Immunotherapeutic Alteration of Tumor-Induced Suppression of Interleukin 2 and 3 Production by *Propionibacterium acnes* Vaccination

by

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(ABSTRACT)

Previous reports indicate that anti-tumor activity arising from systemically injected *P. acnes* is macrophage-mediated, whereas anti-tumor activity arising from locally injected *P. acnes* is T cell-mediated. It is possible these *P. acnes*-induced cytotoxic T cells arise via the Interleukin cascade. Therefore, this study investigated the involvement of Interleukin 2 (IL 2) and Interleukin 3 (IL 3), known components of the Interleukin cascade, in local *P. acnes*-mediated anti-tumor action.

A 500 ug dose of heat-killed stationary phase *P. acnes* given simultaneously with 10^4 tumor cells was found to inhibit tumor formation completely, therefore this amount was used as a standard dose throughout the study. Unvaccinated counterparts developed palpable tumors two weeks after tumor cell administration. Lower doses of vaccine protected animals from tumor growth to a lesser degree. A vaccine prepared from logarithmic phase *P. acnes* exerted a moderate anti-tumor effect in some

cases.

IL 2 and IL 3 levels were measured in vitro in normal BALB/c mice (N), tumor-bearing mice (TBH), normal vaccinated mice (N+V), and mice receiving both tumor cell and vaccine injection (T+V). IL 2 and IL 3 production was maintained in both N and N+V host splenocyte cultures throughout the study. In a similar fashion, levels of IL 2 and IL 3 in T+V host splenocyte cultures were comparable to those of N+V hosts. However, TBH splenocyte production of IL 2 and IL 3 began to decline when tumors became palpable, at Day 14 after tumor cell inoculation. By Day 28, TBH IL 2 and IL 3 levels were <15% of normal control levels.

Causes for this suppression of IL 2 and IL 3 production in TBH were examined. From reports of others it appeared that suppression may be mediated through prostaglandin(s). Addition of the prostaglandin inhibitor indomethacin to splenocyte cultures greatly enhanced IL 2 production by N, N+V and T+V splenocytes, but failed to restore IL 2 production in TBH splenocyte cultures to normal levels. Thus, it appeared prostaglandins were not directly responsible for the majority of suppression seen in TBH. In the non-tumor-burdened host, prostaglandin appeared to play a homeostatic role regarding IL 2 production. Indomethacin-treatment had little effect on IL 3 production.

Nylon wool fractionation of N, TBH, N+V and T+V splenocytes suggested a cell removed by nylon wool treatment was largely responsible for the suppression of IL 2 and IL 3 production in TBH. No obvious presence of functional suppressor cells was noted in N, N+V or T+V splenocytes.

From these results, it appeared that P. acnes administration maintains and/or restores IL 2 and IL 3 production, thus favoring the production of CTL. In addition, the suppression of IL 2 and IL 3 production seen in TBH may be due to a nylon wool adherent suppressor cell. A model describing the effect of P. acnes administration on local anti-tumor activity was presented.

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LIST OF ABBREVIATIONS

Con A	Concanavalin A
CTL	Cytotoxic T lymphocyte
FCS	Fetal calf serum
IL 1	Interleukin 1
IL 2	Interleukin 2
IL 3	Interleukin 3
i.v.	Intravenously
i.m.	Intramuscularly
N	Normal
NK	Natural killer
N+ppv	Normal host vaccinated with partially protective vaccine
N+V	Normal vaccinated host
PGE	Prostaglandin(s) E
PGE_2	Prostaglandin E_2
PHA	Phytohemagglutinin
ppv	partially protective stationary phase vaccine
T	Tumor cell recipient
TBH	Tumor-bearing host
Th	Helper T
T+ppv	Host vaccinated with tumor and partially protective vaccine

LIST OF ABBREVIATIONS (continued)

Ts	Suppressor T
T+V	Vaccinated tumor cell recipient

INTRODUCTION

For the past 20 years, it has been known that the bacterium Propionibacterium acnes (often known as Corynebacterium parvum) is a potent immunostimulant (70). It is capable of inhibiting the growth of transplantable tumors by stimulating the host's reticuloendothelial system (69, 190). For this reason, the organism P. acnes has been extensively studied, with much of the work focusing on determining the host cell population which mediates anti-tumor activity. Inhibition of tumor development and growth resulting from systemic injection of P. acnes has been examined by several groups, and workers have cited P. acnes-activated macrophages as the effector cells in nonspecific anti-tumor activity (159, 11, 23, 100, 140). However, studies employing locally injected P. acnes have suggested that a specific immune response to tumor antigens is responsible for anti-tumor action (10). The specific anti-tumor action, resulting from locally injected vaccine, is T cell-dependent (191, 10, 192, 173) and effected by a short-lived T cell (10, 174). The manner in which P. acnes promotes the development of specific cytotoxic T lymphocytes (CTL) is not known, but it is likely these CTL arise via the Interleukin cascade.

In normal animals, CTL against various stimuli are postulated to develop through a sequential series of molecular and cellular

interactions known as the Interleukin cascade (48). Briefly, a lymphokine designated Interleukin 1 (IL 1), a macrophage-derived soluble product, interacts with antigen- or mitogen-primed helper T (Th) cells leading to the elaboration of both Interleukin 2 (IL 2) and Interleukin 3 (IL 3). IL 2 then stimulates CTL precursor proliferation and immune interferon production (45, 127, 165). The role of IL 3 is not clear. Originally, IL 3 was reported to amplify maturation of a pre-T cell population (79, 80, 82, 84) recruiting T cells into the Interleukin cascade; recent work suggests it may also act on a pre-B cell population (142). In any case, the final step in the cascade is the generation of specific CTL from the pre-CTL population (4, 97, 187).

In tumor-bearing hosts, evidence indicates that IL 2 levels gradually decrease as tumor grows (14, 17). This decrease in IL 2 levels is thought to result in the cessation of cytotoxic activity against the tumor, allowing unrestrained tumor growth (130). Work in our laboratory and that of others provided evidence that formation of a tumor-induced suppressor T (Ts) cell is partially responsible for the perturbation and subsequent decline of IL 2 production, possibly resulting in a lack of tumor-specific CTL (17, 130).

Since anti-tumor action arising from locally injected P. acnes is due to tumor-specific CTL, and CTL arise via the Interleukin cascade, it is feasible that locally injected P. acnes mediates anti-tumor action by

preventing tumor-induced Ts cell development and subsequent shutdown of the Interleukin cascade, or in some manner affects IL 2 and/or IL 3 levels. The goal of this study was to determine the effect of locally injected P. acnes on IL 2 and IL 3 production and/or utilization. I hoped that knowledge of how P. acnes vaccine successfully manipulates IL 2 and IL 3 production in such a way to prevent tumor devlopment and/or growth might someday be applicable in a immunotherapeutic treatment regime employing IL 2 and/or IL 3. Additionally, the determination of IL 2 and IL 3 levels in mice protected against tumor by P. acnes would help to either substantiate or refute the importance of these lymphokines in the development of CTL capable of mediating anti-tumor action.

SUMMARY OF EXPERIMENTAL PLAN

To examine the anti-tumor effect of P. acnes vaccination on IL 2 and IL 3 levels in a controlled manner, up to six different treatment groups were employed: Normal control mice ("N") received an injection of RPMI tissue culture medium only; mice destined to become tumor-bearers were injected with 10^4 viable fibrosarcoma cells suspended in a vehicle of RPMI medium, and were designated tumor-bearing hosts ("TBH", or in some contexts "T"); vaccinated normal control mice received 500 ug stationary phase vaccine in RPMI medium and are referred to as normal vaccinated ("N+V") mice; mice which were challenged simultaneously with an

admixture of 10^4 tumor cells and 500 ug stationary phase vaccine in RPMI medium were designated "T+V". In some experiments, two additional groups were introduced. These consisted of mice injected with log phase, partially protective vaccine (ppv). P. acnes stationary phase vaccine preparations possess the greatest anti-tumor activity, while only slight protection is afforded by log phase preparations. Mice injected with log phase partially protective vaccine and/or tumor cells were designated "T+ppv" and "N+ppv", respectively.

For a 28-day period after injection of vaccine and/or tumor cells, pooled splenocytes from each of the six treatment groups were tested in a number of ways. Parameters measured included ability to proliferate in response to mitogen, IL 2 and IL 3 production, effect of the prostaglandin inhibitor indomethacin on IL 2 and IL 3 production, and the presence or absence of mildly nylon wool-adherent suppressor cells. It was hoped these measurements would begin to define the role of the lymphokines IL 2 and IL 3 in P. acnes-mediated specific anti-tumor action.

LITERATURE REVIEW

Historically, pestilence was among the leading causes of death in the old world. For centuries, diseases such as bubonic plague, typhus and smallpox decimated entire countries and often altered the course of history (194). In less than a century, scientists have eradicated these pandemics of phenomenal magnitude by determining the etiological agents responsible and eradicating them through vaccination programs, improved hygienic standards and education of the populace. However, as Nature would have it, a much more insidious scourge has emerged. Cancer of some variety is a leading cause of death in industrialized nations. Elucidating the causes and providing a cure is probably the most complex and tedious endeavor ever undertaken by the scientific community. It is certainly one of the longest endeavors pursued. Roughly 175 years ago the first plan was proposed for determining the cause(s) of cancer (19). This plan proposed to study the incidence of cancer in relation to factors such as sex, marital status, geographic domicile, occupation and so forth. Since then, voluminous amounts of information pertaining to the origins, varieties, epidemiology and treatment of cancer have been catalogued. In addition, the field of immunology has greatly expanded and new fields such as tumor immunology have arisen as a direct result of cancer research.

Paul Ehrlich advanced the field of tumor immunology in 1909 when he proposed the concept of immune surveillance against neoplasia (36), which was later expanded by others. Basically, as now formulated, this theory proposes that cell-mediated immunity in a normal host is carried out by thymus-derived (T) lymphocytes. The function of T lymphocytes or T cells is deemed to be the recognition and destruction of newly appearing tumor cells in situ. A failure in this system is thought to result in unrestrained tumor growth (134).

The modern version of Ehrlich's immune surveillance theory proposes that immunocompetent cells circulate through the body searching for foreign or altered-self antigens to destroy. In addition, this theory proposes that cancerous cells arise continously, but are promptly eradicated when their neoantigens are recognized. This view asserts cancer formation is a rarity, and occurs only occasionally when aberrant cells are weakly antigenic or otherwise unable to stimulate the protective immune response (63).

