest that tumor cells were killed by toxic oxygen radicals, more than likely by hydrogen peroxide and hydroxyl radicals, which were produced by PMNs during phagocytosis of 48-hr. P. acnes whole cells. To determine whether this protective vaccine elicited the production of a larger amount of oxygen radicals by PMNs than when nonprotective vaccines were used, or whether it elicited the production of certain radicals more toxic than those produced by nonprotective vaccines the amount of oxygen consumed by PMNs during phagocytosis of various vaccine materials was measured.

An oxygen electrode was used for this purpose. It was first calibrated using HBSS-PR buffer, and it was found that 0.0598 ul/ml of oxygen dissolved in the buffer per minute under the experimental conditions used. Table 6 contains the oxygen consumption data in ul/ml per minute of PMNs during phagocytosis of various organisms. There does not seem to be any significant difference in the values obtained when P. acnes was used and those obtained when any

TABLE 6

OXYGEN CONSUMPTION OF PMNs DURING PHAGOCYTOSIS OF VARIOUS VACCINE MATERIALS

Vaccine Used	[O_2] Consumed (in ul/ml/min.)[a]
P. acnes (48-hr. 0009)	40.1
P. granulosum (5888)	40.4
P. avidum (0589)	38.9
P. freudenreichii (0407)	38.1
M. lysodeikticus	37.7
Latex beads	0.0

[a] Data represents the adjusted values of O_2 consumed after stimulation minus O_2 consumed before stimulation (endogenous).

of the other vaccine materials were used. This seems to suggest that, when exposed to equivalent amounts of various vaccine materials, PMNs did not differ in the amount of oxygen that they consumed. If PMNs consume the same amount of oxygen, regardless of the organism being phagocytized, it follows that the total amount of radicals produced by PMNs in all cases should be the same. Since it is known that these vaccines are not all protective against the development of tumors in mice, and it appears that killing of tumor cells is due to the production of oxygen radicals, it seems that the difference between P. acnes and the other vaccines may be in the production of specific radicals rather than in the amount of radicals produced.

DISCUSSION

The experiments described in this thesis were conducted in order to find an answer to the following question: What is the host mechanism, triggered by injection with P. acnes, that is involved in the killing of tumor cells in vivo? From the work done by various investigators, it is evident that the answer to this question is difficult to find. This is principally for three reasons: first, many different immunological mechanisms capable of killing tumor cells are affected or activated by Propionibacterium acnes, which makes it nearly impossible to discern which are directly involved in the killing of tumor cells and which are triggered as a result of the killing of tumor cells (see Literature Review Section). Second, activation of these mechanisms depends on the tumor cell model employed, as well as on the site of vaccine administration, both of which may dictate the type of mechanism that will be activated. Third, the studies performed on these mechanisms, from investigations into the production of cytotoxic T cells to determinations into the activation of macrophages, have all been done by in vitro experiments, thus offering only indirect evidence on the nature of the events involved in the killing of tumor cells in vivo.

In order to identify the mechanisms in our fibrosarcoma tumor cell model, direct evidence was sought through a study on the timing of tumor cell killing by using a direct lesion transfer assay. It was hoped that knowing the time at which the tumor cells are killed *in vivo* would provide a starting point from which the nature of the mechanism could be investigated. As can be seen from the results obtained from the Lesion Transfer Assay, tumor cells that are obtained from the injection site in a mouse previously injected with tumor cells and *P. acnes* are unable to produce tumors in fresh mice even if the cells are obtained from a 12-hour-old lesion. Whether this indicates that the tumor cells are killed, or rather rendered non-tumorigenic 12 hours after being injected into mice together with vaccine cannot be determined from this assay. The fact remains, however, that the killing of tumor cells in the model used here is an event that occurs in less than one day.

These results were confirmed by the ELISA determinations in which tumor cells that had been injected into mice together with *P. acnes* dropped in numbers down to background levels 8 to 12 hours after injection. One curious phenomenon was seen to occur when tumor cells were injected in the abscence of *P. acnes*. The same two-log decrease in numbers 8 to 12 hours after injection that was

seen when P. acnes was used was also detected in this case. However, in the absence of the vaccine, the drop was followed by a rapid increase in tumor cell numbers over the next few hours. It seems that the initial decrease seen in both of these cases is a normal phenomenon, probably representative of a period in which newly injected tumor cells must adjust to the conditions in the muscle tissue. The difference in the two cases is in the ability of tumor cells that are injected in the absence of a protective vaccine to begin logarithmic growth after a few hours, whereas those injected together with P. acnes are somehow killed or inactivated by 12 hours and thus are not able to increase in numbers after that time.