The central role of T cells in the immune system has remained undisputed since Ehrlich's day. Distinct interacting sets of T cells are believed to regulate both humoral and cellular immune responses. These interactions include interactions between different sets of T lymphocytes, between T and B lymphocytes, and T cell-macrophage interactions (8). It is thought T cells are important effector cells in

cell-mediated immunity, and exert cytotoxic activity against foreign antigens, including tumor-specific antigens (134).

THE INTERLEUKIN CASCADE

Recently, the series of interactions resulting in the differentiation and clonal expansion of specific T effector cells, also known as CTL, in response to alloantigen has been partially described (28, 48). It has been proposed that CTL development occurs through a sequential cascade of cellular and molecular interactions involving several cell types and a series of soluble growth factors or activating agents called lymphokines (48, 127, 165, 185). The lymphokines involved in this cascade include IL 1, IL 2 and IL 3. IL 1 is a variably glycosylated (165, 185), 15,000-30,000 MW factor produced by macrophages and formerly known by a variety of names including lymphocyte activating factor (LAF), B cell activating factor (BAF), and mitogenic protein (1, 165, 185). All these activities were deemed to represent one moiety at the Second International Lymphokine Workshop and the name Interleukin 1 was suggested (1).

The second lymphokine of the cascade, IL 2, is a glycoprotein. The MW of cloned human IL 2 is reported to be 15,240 (169). Murine IL 2 has been reported to have a MW of 30,000-35,000 daltons (179, 184), but other workers feel the true MW is 16,000-20,000 (46), and that

glycosylation or aggregation accounts for discrepancies in MW reported values (46, 165, 178, 185). IL 2 was formerly known as T cell growth factor and was also recently renamed at the Second International Lymphokine Workshop (1); Murine IL 3 is also presumably a variably glycosylated (83, 84, 55) glycoprotein with a MW of 28,000-41,000 (83). No MW value for human IL 3 has been reported (55).

Several groups have begun to elucidate the role of the lymphokines IL 1, IL 2, and IL 3 in promoting T cell activation, proliferation, and differentiation. The composite picture arising from these studies has been termed the Interleukin cascade or the lymphokine cascade (45). The Interleukin cascade consists of a series of cellular and molecular interactions that are thought to ultimately result in the differentiation of CTL (182). Most studies contributing to the visualization of the Interleukin cascade model have been _in vitro_ studies, and there is a definite need for _in vivo_ corollaries of these experiments to yield _in vivo_ confirmation of many portions of this model.

Critical to the understanding of the Interleukin cascade of lymphokines is the second signal hypothesis (4, 161, 182, 185). The crux of this hypothesis is that T cells require two signals in order to be activated. Stimulation of naive T cells by a lectin or alloantigen alone is not sufficient stimulus for the activation of T cells and will

not result in T cell proliferation. The ligand stimulus must be combined with a second stimulus such as a lymphokine. This two-step mechanism seems to be important in activating many types of T cells, including Th cells and CTL (94, 161).

Dissection of the Interleukin cascade in detail reveals several interdependent steps in the circuit, with additional regulatory steps superimposed on the circuit. The first step in the generation of CTL via the lymphokine or Interleukin cascade involves the macrophage. The Ia+ subpopulation of macrophages bind, process, and present antigen to T cells in a genetically restricted manner (155). Macrophages also elaborate IL 1 (110, 121, 162). The target cell for IL 1 is a T cell population, and work is currently being done to isolate a T cell receptor for IL 1 (133). IL 1 is thought to have a stimulatory effect on this T cell population by encouraging the production of IL 2 by ligand-stimulated T cells (61, 109). Evidence suggests that antigen-specific T cell proliferation is dependent on two macrophage functions, one being the generation of the IL 1 signal, the other being presentation of the antigen to the T cell (133).

The T cell population believed to receive the amplifying signal of IL 1 expresses the cell surface phenotype Lyt 1+23- and is designated a Th cell, and it is this Th cell population which is responsible for IL 2 production (106, 145, 179). The precise mechanism by which IL 1

amplifies the production of IL 2 is not known. Interleukin 2 synthesis is not stimulated by IL 1 unless another signal such as a specific antigen or T cell mitogens such as PHA or Con A are present (133). It has been suggested that IL 1 converts Th cells to a state where ligand triggering causes the specific release of IL 2 (133, 185). This step may involve the induction of cell surface receptors for antigen on the T cell (133).

Several groups have investigated IL 2 production by Th cells and their findings are summarized immediately below. Not all Th cells are involved in IL 2 production; at least two subsets are reported to exist. One subset is involved in assisting the macrophage in IL 1 production, while the other subset actually produces IL 2 (91, 114). Evidence indicates that DNA synthesis is not necessary for IL 2 production (179). Production of IL 2 takes place in the late G1 phase of the cell cycle. Once produced, IL 2 is thought to act on another T cell population (46).

Studies indicate that IL 2 is most likely the essential substance responsible for a majority of the mitogenic action present in lymphocyte culture supernatants (161, 163). Researchers have proposed a model for IL 2-induced T cell proliferation. This model suggests pre-effector T cells are activated by exposure to a ligand stimulus such as alloantigen or lectin, identical to the ligand triggering the release of IL 2 by Th cells. In response, pre-CTL enter late G1 phase and generate receptors

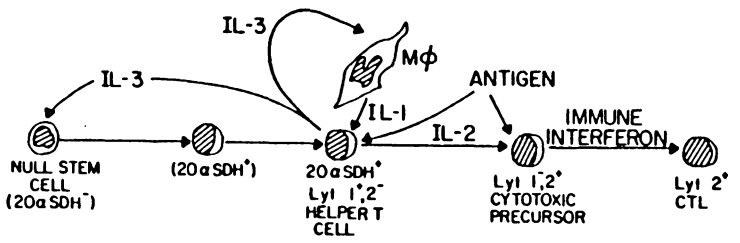
for IL 2 (161, 165, 185). Adsorption experiments suggest sensitivity to IL 2 is mediated in part by the appearance of membrane receptors for IL 2 (63). It is thought that the second signal to proliferate is delivered when IL 2 binds to the receptor and undergoes receptor-mediated endocytosis (45). Interaction of IL 2 with its receptor drives the T cells to division, and continuous proliferation can be maintained if sufficient IL 2 is available (154, 166, 185). The target cell population driven to proliferation by IL 2 is pre-CTL (25, 107, 130, 193). Several groups have reported IL 2 regulates CTL generation (3, 4, 97, 111, 112, 115). Specifically, the cell type responsive to IL 2 has been demonstrated to be a Lyt 2+ population (68). Furthermore, evidence indicating an IL 2 receptor is present on CTL has been presented (2). In addition to inducing CTL, IL 2 has been shown to augment natural killer (NK) cell activity (113).

It is believed that IL 2 in conjunction with immune interferon, also known as gamma interferon, acts on a pre-CTL population to cause the ultimate generation of mature CTL (25, 46, 107, 126). The exact mode of interaction is unclear, but there is evidence IL 2 regulates gamma interferon production (45, 46, 93, 96, 171). Production of gamma interferon is thought to be regulated by a triad of interactions between IL 2-secreting Lyt 1+2- Th cells, Lyt 1+2+ Ts cells and Lyt 1-2+ gamma interferon-producing cells (88). The Lyt 1-2+ cell has been identified as the principal immune interferon producer (65, 181) ; however one

group later reported that some cloned cultures derived from single cells of the Lyt 1+2- phenotype also produced interferon (66). There is evidence that gamma interferon is necessary for the expression of IL 2 receptors on T cells (45, 87). In summary, it is well-documented that IL 2 regulates immune interferon production and that these molecules interact in some manner to engender mature CTL production (9, 25, 43, 48, 93, 101, 107, 171).

A further level of complexity in regulation of the Interleukin cascade is added by IL 3. Formerly, this molecule was believed to be released by a Th cell and act early on in the cascade to trigger the release of IL 1 from macrophages (179) and to promote the maturation of pre-T cells into IL 2-producing Th cells. In addition, IL 3 has been shown to augment CTL activity (113). However, recent evidence was presented indicating IL 3 supports the growth of pre-B cells (142). It is likely this molecule is multifunctional (55); however at present the precise function(s) of IL 3 remains unclear.

The proposed development of CTL via the Interleukin cascade is summarized in the following schematic:



In the TBH, hyporesponsiveness of the immune system to various immunologic stimuli is manifested as a decline in functional T lymphocytes (37, 98). This tumor-induced immunosuppression is the subject of intense study. Several mechanisms have been proposed to explain immune suppression, including: i) the triggering of suppressor cells in response to tumor antigens (63); ii) antibody-mediated interference of the protective immune response (88); and iii) immunogenetic factors such as major histocompatibility restriction or lack of determinants on tumor cells may render the host unable to respond (63). However, patients in late stages of tumor progression have been repeatedly shown to have a decreased immune response to a variety of nonspecific antigens, whereas in early stages of tumor growth normal immune reactivity is observed. There are apparently many interrelated causes of tumor-induced immune suppression. In certain tumor systems, tumor cells themselves have been found to elaborate substances capable of depressing the immune response. These immunosuppressive soluble products include soluble antigens, prostaglandin-like factors, alpha-fetoprotein and viral particles (63). In addition, a tumor cell-derived factor inhibiting IL 2 production has been reported (74). Suppression of the immune system by tumor cells has been reviewed by Giogi and Warner (63).

In addition to tumor cells themselves, other types of cells have been implicated in suppression. Suppressor T cells (6, 38, 58) as well

as suppressor macrophages (167) have been cited as the cause of immunodepression. We have reported dualistic inhibition of the immune response by both suppressor macrophages and Ts cells (38). The role of suppressor cells of both leukocytic and monocytic lineage in immunodeficiency associated with cancer has been recently reviewed (6).

At least one human study has indicated a factor secreted by tumor cells induces suppressor cells. It remains to be established whether this is a general mechanism for suppressor cell induction (95). Soluble suppressive factors secreted by suppressor cells are well documented (105, 146). Suppressor T cell inhibition of cytotoxic activity has been documented in several systems including murine fibrosarcoma, Lewis lung carcinoma, teratocarcinoma and plasmacytoma (summarized in reference 6). Studies in our laboratory (17) and that of others (67) indicate that Ts cells are capable of inhibiting IL 2 production. In addition, investigations have indicated the that cessation of cytotoxic activity against tumor cells may be due to the absence or perturbation of IL 2, and consequent CTL production (67). Thus, it appears that at least in some systems, the decline of IL 2 production and the presence of suppressor cells may be largely responsible for tumor growth and immunosuppression.

The immunosuppression associated with the growth of methylcholanthrene-induced fibrosarcomas, such as the one used in this

study, has been well-documented (25, 38, 47), and can be divided into two phases (57). Initially, suppressor macrophages and T cells differentiate, followed by the production of suppressor factors (38, 52, 58, 147), which may be nonspecific (116) or specific (64, 144). It has been proposed that macrophages may inhibit normal T cell proliferation via prostaglandin(s) E (PGE) (57). Other work indicates macrophage-derived PGE may stimulate Ts cells (54), or may facilitate the induction of Ts interaction molecules (51).

The macrophage-derived arachidonic acid breakdown product prostaglandin E2 (PGE2) has been shown to inhibit the production of IL 2 (73, 151, 188, 170, 183). PGE has also been implicated in the induction of Ts cells (21, 22). Furthermore, direct evidence of the strong suppressive effect of PGE on CTL activity has been presented; the authors felt inhibition of the cytotoxic response was partially due to obstruction of IL 2 production by Th cells (188). Cumulatively, these data suggest at least three phenomena are involved in tumor growth and immune suppression: i) PGE-mediated inhibition of IL 2 production; ii) PGE-induced suppressor cell formation; and iii) tumor-induced Ts cell-mediated inhibition of IL 2 production.

IMMUNOTHERAPY

Knowledge of parameters involved in tumor-induced immunosuppression

has led researchers to attempt various types of immunotherapy in hopes of preventing or reversing tumor-induced immunosuppression. In this discussion, the subject of immunotherapy will be divided into two sections: i) immunotherapy utilizing material of either syngeneic or allogeneic mammalian origin, such as lymphocyte transfer and/or lymphokine administration and ii) immunotherapy employing immunostimulants or immunopotentiators totally foreign to the host, in particular, bacterial immunostimulators.

Lymphocyte Transfer and Lymphokine Administration

The possibility of employing lymphokines and/or lymphocyte transfer as an immunotherapeutic treatment regime for augmenting the immune response in the TBH has often been suggested (49, 75, 153, 186). IL 2 has been found to be capable of sustaining cytotoxic cell lines *in vitro* (4, 59, 184), inducing the generation of CTL from athymic nude mice (60), and amplifying CTL production in normal mice (26, 141). Thus, it is possible IL 2 may be used to augment specific CTL production in the compromised host and therefore is a likely candidate for use in lymphokine-mediated tumor therapy.

Studies employing *in vivo* administration of IL 2 report several positive effects of IL 2 administration, including enhanced CTL activity, enhanced NK cell activity, reduced tumor growth and enhanced

survival (26, 141). There are problems with this form of treatment, however. The clearance of i.v. administered IL 2 is rapid (34, 136, 141), and some groups have detected serum inhibitors of IL 2 (119, 180, 181).

Another type of experimental immunotherapy which has recently experienced a resurgence of interest is lymphocyte transfer (49, 50). This therapy consists of the infusion of syngeneic or allogeneic viable cells cytotoxic for tumor antigens into the compromised host. Lymphocyte transfer has been practiced with limited success for over a decade but recent advances have made it much more feasible (50). Increased knowledge of T cell subsets and their interactions plus the discovery of IL 2 and the development of IL 2-dependent long term T cell culture techniques are two such advances. Both lymphocyte transfer experiments employing CTL activated in vitro by IL 2 (153), and those employing CTL supplemented with in vivo administration of IL 2 have reported augmentation of anti-tumor action (20, 124). At least one reviewer speculates that lymphocyte therapy may someday have therapeutic value in treating human malignant disease (50).

Bacterial Immunostimulants

A second area of immunotherapy which was in vogue in the 1970's is the use of immunostimulants such as microbial augmenting agents or

chemical substances. My research has examined the effect of one such bacterial stimulant, P. acnes, on IL 2 and IL 3 levels. Immunopotentiators or immunostimulants are biologically active substances that when administered to a particular host are capable of augmenting the host's specific or nonspecific immune response. Much research has been done on immunopotentiating substances in search of clinical applicability for these substances (7). The impetus for this research has been two-fold. The major goal has been to develop immunotherapeutic methods in which immunostimulators may be used to augment or restore the immunologic response of a TBH. Many immunostimulators are known to be capable of stimulating the reticuloendothelial system to a degree facilitating the regression of malignant growths. A second goal has been to develop methods to use immunostimulators to enhance the ability of an immunologically depressed host to respond to the antigens of infectious agents, such as bacteria and viruses, thereby preventing opportunistic infections common in the immunocompromised host (75, 95).

In addition to immunotherapy, much recent work on immunostimulants has focused on elucidating the mechanism of immunostimulant action, thereby gaining a detailed view of precisely how the immunostimulant prevents tumor formation, inhibits tumor growth, or mediates tumor regression (5). Workers hope that by unravelling this riddle, light will be shed on the process by which tumor-mediated suppression of the

immune system occurs. The goal of many researchers is to gain sufficient understanding of tumor-induced immunosuppression to exogenously reverse and control the neoplastic process (5).

Knowledge of the existence of bacterial immunostimulants is not new. It was recognized well before the turn of the century that bacterial infections, especially streptococci and staphylococci, in cancer victims often correlated with the subsequent spontaneous regression of cancerous tumors (138). Deliberate immunotherapy with bacterial stimulants was practiced with some success over 90 years ago (24). Interest in immunostimulants waned because of various technical problems, such as varying potency of batches, probably due to the uncertain composition of preparations, which resulted in unpredictable cure rates or often total failure (172).

An incredible number of substances are capable of immunostimulation. Many chemical compounds have been found to enhance cell-mediated immunity and thereby induce an anti-tumor effect. Inorganic substances such as alum, beryllium, and silica, and organic substances such as fatty acids, lipids, and nucleic acids have been found to have immunostimulatory capabilities under certain conditions (7, 75, 95, 152). Synthetic polymers consisting of repeating units of polyinosinic acid and polycytidylic acid or polyadenylic acid and polyuridylic acid have also been shown to possess immunomodulatory capabilities (131). A

seemingly inexhaustible array of microorganisms are known to mediate anti-tumor effects by augmenting the host response. Many of the mycobacteria such as Mycobacterium bovis (Bacillus Calmette-Guerin, BCG), M. tuberculosis, M. kansasii, and M. phlei are immunostimulatory (7, 108). Propionibacterium acnes, Bordetella pertussis, Brucella abortus, Haemophilus influenzae, Nippostongylus brasiliensis, Micrococcus lysodeikticus, Lactobacillus casei (7, 13), as well as several species of staphlococci (12), and fungi such as Peziza vesiculosa (168) have been shown experimentally to mediate an anti-tumor effect, either by causing actual tumor regression or by suppression of initial tumor development. In addition, subcellular fractions of bacteria are often sufficient to produce anti-tumor activity. Serratia marcescens polyribosomes (176), culture filtrates of Streptomyces olivoreticuli, zymosan, glucans, many bacterial endotoxins, polysaccharides from both Gram positive and Gram negative organisms, as well as cell wall products are capable of immunostimulation (7, 75, 122).

Modern immunotherapy is also plagued by a host of problems (152). Several factors affect the immunostimulatory action of bacterial or synthetic immunopotentiators on tumor growth. The route of administration, frequency, and dose of immunopotentiator given are critical. Often an immunopotentiator will suppress tumor growth when given by one route but promote tumor growth when given by another route

(7). For example, systemic administration of B. pertussis has been found to stimulate tumor growth, whereas other routes of administration inhibit tumor growth (7). In many situations direct contact between tumor cells and immunostimulant results in higher rates of tumor regression or suppression of growth. Generally, direct administration of vaccine into the tumor gives the highest cure rates. Systemic therapy, consisting of intravenous administration of immunostimulant sometimes results in complete regression of the tumor, but this is rare. Usually systemic administration simply suppresses further tumor growth (7).

The method of immunostimulant preparation for injection can effect the resultant anti-tumor activity or lack thereof. For example, ten times more heat-killed L. monocytogenes than viable L. monocytogenes is necessary to produce anti-tumor activity (7). Tumor size or number of tumor cells transferred is a critical consideration. It has been shown experimentally that if the amount of tumor injected exceeds a certain number of viable cells, the tumor cure rates begin dropping drastically, regardless of the amount of immunostimulatory agent administered. The threshold level of tumor cells destroyed is dependent on the type of cancer and also on the efficacy of vaccine. Regardless of tumor type, in an experimental situation, usually no more than a million cells can be overcome by immunostimulatory agents (7).

Tumor load becomes especially significant when considering immunotherapy of spontaneously arising human cancer. In this situation, it is very important to initiate therapy while tumor size is quite small. In practice, this is usually not possible. For this reason, in clinical use, immunostimulants are not usually used as a primary means of treatment but instead are used to treat microscopic metastases which may be found throughout the body following removal of the primary tumor mass (75, 128). In clinical immunotherapy other factors must also be considered before embarking on clinical trials. Genetic polymorphism in the human population may play a role in the host response. Side effects such as chronic toxicity of immunostimulant, and potential pathogenicity of a live vaccine must be considered as well as the risk of exhaustion or overstimulation of the already compromised immune system (152).

Two often employed immunostimulants of bacterial origin are BCG and C. parvum, more accurately known as P. acnes (75). Both are frequently used as both a research tool to help determine the mechanisms of anti-tumor action, have also been used in clinical therapy. This review will focus primarily on P. acnes, since it is the immunostimulant employed in my studies. Generalizations about immunostimulators, made earlier in this discussion, concerning the dependence of anti-tumor action on route of immunostimulant administration, size of tumor, frequency of immunostimulant administration, and mode of vaccine preparation also apply to P. acnes.

Interest in P. acnes was greatly boosted in 1963 when Halpern and colleagues demonstrated this organism possessed great ability to stimulate the reticuloendothelial system (70). Shortly thereafter, Halpern and others demonstrated that P. acnes (then called C. parvum) was capable of inhibiting growth of transplantable tumors in mice (69, 190). Both clinical trials employing P. acnes and studies designed to determine the mechanism of anti-tumor action of the vaccine mushroomed from then on (128).

NATURE OF THE P. ACNES VACCINE

P. acnes is a non-sporulating, immotile Gram (+) slender pleomorphic rod. It is a member of the anaerobic coryneform group. Originally it was placed in the Corynebacterium group because of superficial resemblance to this group, but later biochemical data and DNA homology studies indicated this organism belonged in the Propionibacterium group (30). However, in a great majority of the literature the archaic name of Corynebacterium parvum persists and continues to be published.