Since ELISA determinations like the ones performed here are based on antigen-antibody interactions, one question that must be considered is whether the decreases and increases detected by this assay accurately represent changes in the number of tumor cells, or whether they are actually changes in the cells' antigenic configuration. If the former case is to be assumed, then it can be concluded that tumor cells are killed as early as 12 hours after injection into Balb/c mice in the presence of the vaccine. However, if the changes detected by ELISA are due to changes in the tumor cells' antigens, then it could be concluded that concomitant injection of tumor cells with P.

acnes into mice results in the alteration of the tumor cells' surface antigens in such a way as to render them non-oncogenic. This would then result in the cells being recognized as foreign by the host's immune system, causing them to be promptly destroyed and subsequently eliminated from the lesion area by a host mechanism. It is a known fact that during serial transplantation, tumors frequently seem to decrease in specificity, developing the ability to grow in foreign strains and sometimes even in foreign species. This has been attributed to a loss in tumor cell antigens due to somatic mutation (161). Some investigators have even found that treatment with enzymes that attack the outer surface of tumor cells, such as neuraminidase, can cause the tumor cells to be rejected by tumor-compatible hosts, suggesting that removal of cell surface substances like sialic acid exposes histocompatibility antigens to destruction by host immunological mechanisms (136). Thus, it is entirely possible that, in our model, tumor cells are made recognizable to the host and that this is what results in their death. Whether tumor cells are killed directly or are first rendered non-oncogenic and then killed, it is clear from our results that the tumor cells are no longer able to produce tumors as early as 12 hours after injection.

It has been suggested by investigators using the same fibrosarcoma tumor model that injection with P. acnes results in an increase in the production of Interleukin 2 and 3, substances necessary for the production of cytotoxic T cells. Normally, the level of these Interleukins is high, but injection with tumor cells causes them to decrease. They contend that injection with P. acnes results in the maintenance of adequate levels of these substances by their increased production by the host in the presence of the vaccine (126). In view of the results obtained in our laboratory using the same tumor model, however, it appears that perhaps the maintenance of high levels of the Interleukins after injection with tumor cells and P. acnes is due to the early destruction and/or elimination of tumor cells, which then results in the resumption of normal Interleukin levels, rather than to an increase in the production of these substances.

The histological studies carried out for this thesis indicate that 12 hours after injection with P. acnes, the majority of the immune cells present in the lesion are polymorphonuclear leukocytes. Weiss et al. as well as other investigators have found that stimulated PMNs can produce various short-lived oxygen-derived radicals capable of killing tumor cells (148, 31, 173). Although the primary role of PMNs is phagocytosis, secretion of these

toxic agents can occur with the potential for damage to adjacent cells. It is possible, then, that through the production of these radicals, PMNs are able to kill tumor cells in our fibrosarcoma tumor model. However, latex beads, which do not protect mice against the development of tumors, are also capable of eliciting the migration of vast numbers of PMNs to the lesion. Therefore, the role of PMNs in the production of toxic oxygen radicals was examined through comparisons between protective and non-protective vaccine materials.

Chemiluminescence studies revealed a marked difference between stimulation of phagocytosis of PMNs by latex beads and other vaccine preparations with the former failing to produce any response. Of the other vaccine materials tested, 48-hr. P. acnes, a protective vaccine, did not differ greatly from P. acnes cell walls, a non-protective vaccine, in the initial response. Rather, a difference was detected in the production of a secondary peak by the protective vaccine preparation which was much larger than any produced by any of the other vaccines. The nature of this second peak is unknown, but it has been suggested that it represents the subsequent formation of secondary products of the respiratory burst, such as hydrogen peroxide, hydroxyl radical, and hypochlorous acid, with superoxide radical and singlet oxygen having been already

produced in the initial few seconds of the reaction (154). It appears, thus, that there is a detectable difference in the production of radicals between protective and non-protective vaccine materials. Whether this difference is in the amount of radicals produced by PMNs after stimulation or whether it represents the production of a particular radical or radicals will be discussed later.