The component of the P. acnes organism responsible for the anti-tumor activity is not known and is presently the subject of study (32). Evidence suggests the active component is carbohydrate in nature and associated with the cell wall (148, 149). Broken cells lack immunostimulatory activity, as do exponentially growing cells. The

reticulostimulatory ability has been shown to develop between the transition from exponential to stationary growth phase (148). This has been demonstrated by experiments employing antibiotics with differing modes of action. The only major activity of the bacterial cell not inhibited by chloramphenicol treatment is cell wall synthesis. Choramphenicol treatment of log phase cells does not prevent cell wall synthesis, and stimulatory ability develops. Penicillin treatment, however inhibits cell wall synthesis and results in lack of development of immunostimulatory properties, indicating cell wall synthesis is involved and necessary for generation of activity (148).

It is not known how the component responsible for anti-tumor activity stimulates the reticuloendothelial system. Several researchers have suggested that immunostimulatory bacteria and tumor cells share antigens, and that the host response to tumor is enhanced by cross reaction between tumor antigens and bacterial antigens (76). Others have suggested some portion of the bacterium may become adsorbed onto the surface of the tumor cells (128). In vitro evidence has shown P. acnes readily adsorb to erythrocytes (29).

EFFECT OF P. ACNES ADMINISTRATION ON THE IMMUNE SYSTEM

Since P. acnes can have either immunoenhancing effects or immunosuppressive effects, it is probably more correct to think of P.

acnes as an immunomodulating agent rather than a strict immunostimulatory agent (128). When summarizing studies examining the mechanism of action of P. acnes, it is probably best to divide the studies into two groups -- those employing systemic P. acnes treatment and those employing locally injected P. acnes. It appears there is a difference in the mechanism of action depending on whether vaccine is locally or systemically injected (128).

SYSTEMIC ADMINISTRATION OF P. ACNES VACCINE

P. acnes when systemically administered to normal animals causes splenomegaly, manifested as changes in the red and white pulp, including cellular proliferation, influx of macrophages, and an increase in the general hemopoietic activity of the spleen. P. acnes administration increases the number of macrophage precursors and also the numbers of macrophages present. Phagocytic capabilities of these P. acnes-activated macrophages is greatly enhanced (128). Hepatomegaly is also a prevalent response to P. acnes administration and the ability of P. acnes to induce hepatosplenomegaly has often been used to screen strains of P. acnes for anti-tumor activity. However recent evidence suggests hepatosplenomegaly is not necessarily a good index for judging anti-tumor activity. Treatments of vaccine preparations which abolish hepatosplenomegaly induction often still retain anti-tumor activity, suggesting the activities are distinct and caused by two different

moieties (30). The vaccine used in this study effects little hepatosplenomegaly in BALB/c mice, although hepatosplenomegaly is often seen in CBA mice (30).

Much of the work done employing systemic or intraperitoneal administration points to activated macrophages as being the effector cells responsible for anti-tumor activity (11, 100, 140, 159, 172). *In vitro* evidence indicates activated macrophages are cytotoxic to tumor cells (135). It has been often suggested that *P. acnes*-activated macrophages are the effector cell in *P. acnes*-mediated nonspecific anti-tumor activity. The manner in which *P. acnes*-activated macrophages become activated is not known, however a unique cell surface antigen is expressed on these macrophages (92). *P. acnes* organisms persist in macrophages for considerable periods of time, resisting degradation (150). *In vitro* evidence suggests there is a T cell-dependent step in activation (23, 156). However, *in vivo* evidence suggests activation can occur in the presence or absence of T cells since macrophages can be activated to the same extent in T cell-deprived and normal mice (11).

The manner in which activated macrophages exert their tumoricidal effect is not completely understood, but direct contact is thought to be necessary (128). Tumoricidal activity of macrophages have been shown to increase in the presence of phorbol myristate acetate or if target cells are sensitized with alloantiserum. Hydrogen peroxide production is also

increased by phorbol myristate acetate treatment and/or P. acnes treatment and it has been suggested that P. acnes-activated macrophages kill through toxic oxygen intermediates (137).

Systemic P. acnes injection generally inhibits T cell-mediated immune responses (128) although there is one report in which systemic P. acnes enhanced T cell-mediated anti-tumor activity in fibrosarcoma-bearing mice (125). Systemic P. acnes treatment results in decreased T cell-mediated responses (99, 117, 156, 157, 172). Suppressor macrophages and Ts cells are thought to be responsible for the inhibition of T cell-mediated immune responses in systemically injected animals (77, 99, 156); specifically this inhibition affects the development of effective CTL (99). Suppression mediated by suppressor macrophages has been shown to occur by the liberation of a soluble factor (118). Macrophages activated by P. acnes produce increased amounts of PGE; this may be one mechanism by which suppressor cells are induced (56). In addition, P. acnes-activated macrophages are capable of inhibiting the production of migration inhibition factor (178), and T cell protein synthesis (177).

LOCAL ADMINISTRATION OF P. ACNES VACCINE

In contrast to systemically administered P. acnes, which results in macrophage-mediated anti-tumor action, P. acnes anti-tumor activity

resulting from local injection is thought to be T cell-mediated. *In vivo* and *in vitro* evidence supports the idea that the anti-tumor activity of locally injected *P. acnes* is due to a specific cell-mediated mechanism. Local injection of *P. acnes* inhibits tumor cell growth in normal mice but not in T cell-deprived mice, indicating T cells are involved in the lack of tumor formation (160, 191, 192). Adoptive transfer studies show anti-Thy 1.2 serum can completely abrogate the specific immunity which can be transferred from immune animals to normal recipients, indicating the T cell is at least one important component in transferring protection (35, 174). Evidence has been presented for a short-lived CTL possessing anti-tumor activity which operates in the local destruction of tumor cells following intralesional injection of *P. acnes* (173). Studies employing antisera against various cell markers indicate CTL are responsible for the specific cell-mediated immunity (129, 175). Others have also suggested local *P. acnes* therapy is T cell-dependent (158, 191). Thus, it appears that in the case of locally injected *P. acnes*, the production of CTL effective against tumor cells may be the primary method of anti-tumor action.

As mentioned above, several studies implicate CTL as the final effectors of anti-tumor action. This implies the Interleukin cascade is involved in local *P. acnes* anti-tumor activity, since CTL arise via the Interleukin cascade. However little is known about the effect of *P. acnes* on the events leading to the development of functional specific

CTL. Studies to delineate the effect of P. acnes on soluble factors of the Interleukin cascade are rare. No studies examining the effect of P. acnes on IL 2 or IL 3 levels have been reported, although there are some reports describing the bacterium's effect on mediators such as gamma interferon (76, 103, 139) and IL 1 (189).

In summary, the literature suggests cancer-induced hyporeactivity may be due at least in part to tumor-induced perturbations in lymphokine levels, resulting in the shutdown of the Interleukin cascade and failure of CTL development. In addition, it appears P. acnes-mediated anti-tumor action in some instances is T cell-mediated and therefore may involve the Interleukin cascade. Therefore, studies examining the effect of P. acnes on lymphokine levels appear relevant and would yield a more detailed understanding of the relationship between the Interleukin cascade and P. acnes-mediated anti-tumor action. Knowledge of how P. acnes successfully manipulates the immune response may someday have therapeutic value and allow the external modulation of the immune response in compromised patients.

MATERIALS AND METHODS

MICE

Six to eight week old male BALB/c mice weighing 13-16 g were obtained from Dominion Labs (Dublin, VA). Mice were shipped in a group container, and upon receipt, were housed 4-6 mice per cage. Tap water and Purina Mouse Chow (Dominion Labs) was supplied *ad libidum*. Wood-chip bedding was changed once a week. Cages containing mice were kept in a vivarium, with an alternating light cycle consisting of 12 hr of illumination followed by 12 hr of darkness. A constant temperature of 20 C and unidirectional filtered air flow was maintained.

VACCINE

Lyophilized vaccine was obtained from Dr. Cecil Cummins at the VPI&SU Anaerobe Laboratory. The vaccine was prepared in his laboratory using anaerobic culture techniques described in the VPI&SU Anaerobe Laboratory Manual. For specific method of vaccine preparation see reference 31. The "*C. parvum*" vaccine used in all studies was *Propionibacterium acnes* type I strain 0009 from the VPI&SU Anaerobe Laboratory Culture Collection.

Protective Vaccine

Early stationary growth phase cells (48 hr growth at 35-36 C) were used to prepare a vaccine protective against tumor formation. The protective vaccine consisted of whole cells which were heat-killed, washed, filtered through cotton, re-washed and lyophilized to yield the the protective vaccine (31). This vaccine is subsequently referred to as stationary phase or protective vaccine.

Partially-protective Vaccine

Partially-protective vaccine was prepared in an identical manner, with the exception that early exponential phase cells (12-14 hr growth) were used. This vaccine is referred to as partially protective vaccine or early log phase vaccine.

TUMOR

A transplantable methylcholanthrene-induced fibrosarcoma was used in all studies. This tumor has been passaged in our laboratory for several years by the method of Smith and Konda (164). The tumor is routinely transplanted every two weeks into the left hind flank of normal BALB/c mice via intramuscular injection of a single cell suspension of 5×10^5

- 1 x 10^6 viable cells prepared from 2-week old excised tumors.

MEDIA

The medium used in all procedures, including routine tumor transfer, tumor injection, supernatant preparation, mitogen-induced proliferation assays, and assaying for IL 2 and IL 3, was RPMI 1640 tissue culture medium, and was prepared as described elsewhere (40). Specifically, this medium consisted of powdered RPMI 1640 with 2 mM glutamine (Flow Laboratories, McClean, VA), 25 mM HEPES buffer (No. H-3375, Sigma Chemical Co., St. Louis, MO), 2 g/l $NaHCO_3$ (Sigma), $4x10^{-5}$ M 2-mercaptoethanol (Sigma), and 50 mg/l Gentamycin (Gibco, Grand Island, NY). Depending on the procedure in which the medium was used, between 0-10% fetal calf serum (FCS, Flow Laboratories) was also added.

VACCINATION PROCEDURE

A single cell suspension of tumor cells was prepared by passing excised tumors through a 50-gauge wire mesh. After three washes in RPMI medium, tumor cell viability was determined by Trypan Blue dye (Gibco) exclusion, and viable cells were enumerated by direct counting in a hemocytometer (American Optical, Buffalo, NY). Tumor cells were brought to a concentration of 10^5 cells per ml. Lyophilized vaccine was weighed out using a Cahn Electrobalance (Cahn Instrument Co., Cerritos, CA) and

brought to a final concentration of 5 mg/ml with either RPMI medium or RPMI medium containing tumor cells.

Mice were confined in a "Mouse-vise" rodent restraining apparatus (Fisher Scientific, Raleigh, NC) and injected intramuscularly in the left hind flank using an insulin syringe (Becton-Dickenson, Rochelle Park, NJ) and needle. All mice received an injection volume of 0.1 ml containing tumor and/or vaccine, or medium alone. Larger injection volumes were not well tolerated, as they tended to distend the tissue, and the injected material seeped out of the site immediately following injection. Normal mice (N) received a sham inoculation of 0.1 ml RPMI medium alone. Mice destined to become TBH received a 0.1 ml injection of RPMI medium containing 10^4 viable tumor cells. A third group of normal mice received 500 ug injections of vaccine suspended in RPMI medium. These mice were designated normal vaccinated (N+V) mice. The last group of mice received a 0.1 ml injection containing an admixture of 500 ug vaccine and 10^4 tumor cells. These mice were designated tumor-bearing vaccinated (T+V) mice. One to four mice were then housed per cage until sacrificed.

TUMOR MEASUREMENT

Tumor progression or lack thereof was followed by periodic measurement of hind leg diameter. Two planes of the injected leg were

measured using Manostat calipers (Fisher Scientific). These measurements were then averaged. This procedure was repeated for the uninjected leg. The average leg diameter value from the uninjected leg was subtracted from the average leg diameter of the injected leg. The difference was said to be tumor diameter.

SACRIFICE AND SPLENECTOMY

Three to five mice from each group (N, T, N+V, T+V, N+ppv, T+ppv) were chosen at random and killed by cervical disclocation. Each mouse was then wetted with 95% ethanol. Using sterile scissors and forceps, an opening was made to permit the removal of the spleen. Removed spleens from each treatment group were quickly placed in a beaker containing RPMI medium and were immediately further processed.

PREPARATION OF IL 2 AND IL 3 CONTAINING SUPERNATANTS

Concanavalin A-stimulated cultures

At appropriate intervals after tumor cell and/or vaccine inoculation, three mice per group (N, TBH, N+V, T+V, N+ppv, T+ppv) were sacrificed and splenectomized, as previously described (38). Spleens were pooled and a single cell suspension was prepared by forcing the spleens through a 50-gauge wire mesh. Erythrocytes were lysed by

briefly suspending cells in 0.85% NH_4Cl (Sigma). Cells were then washed 3 times in RPMI medium, and then enumerated using a Coulter Counter (Coulter Electronics, Hialeah, FL). Cells were suspended at 10^7 cells/ml in RPMI medium supplemented with 5% FCS and 2.0 ml of this suspension was then aliquoted into culture wells (24 well plates, Flow Laboratories). Two micrograms of the plant lectin Concanavalin A (Con A, Difco Laboratories, Detroit, MI) were added per 10^7 cells. Cultures were incubated 24 hr at 37 C in a 5% CO_2 humidified incubator.

Concanavalin A plus indomethacin-treated cultures

Some cultures were treated with indomethacin (Sigma) in addition to Con A. Indomethacin was dissolved in absolute alcohol, filter sterilized, diluted in RPMI medium, and added to cultures in addition to Con A at time of preparation. Cultures treated with both indomethacin and Con A contained a final concentration of 1×10^{-7} M indomethacin. Cultures treated with indomethacin only were generally not prepared, since preliminary studies showed these cultures, not stimulated by mitogen, produced minimal amounts of IL 2 and IL 3, equivalent to background levels.

Nylon Wool Fractionation of Splenocytes

In some instances, splenocytes were fractionated into subpopulations

via the nylon wool fractionation technique of Julius et al. (89). Nylon wool (Fenwal Laboratories, Deerfield, IL) was incubated four days at 37 C to leach out any toxic materials. During this period the wool was washed several times with double distilled water, and then dried at 75 C for 48 hr. Syringe barrels were then packed with 1.2 g of detoxified nylon wool, autoclaved, and used as a column. Before applying cells to the column, the column was washed three times with RPMI medium, and then pre-incubated 45 min with RPMI medium containing 5% FCS.

For each group (N, TBH, N+V, T+V) a single cell suspension of splenocytes was prepared as described in "Preparation of IL 2 and IL 3 Containing Supernatants" (p. 34), with the exception that the erythrocytes were not lysed prior to column application. Erythrocytes serve as a visible marker to indicate the progress of the cell suspension into the column. Two ml of a whole spleen cell suspension not exceeding a total 3 x 10^8 cells, was washed into the wool using RPMI medium supplemented with 5% FCS and then incubated at 37 C for 45 min. Higher cell concentrations overload the column and result in poor separation. An aliquot of whole spleen cells for each group was held in reserve and not fractionated, but treated identically to fractionated cells in all other respects.

After incubation, nylon wool nonadherent cells were collected as described previously (37). Nonadherent cells were eluted by washing the

column with 30 ml of warmed RPMI medium.

Both the nonadherent cells and the unfractionated spleen cell aliquots which had been reserved, were then pelleted by centrifugation and resuspended in NH_4Cl to lyse erythrocytes, washed three times with RPMI medium and adjusted to the desired concentration for culture, usually 1 x 10^7 cells/ml. These cells were then cultured for 24 hr with Con A or with Con A plus indomethacin in a manner identical to the method described in "Preparation of IL 2 and IL 3 Containing Supernatants". After the 24 hr incubation period, cultures were centrifuged and the supernatants were drawn off. The supernatants were then stored up to 12 months at -70 C in polystyrene (Fisher Scientific) tubes prior to testing. No diminution of counts was observed with prolonged storage at -70 C.

MICROCULTURE ASSAY OF SUPERNATANTS FOR IL 2 AND IL 3

The supernatants were assayed for IL 2 and IL 3 content using lymphokine-dependent cell lines. The degree of proliferation of the dependent cell line is proportional to the amount of Interleukin present in the sample. IL 2 content was measured using the IL 2-dependent cell line HT-2 or CT-6. IL 3 content was determined using the IL 3-dependent cell line FDC-P1. Briefly, dependent cell lines were grown to sufficient numbers (see sections dealing with culturing of cells pp.

38-40). Samples to be tested for IL 2 or IL 3 content were then diluted and dependent cells were added (see "Assaying for IL 2 and IL 3 pp. 40-41) and incubated. Dependent cells were then radiolabelled, harvested and radioactivity was counted as described on pp. 41-42, and units of Interleukin were determined (pp. 41-42).

Culturing CT-6 cells

CT-6 cells were obtained from Dr. James Ihle (Frederick Cancer Research Center, MD) and were maintained by a modification of Ihle's technique (85). CT-6 cells were continuously cultured for routine use in assays. Cells were grown in 75 cm^2 flasks standing upright. Medium consisted of 5% heat-inactivated FCS, 50% rat factor, 4 x 10^{-5} M 2-mercaptoethanol solution (Sigma) and the remainder RPMI medium. Approximately 50 ml of this medium containing a 25 ml of pelleted cells were placed in flasks and incubated in a 5% CO_2 incubator. Cells were split 1:2 twice weekly. Rat factor was prepared periodically and frozen until needed. Rat factor was prepared by homogenizing 10-12 rat spleens, lysing erythrocytes, washing and then resuspending in RPMI medium supplemented with 2% FCS and 4 x 10^{-5} M 2-mercaptoethanol, at 10^7 cells/ml. Cells were then stimulated with 5 ug of Con A per ml and incubated overnight in a tissue culture flask. Supernatant was then harvested, filtered and stored.

Culturing HT-2 cells

HT-2 cells were grown in 30% rat factor, 10% FCS, RPMI medium and 4 x 10^{-5} M 2-mercaptoethanol. Cells were suspended at an volume of 50 ml at an initial concentration of 2 x 10^4 cells per ml in a tissue culture flask placed on its side. Cells were split every other day.

Culturing FDC-P1 cells

Culture of FDC-P1 cells was a modification of Ihle's technique (85). FDC-P1 cells were grown in medium consisting of 10% FCS, 4 x 10^{-5} M 2-mercaptoethanol, 25% WEHI-3 conditioned media and RPMI in 75 cm^2 tissue culture flasks incubated on their side. Cells were split 1:5 - 1:10 twice weekly.

WEHI-3 conditioned media, used to supplement FDC-P1 cell culture media, was prepared by culturing a heavy monolayer of WEHI-3 cells in 5% FCS, 4 x 10^{-5} M 2-mercaptoethanol and RPMI medium for 3-4 days. Flasks were placed on their side during incubation, and split twice weekly. Cells were scraped off the wall of the flask when cultures were to be split using a sterile squeegee. Spent culture supernatants were then centrifuged and filtered through 115 ml Nalgene disposable filters (Sybron Nalge) and then either used to supplement FDC-P1 cultures or

frozen for later use.

Assaying for IL 2

IL 2 assay was performed by a modification of the technique originally described by Gillis et al. (62). Supernatants to be tested for IL 2 content were thawed, vortexed and then plated in serial 1:2 dilutions in a 96 well flat-bottom plate (Linbro, Flow Laboratories). RPMI medium supplemented with 10% FCS was used as diluent. Dilution was usually carried out to 1:32 or 1:64. Dilutions were made by mixing 0.1 ml sample with 0.1 ml supplemented RPMI medium, and then removing 0.1 ml of this mixture with a Titertek (Flow Laboratories) serial diluter to be used for further dilution. After all dilutions were made, 2×10^4 twice-washed HT-2 cells suspended in 0.1 ml RPMI medium with 10% FCS were added to each well. Assays in which CT-6 cells were used to assess IL 2 content were carried out in an identical manner, with the exception that 10% of the Con A competitor alpha-methyl-mannoside (Sigma) was included in the RPMI media. Plates were then incubated for 24 hr at 37 C in a 5% CO_2 humidified atmosphere, and then further processed as described on pp. 41-42.

Assaying for IL 3

IL 3 content of samples was determined using the IL 3-dependent cell

line FDC-P1, as described elsewhere (83). The IL 3 assay is very similar to the IL 2 assay. Sample dilution and assay was performed identically to the scheme used in the IL 2 assay, with the exceptions that RPMI medium supplemented with 5% FCS was used and 5 x 10^4 cells were added to each well containing samples, and that dilution of samples was carried further, often to 1:128. Samples were then further processed as described immediately below.

Isotope Labelling and Counting of Lymphokine-Dependent Cells

To determine the degree of proliferation of the IL 2- or IL 3-dependent cells, after 18 hr of incubation, each well received a pulse of medium containing 1 mCi of tritiated thymidine (^{3}H-TdR; specific activity 6 Ci/mmol, Amersham Corp., Arlington Heights, IL). Once pulsed with radiolabel, the samples were allowed to incubate an additional six hr.