There is a possibility that differences between vaccine materials in their ability to protect mice against tumors in vivo are actually based on physiological differences between PMNs: in other words, that PMNs produced in the host after injection with a protective vaccine are somehow different from those produced after injection with a non-protective vaccine. This possibility, however, seems to be eliminated by the fact that differences in the chemiluminescence profile of the various vaccines occurred even though all PMNs used were obtained from the peritoneal cavity of mice after injection with the same substance (glycogen). Also, the possibility that injection with P. acnes elicits more PMNs to the lesion than injection with non-protective materials, and that this is the basis for the difference in their protective ability has also been disproved since the same number of PMNs was used in all experimental groups. However, it may be that in vivo, injection with P. acnes does attract more PMNs to

the site, adding to the effectiveness of whatever anti-tumor mechanism is triggered by injection with this vaccine.

Through the Phagocytosis and CHO Cytotoxicity Assays, it was determined that the mechanism responsible for the killing of tumor cells in our model could be a substance produced during phagocytosis of P. acnes by PMNs since the supernatant obtained from this mixture was capable of killing tumor cells in both assays. That this substance is an oxygen-derived radical was shown by the ability of azide and catalase to completely prevent the killing of tumor cells when added to the PMN-P. acnes mixture at the start of phagocytosis. Since catalase breaks down hydrogen peroxide into non-toxic radicals, it appears that hydrogen peroxide is responsible for the killing of tumor cells. Nathan and others found that extracellular cytolysis by activated granulocytes after stimulation with various pharmacological agents strongly correlate with the cells' ability to release hydrogen peroxide (106).

When catalase was added 30 minutes after the start of phagocytosis in our model, however, it had almost no effect in inhibiting the cytotoxic effect of the supernatant. In addition, mannitol, which had a very minor effect in reducing the cytotoxic effect of the supernatant when added at the start of phagocytosis, and which is known to quench

hydroxyl radicals, was able to markedly reduce the effect of the supernatant when added to the mixture 30 minutes after the start of phagocytosis. These results strongly suggest that hydrogen peroxide is produced early on during phagocytosis of P. acnes by PMNs but that it subsequently disappears while hydroxyl radicals begin to be produced. Since hydroxyl radicals are known products of the break down of hydrogen peroxide by a metal-catalyzed Haber-Weiss-type reaction (most commonly, iron), the appearance of hydroxyl radicals after the disappearance of hydrogen peroxide is probably due to the break down of hydrogen peroxide into these more toxic radicals which may then be directly or indirectly responsible for the killing of tumor cells.

The one difficulty about this conclusion is that hydroxyl radicals are known to be very short-lived and thus it is unlikely that they are around long enough in the supernatant to exert a cytotoxic effect on tumor cells when incubated with it. In fact, most investigators have found through the use of methionine and other scavengers that hypochlorous acid, not hydroxyl radical, is the material responsible for the killing of tumor cells in vitro (148, 173). However, these investigators used phorbol myristate acetate (PMA) as the stimulant of phagocytosis. Since this substance has not been shown to prevent the growth of

tumors in our fibrosarcoma model, it may not be appropriate to compare the results obtained when it is used vs. those obtained when P. acnes is used. A shortcoming of the theory that hydroxyl radicals are formed in our model and that they are responsible for the killing of tumor cells is that the metal-catalyzed Haber-Weiss reaction, needed to form these radicals, will only occur if it can outcompete alternative reactions of the hydrogen peroxide or superoxide produced by the cells. Winterbourn found that myeloperoxidase can divert hydrogen peroxide from hydroxyl radical to hypochlorous acid production (178). If this is the case, then release of myeloperoxidase may play a role in inhibiting hydroxyl radical production. However, it was found in our model that mannitol, rather than methionine, had an effect on the cytotoxic ability of the supernatant. Thus, it may be suggested that at least in our model, myeloperoxidase may not be released in great enough quantities to inhibit the production of hydroxyl radicals.

It is clear from the results presented that PMNs stimulated with a protective vaccine produce toxic oxygen-derived radicals capable of killing tumor cells. This would seem to suggest that non-protective vaccines are either not able to induce the production of these radicals in as high amounts as when protective vaccines are used, or that non-protective vaccines are not able to induce the

production of a particular kind of radical. Oxygen consumption experiments were carried out so as to attempt to determine indirectly which of these theories is correct. It was found that when both protective and non-protective vaccines are used, the concentration of oxygen consumed by the PMNs was very similar. In fact, the values obtained using the various vaccine preparations were very close to those obtained by Root et al. who used Staphyloccocus aureus cells in their experiments (128).

If the amount of oxygen consumed by PMNs is the same, regardless of the protective ability of the vaccine, it follows that the difference between protective and non-protective vaccines may not be in the total amount of radicals produced but rather in the specific kinds of radicals produced. In such an event, it could be assumed that stimulation by P. acnes somehow dictates the pathway(s) to be followed within the radical-producing mechanism of PMNs in order to produce the toxic substance responsible for the killing of tumor cells. This may involve the promotion or inhibition of a certain enzymic reaction within the PMNs by a component of the P. acnes cell released during phagocytosis. Root and co-workers found a direct relationship between the amount of oxygen consumed and the concentration of extracellular hydrogen peroxide. However, through work with various metabolic

inhibitors, they found that hydrogen peroxide release is increased when other metabolic pathways of the PMN that utilize hydrogen peroxide, such as the glutathione peroxidase pathway, are inhibited (128). Thus, it is conceivable that a component of P. acnes may somehow block certain hydrogen-peroxide-consuming pathways, resulting in more hydrogen peroxide being released by PMNs without necessarily consuming more oxygen than when non-protective vaccines are used (see Figure 13). In any event, the experiments of Root et al. did not involve P. acnes or any other anti-tumor material as a stimulant of phagocytosis, thus the relationship between hydrogen peroxide produced and oxygen consumed may not be the same as in their model.

There is also the possibility that SOD from P. acnes cells may be released and that it, in turn, acts on the radical-producing mechanism of the PMNs to produce hydrogen peroxide. This is unlikely since all vaccine preparations are heat-killed at 60 C before use in all of the assays, and this would probably inactivate or denature any such enzyme within the microorganism.

We have determined that the mechanism responsible for the killing of tumor cells in our model is the production of oxygen-derived radicals produced during phagocytosis of P. acnes. However, the exact nature of the radical involved is not yet known. Electon Spin Resonance

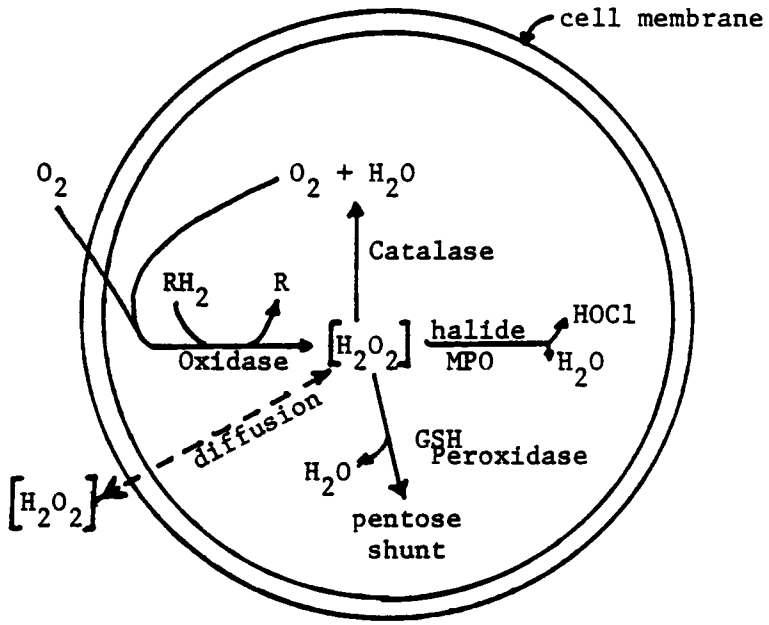

Figure 13: Postulated events involved in H_2O_2 formation, catabolism and release by human PMN's. (From Root, R.K. et al. 1975. J. Clin. Invest. p. 953).

Spectroscopy (EPR) as well as other methods such as ethylene formation to detect hydroxyl radical production could help to determine the role that this material plays in the destruction of tumor cells. In addition, experiments concerned with determining the nature of the component of P. acnes that triggers the mechanism of radical production will be necessary. Knowing that PMNs are the effector cells directly responsible for this, it may now be possible to determine the role that the vaccine plays in causing the production of hydrogen peroxide, based on the above speculations. By concentrating efforts towards the known mechanisms of production of hydrogen peroxide, and the possible interactions between them and the components of P. acnes cells known to play a role in the anti-tumor activity of the vaccine (such as peptidoglycan, or cell wall polysaccharides), it may be possible to unravel the mystery behind the cell component responsible for the destruction of tumor cells in vivo.

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