Harvesting of Samples

Six hr after the pulse, samples were harvested using a Multiple Automated Sample Harvester II (MASH II, Microbiological Associates, Bethesda, MD). This device aspirates cells out of each well, deposits the cells onto a glass fiber filter paper and repeatedly rinses each well with distilled water. Strips were allowed to air-dry for 24 hr.

Counting of Samples

After drying, each sample spotted onto glass fiber filter paper by the MASH unit was placed into a filmware bag (Sybron Nalge, Rochester, NY) containing 2 ml Scintilene scintillation fluid (Fisher Scientific). The bag was then sealed and placed into a plastic scintillation vial. Vials were then loaded into a Beckman LS-230 Liquid Scintillation Counter and counted for one minute to determine the radioactivity of each sample, expressed in counts per minute (cpm).

MICROCULTURE ASSAY DATA ANALYSIS

Raw data expressed in cpm was converted to units of Interleukin activity using a program written for an HP 97 calculator. This program determines units of Interleukin activity in the following manner: A standard source of Interleukin is chosen and arbitrarily said to contain 100 units of Interleukin per ml. Con A-induced rat splenocyte culture supernatant was used as a standard for IL 2. For IL 3 assays, culture supernatants of the cell line WEHI-3, which constitutively produces high amounts of IL 3, was used as the standard. In each experiment, a standard was titrated out to extinction in addition to the samples. A log regression line was then generated for the standard using the log transformation of the cpm and the log transformation of the dilution values. The dilution giving 50% maximum response was determined. A

regression line for each sample was similarly determined. Units of Interleukin were then calculated by the following equation:

$$\text{Interleukin Units/ml} = \frac{\text{Sample dilution}}{\text{cpm 50\% maximum}} \Bigg/ \frac{\text{Standard dilution}}{\text{cpm 50\% maximum}}$$

Where indicated, units generated by the HP program were tested for statistical significance using a program written for an IBM personal computer, which generates confidence intervals at the 95% level by Finney's parallel line assay, employing Analysis of Variance (ANOVA) and regression analysis (18).

MITOGEN-INDUCED PROLIFERATION ASSAY

The ability of splenocytes to proliferate in response to mitogen stimulation was assessed at periodic intervals after treatment. Three to five mice per group were sacrificed and splenectomized as described before. For each group, six replicates of a single cell suspension of 2 x 10^5 spleen cells were cultured in RPMI medium supplemented with 5% FCS in a 96 well flat bottom plate. Wells received optimal stimulatory concentrations of either Con A or PHA (Difco). Con A was used at a concentration of 0.8 ug per 2 x 10^5 cells. Optimal concentrations for each lot of PHA must be determined by titration before use in proliferation assay. Fifty ul of a 1:250 - 1:500 dilution of rehydrated

stock PHA obtained from Difco was used in all experiments. After 66 hr of incubation at 37 C in a 5% CO humidified incubator, each well was pulsed with radioactive ^{3}H-TdR as discussed above, incubated an additional six hr, harvested and counted as before. Average cpm were determined by an IBM PC program which Q-tested six replicate samples. The Q-test statistically determines whether the highest and/or lowest value within a series of replicates should be accepted or rejected.

RESULTS

The Effect of Vaccine Dose on Tumor Inhibition

In order to determine the vaccine dose offering complete protection against tumor development, varying amounts of heat-killed stationary phase vaccine were added to a preparation of tumor cells and administered to groups of three mice. Mice were injected intramuscularly in the hindleg with 50 ug, 100 ug, 150 ug, 200 ug or 500 ug of vaccine admixed with 10^4 tumor cells. Control mice received 10^4 fibrosarcoma cells alone. Mice were followed for a 5-week period post inoculation and observed for tumor formation. The time period between initial inoculation and the appearance of a measurable, palpable tumor was noted.

Results of the 5-week study are shown in Table 1. All control animals (TBH) receiving 10^4 tumor cells developed a measurable tumor two weeks post injection. This tumor eventually engulfed the entire hindleg, resulting in immobility and slight necrosis. These animals became emaciated and dehydrated as a result of tumor burden and usually succumbed to the tumor within 30 days after tumor cell injection.

Larger doses of vaccine, in the 300-500 ug range, were completely

Table 1. Determination of vaccine dose offering complete protection against tumor[a]

Material Injected	Time Post Tumor Cell Injection					Comments
	Day 7	Day 14	Day 21	Day 28	Day 35	
0.1 ml RPMI medium (Negative control)	-	-	-	-	-	Presence of measurable tumor
10^4 tumor cells (Positive control)	-	+	+	+	+	Presence of measurable tumor
		3/3	3/3	3/3	3/3	Number of mice positive
		5.3	10.8	14.2	20.0[b]	Average tumor diameter (mm)
50 ug vaccine + 10^4 tumor cells	-	+	+	+	+	Presence of measurable tumor
		1/3	2/3	2/3	2/3	Number of mice positive
		2.7	4.0	6.1	10.7	Average tumor diameter (mm)
100 ug vaccine + 10^4 tumor cells	-	-	+	+	+	Presence of measurable tumor
			2/3	1/3	1/3	Number of mice positive
			2.4	7.5	9.3	Average tumor diameter (mm)
150 ug vaccine + 10^4 tumor cells	-	-	+	+	+	Presence of measurable tumor
			1/3	2/3	2/3	Number of mice positive
			2.6	7.2	11.7	Average tumor diameter (mm)
200 ug vaccine + 10^4 tumor cells	-	-	+	+	+	Presence of measurable tumor
			2/3	2/3	2/3	Number of mice positive
			2.4	5.0	7.5	Average tumor diameter (mm)
300 ug vaccine + 10^4 tumor cells	-	-	-	+/-[c]	-	Presence of measurable tumor
				2/3		Number of mice positive
				1.0		Average tumor diameter (mm)
500 ug vaccine + 10^4 tumor cells	-	-	-	-	+/-	Presence of measurable tumor
					1/3	Number of mice positive
					0.90	Average tumor diameter (mm)

[a] Three mice per group were used. Mice were injected intramuscularly with 10^4 viable tumor cells and/or HK WC 0009 stationary phase P. acnes vaccine suspended in RPMI medium. Average of measurements from RPMI-injected leg subtracted from average of measurements of tumor and/or vaccine injected leg. The difference of these measurements is reported as tumor diameter.

[b] Tumor measurements based on one mouse only. Two of three mice died due to tumor.

[c] Probably not indicative of tumor. Small aberrations in measurements may be due to experimental error such as positioning of leg, calipers, etc.

effective in preventing tumor formation during the 5-week study. No animals receiving 300 ug or more developed a detectable tumor, whereas all unvaccinated TBH had large tumors by the end of the study or had died as a result of tumor burden.

Midrange doses of vaccine (100-250 ug) afforded partial protection against tumor formation, delayed tumor formation, and reduced tumor size compared to unvaccinated TBH controls. Unvaccinated TBH had an average tumor diameter of 5 mm at Day 14 post injection. Tumor formation was delayed by one week in mice vaccinated with midrange doses. In addition, roughly one-third of the animals vaccinated with a midrange dose of vaccine were completely protected by this dose and did not develop a detectable tumor during the observation period. The animals vaccinated with midrange doses that did develop tumors consistently bore smaller tumors than their unvaccinated counterparts.

Vaccination with as little as 50 ug vaccine exerted a partially protective effect. This small dose did not delay tumor formation in 100% of mice injected, however roughly 30% of mice injected were completely protected throughout the study by 50 ug of stationary phase vaccine. This small dose also effectively reduced tumor size compared to unvaccinated counterparts. Based on these results, a 500 ug dose of stationary phase vaccine was chosen as a standard dose of vaccine in all subsequent experiments.

The Anti-Tumor Activity of a Log Phase Vaccine

P. acnes does not possess anti-tumor activity at all phases of the life cycle nor do all manners of vaccine preparation result in anti-tumor action. Stationary phase whole cell preparations possessed the most anti-tumor activity. It has been reported that early log phase cultures of P. acnes do not possess significant anti-tumor activity (148). In later sections the effect of this inactive early log phase vaccine versus the effect of active stationary phase vaccine on IL 2 and IL 3 levels will be presented. Results of a study of the anti-tumor activity of a early log phase culture versus the activity of a stationary phase culture of P. acnes are presented here. Early log phase vaccine or stationary phase vaccine were injected either alone or admixed with an aliquot of a tumor cell preparation in such a way to deliver 500 ug vaccine and/or 10^4 tumor cells.

Early log phase vaccine was not completely devoid of anti-tumor activity (Table 2), since it delayed tumor production by 7-10 days compared to unvaccinated TBH controls. In addition, early log phase vaccinated animals developed smaller tumors than unvaccinated TBH. Approximately 60% of animals injected with early log phase vaccine were completely protected against tumor development, in contrast to stationary phase vaccine which protected 100% of the injected animals.

Table 2. Comparison of the effect of partially protective log phase vaccine vs. protective stationary phase vaccine on tumor formation[a]

Group - Material Injected	Time Post Tumor Cell Injection						Comments
	Day 4	Day 7	Day 11	Day 14	Day 21	Day 28	
N (0.1 ml RPMI medium)	-	-	-	-	-	-	Presence of measurable tumor
T (10^4 tumor cells)	-	-	+	+	+	+	Presence of measurable tumor
			3/3	3/3	3/3	3/3	Number of mice positive
			1.8 ± 3.0	6.3 ± 2.5	10.2 ± 0.6	15.3 ± 3.0	Average tumor diameter (mm)
N+V (500 ug vaccine)	-	-	-	-	-	-	Presence of measurable tumor
T+V (500 ug vaccine + 10^4 tumor cells)	-	-	-	-	-	-	Presence of measurable tumor
N+ppv (500 ug vaccine)	-	-	-	-	-	-	Presence of measurable tumor
T+ppv (500 ug vaccine + 10^4 tumor cells)	-	-	-	-	+	+	Presence of measurable tumor
					1/3	2/3	Number of mice positive
					4.0 ± 0	12.7 ± 1.7	Average tumor diameter (mm)

[a] Three mice were used per group. Either early log phase (ppv) or stationary phase (V) P. acnes vaccine (500 ug) and/or 10^4 viable tumor cells were suspended in RPMI medium and injected intramuscularly. Tumor diameter was determined by subtracting control leg measurements from injected leg.

In the following studies, the effect of early log phase vaccine in addition to stationary phase vaccine on IL 2 and IL 3 production and blastogenic ability in response to mitogen was investigated. Due to the slight protection afforded by the early log phase vaccine it is referred to in subsequent studies as "partially protective vaccine" (ppv).

Splenomegaly in Vaccinated and Unvaccinated Mice

Splenomegaly is often seen as a result of either allocthonous stimulation of the reticuloendothelial system or as a result of a disease process. Many investigators have used splenomegaly as a tool to screen for possible anti-tumor activity, for many compounds possessing anti-tumor activity also possess reticuloendothelial stimulatory capabilities resulting in splenomegaly. However, this is not always the case and often the two phenomena are separate.

Spleens removed from animals in all subsequent studies were examined upon removal for enlargement and general appearance of each spleen was noted (Table 3). Spleens from all groups (N, T, N+V, T+V, N+ppv and T+ppv) were normal, reddish-brown and smooth in appearance. No splenomegaly was observed in animals receiving vaccine alone (N+V or N+ppv). Unvaccinated TBH developed moderate splenomegaly by Day 14 post tumor cell injection and gross splenomegaly by Day 21. Such spleens were pinkish-grey in color, the outer capsule rough and pebbled in

Table 3. Splenomegaly in vaccinated vs. unvaccinated mice[a]

Group	Days Post Injection					
	4	7	11	14	21	28
N	-	-	-	-	-	-
T	-	-	-	++	+++	+++
N+V[b]	-	-	-	-	-	-
T+V	-	-	-	-	-	-
N+ppv[c]	-	-	-	-	-	-
T+ppv	-	-	-	-	+	++

[a] - = no splenomegaly (normal spleen); ++ = moderate splenomegaly; +++ = gross splenomegaly. Three mice per group were used. Mice were injected with 500 ug vaccine and/or 10^4 viable tumor cells suspended in RPMI medium.

[b] Stationary phase P. acnes vaccine was used.

[c] Early log phase P. acnes vaccine was used.

appearance, and the spleen fibrous in nature. Splenomegaly was only observed in animals bearing tumor, namely TBH and some T+ppv which developed tumor in spite of vaccination. Animals injected with tumor and ppv that did not develop tumor also did not exhibit splenomegaly.

Proliferative Response of Whole Spleen Cells to Mitogen

Splenocytes of animals from each of the six groups (N, T, N+V, T+V, N+ppv, and T+ppv) were assessed for ability to proliferate in response to the mitogens, Con A and PHA, at intervals throughout a 28-day period post vaccine and/or tumor cell injection. Normal murine splenocytes typically proliferated intensely in response to the proper dose of a mitogenic stimulus. In the murine system, Con A was usually a "better" mitogen than PHA, resulting in higher level of proliferation.

Shown in Table 4 are the average cpm for each treatment group of ^{3}H-TdR incorporation for six replicate wells of splenocytes stimulated with Con A (Q test applied) at various timepoints after tumor cell and or vaccine inoculation. Average cpm of ^{3}H-TdR incorporation in response to PHA stimulation is shown in Table 5.

Through Day 14 post initial tumor cell and/or vaccine injection, all treatment groups responded normally to Con A or PHA. N+V, T+V and N+ppv continued to respond normally to mitogen throughout Day 28. However,

Table 4. Con A-responsiveness of splenocytes from vaccine and/or tumor cell recipients.

Source of Splenocytes[a]	Mitogen Responsiveness (^{3}H-TdR Incorporation)[b]		
	Day 7	Day 14	Day 28
	Δ cpm	Δ cpm	Δ cpm
N	303,792	231,284	265,269
T	322,031	202,345	778
N+V[c]	274,435	264,277	285,444
T+V	222,052	249,994	288,278
N+ppv[d]	259,146	224,163	234,828
T+ppv (tumor)	303,639	211,418	22,736
T+ppv (no tumor)	-	-	277,804
T+ppv + T+ppv (tumor)	-	-	162,472

[a] Splenocytes from 3-5 mice per group were suspended in RPMI medium and Con A-stimulated as described in Materials and Methods.

[b] Proliferation is expressed as the difference (Δ cpm) between the average cpm of 6 replicate wells of whole spleen cells stimulated with Con A minus the average cpm of nonstimulated cells, Q Test applied. Standard deviations were less than 10% for all samples except Day 28 T and Day 28 T+ppv (tumor). Day 28 T average cpm was 5495 $\pm$ 1728, with a background of 4717 $\pm$ 1893. Day 28 T+ppv (tumor) average cpm was 24,734 $\pm$ 5331 with a background of 1998 $\pm$ 400.

[c] N+V and T+V mice received 500 ug stationary phase *P. acnes* vaccine and/or 10^4 tumor cells on Day 0.

[d] N+ppv and T+ppv mice received 500 ug log phase *P. acnes* vaccine and/or 10^4 tumor cells on Day 0.

Table 5. PHA responsiveness of splenocytes from vaccine and/or tumor cell recipients

Source of Splenocytes[a]	Mitogen Responsiveness (^{3}H-TdR Incorporation)[b] Day 14	Day 28
	Δ cpm	Δ cpm
N	50,273	86,737
T	33,927	8,551
N+V[c]	54,389	112,583
T+V	38,788	75,894
N+ppv[d]	49,071	110,691
T+ppv (tumor)	50,228	4,274
T+ppv (no tumor)	N.D.	129,853
T+ppv + T+ppv (tumor)	N.D.	50,570

[a] Splenocytes from 3-5 mice per group were suspended in RPMI medium and PHA-stimulated as described in Materials and Methods.

[b] Proliferation is expressed as the difference (Δ cpm) between the average cpm of 6 replicate wells of whole spleen cells stimulated with PHA minus the average cpm of nonstimulated cells, Q Test applied. Standard deviations were less than 10% for all samples except for Day 28 N+V (standard deviation less than 12%), Day 28 N+ppv (standard deviation less than 14%), Day 28 T (standard deviation less than 20%) and Day 28 T+ppv (tumor) (standard deviation less than 14%).

[c] N+V and T+V mice received 500 ug stationary phase *P. acnes* vaccine and/or 10^4 tumor cells on Day 0.

[d] N+ppv and T+ppv mice received 500 ug log phase *P. acnes* vaccine and/or 10^4 tumor cells on Day 0.

after Day 14, T+V and T+ppv splenocytes began to deviate from normal. Past this time point, the proliferative ability of TBH splenocytes began decreasing and by Day 28 post tumor cell inoculation, the proliferative ability of TBH splenocytes was abolished. At Day 28, Con A-stimulated TBH splenocytes were not capable of proliferating above background levels (Table 4). PHA-stimulated TBH splenocytes showed a weak blastogenic response at Day 28 (Table 5).

As mentioned in previous sections, not all animals injected with tumor cells and partially protective vaccine (T+ppv) developed a tumor. T+ppv animals bearing no measurable tumor responded normally to Con A and PHA throughout the study. Spleen cells from animals vaccinated with ppv which subsequently developed tumor had a sharply suppressed response to mitogen, analogous to unvaccinated TBH splenocytes. Admixture of splenocytes from T+ppv treated animals bearing tumor and T+ppv treated animals protected from tumor responded in an intermediate fashion (Table 5). Average cpm of these cells were less than normal but significantly greater at the same timepoint than either TBH splenocyte cpm or cpm of splenocyte cultures from T+ppv animals which developed tumor in spite of vaccination.

IL 2 Production by Splenocytes

At various intervals after tumor and/or vaccine injection, spleens from

each treatment group were pooled and splenocyte cultures were prepared. In vitro IL 2 production by Con A-stimulated splenocyte cultures was then examined by the method outlined above in "Assaying for IL 2". Figure 1 depicts IL 2 production in units of IL 2 per ml by Con A-stimulated N, TBH, N+V, and T+V splenocyte cultures over a 28 day period after injection of tumor cells and/or vaccine. The most striking result was the drastic decline in units of IL 2 exhibited by TBH splenocytes from Day 14 onwards. This decline usually accompanied palpable tumor formation, although occasionally in animals bearing small, freshly formed tumors this decline was sometimes not yet apparent. At Day 21 tumor formation was full-blown and was accompanied by sharply suppressed levels of IL 2 compared when compared to normal counterparts. This decline in IL 2 production continued until IL 2 production was virtually extinct at Day 28 by TBH splenocytes. Declining IL 2 levels were not observed in T+V splenocytes. Both N+V and T+V splenocyte cultures maintained normal or near normal levels of IL 2 throughout the study. T+V splenocytes demonstrated a greatly restored IL 2 response compared to TBH.

Production of IL 2 by animals vaccinated with ppv was also examined (Fig. 2). Normal animals vaccinated with ppv maintained normal IL 2 levels throughout the study. Cultures of splenocytes from T+ppv animals demonstrated a decline in IL 2 production from Day 14 onwards, similar to that seen in TBH splenocytes. Levels of IL 2 did not decline quite

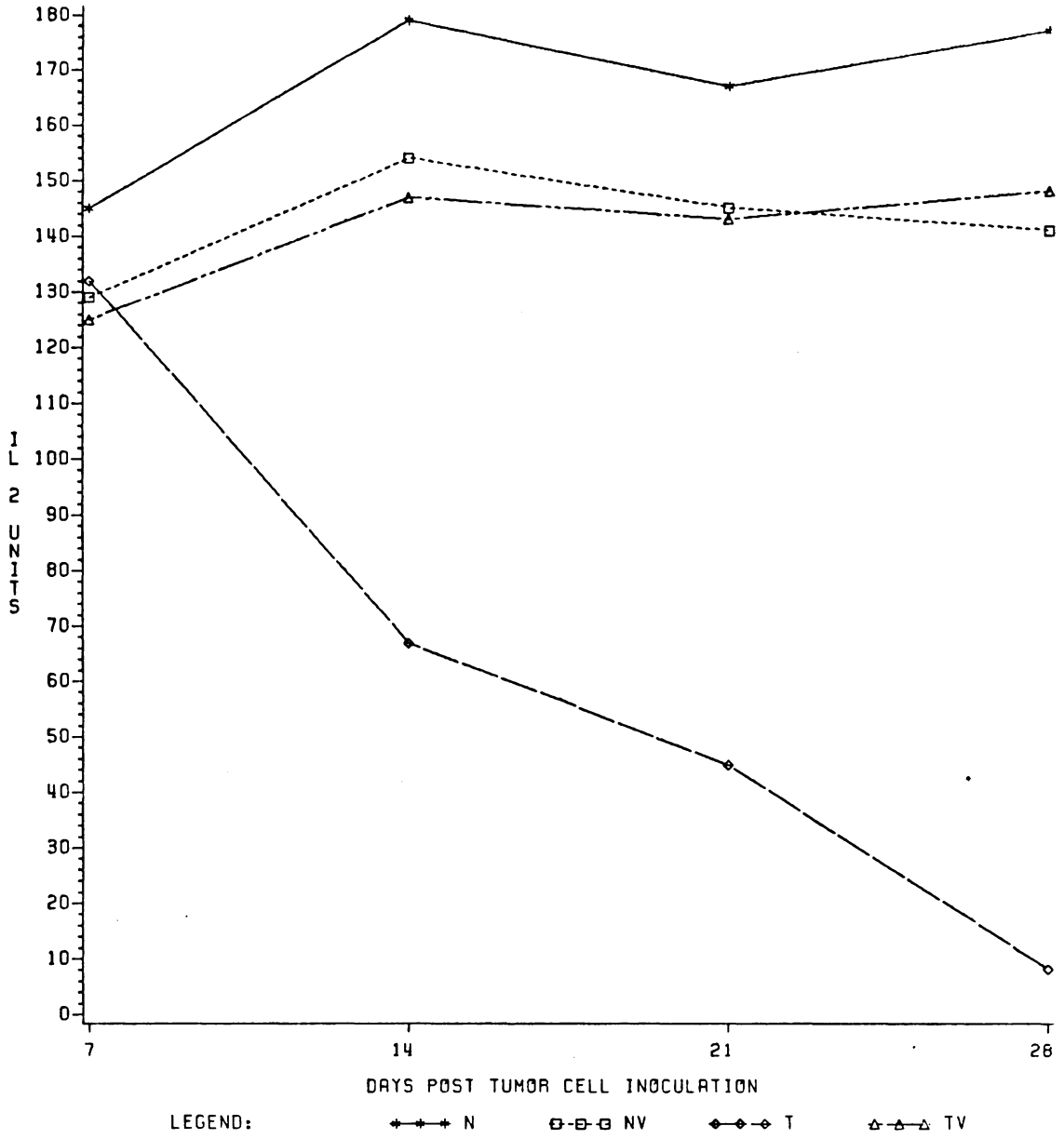


Figure 1. Kinetic study of IL 2 production by Con A-stimulated splenocytes from N, N+V, T, and T+V hosts. Splenocyte cultures containing 10^7 cells/ml and 2 ug Con A were prepared at 7, 14, 21 and 28 days after tumor cell and/or vaccine administration. Supernatants were harvested at 24 hr and assayed for IL 2 content. The parallel line assay revealed no significant difference ($p \doteq .05$) between N, N+V and T+V units for the duration of the study. TBH IL 2 units could not be tested for significance due to nonparallelism of sample vs. standard from Day 14 to Day 28.

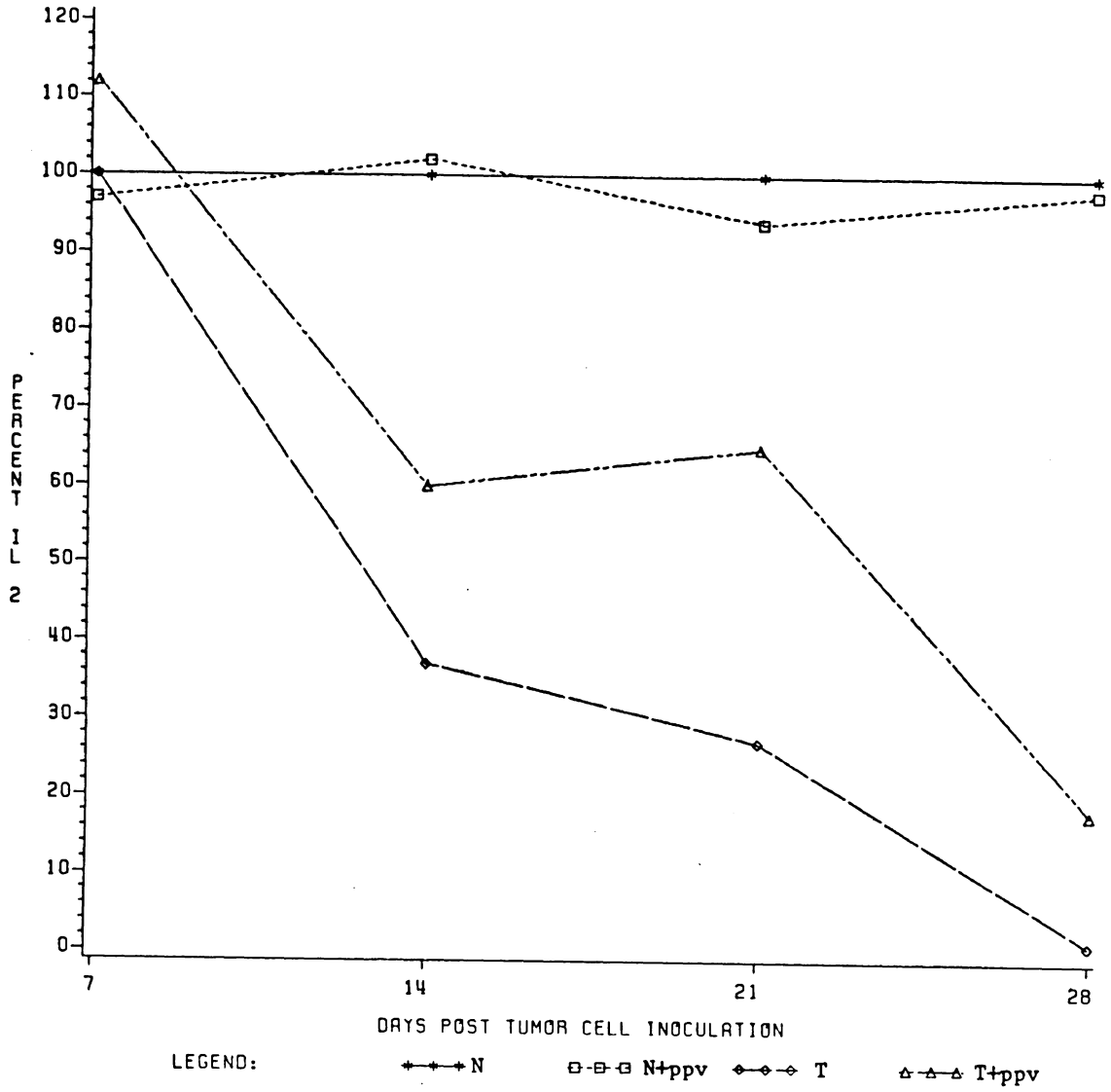


Figure 2. Kinetic study of IL 2 production by Con A-stimulated splenocytes from N, N+ppv, T and T+ppv hosts. Splenocyte cultures containing 10^7 cells/ml and 2 ug Con A were prepared at 7, 14, 21 and 28 days after tumor cell and/or vaccine administration. Log phase partially protective vaccine (ppv) was used. Supernatants were harvested at 24 hr and tested for IL 2; IL 2 content is expressed as percent of N control. No significant difference (p=.05) between N and N+ppv units of IL 2 was noted. Regression lines of T and T+ppv samples were not parallel compared to standard line and generally could not be tested for significance. Splenocytes from both T+ppv animals with tumor and without were used to prepare T+ppv cultures.

as sharply in T+ppv splenocyte cultures as in TBH splenocyte cultures. This phenomenon was further investigated. In previous sections, it was mentioned that ppv delays tumor formation until Day 21 and that not all animals vaccinated with tumor and ppv develop tumor. If spleens from Day 28 T+ppv animals bearing no tumor were cultured with Con A, normal (92% of control) levels of IL 2 were observed. Conversely, if spleen cells from Day 28 T+ppv-treated animals bearing tumor were cultured with Con A, IL 2 production was greatly suppressed (<1% of normal), as in Day 28 T WSC. If, however, spleen cells from Day 28 T+ppv which developed tumor were pooled with spleens from Day 28 T+ppv animals bearing no tumor, some IL 2 production was restored, thus accounting for the more gradual decline in IL 2 production seen in T+ppv pooled splenocyte cultures.

IL 2 Production after Indomethacin Treatment

Since there is an increase in splenic macrophages during tumor growth and macrophages secrete PGE, a known inhibitor of IL 2 production (151, 183), it was possible tumor-induced suppression of IL 2 production was due to PGE. To explore this avenue, the PGE-synthetase inhibitor indomethacin was added, in addition to Con A, to splenocyte cultures at time of culture preparation. Units of IL 2 produced by these cultures were compared to identical cultures lacking indomethacin, but stimulated with Con A. Indomethacin addition in the absence of Con A did not

effect IL 2 production. Results are shown in Table 6. Indomethacin treatment had a similar effect on N, N+V, and T+V splenocyte cultures throughout the study. Levels of IL 2 in these cultures were generally increased 100-200 units following indomethacin treatment. Through Day 14, indomethacin treatment of TBH splenocyte cultures restored any suppression of IL 2 to normal or near normal levels. However, from Day 14 onward, while indomethacin did enhance IL 2 production somewhat, the addition of indomethacin did little to restore IL 2 production by TBH splenocyte cultures to normal levels.

IL 2 Production after Nylon Wool Fractionation of Splenocytes

In order begin to determine the contribution of different cell populations to IL 2 production, nylon wool fractionation was carried out. Nylon wool filtration is a crude technique that separates splenocytes into two fractions, nylon wool adherent cells and nylon wool nonadherent cells. Nylon wool adherent cells consist primarily of macrophages, B cells and some T cell populations. Nylon wool nonadherent cells are primarily T cells, and in a normal animal, this population is primarily responsible for IL 2 production. Nylon wool is an approximate technique however, and neither cell fraction is free of contaminating constituents from the other fraction.

Fractionation of splenocytes by nylon wool demonstrates the intense

Table 6. The effect of Indomethacin treatment on IL 2 production.[a]

Group	Days Post Injection 7 Con A	7 +Indo	14 Con A	14 +Indo	21 Con A	21 +Indo	28 Con A	28 +Indo
N	145[b]	271	179	313	167	372	177	412
T	132	248	67[c]	229	45	81	8	36
N+V	129	218	154	290	145	329	141	334
T+V	125	206	147	315	143	273	148	266

[a] CT-6 cell assay of wsc supernatants. Control supernatant made from RPMI medium and Con A and Indomathacin (no spleen cells added) did not induce proliferation in CT-6 cells.

[b] Units calculated by probit analysis. Indomethacin treated culture consistently had higher raw cpm at each point of serial dilution.

[c] Slope of T wsc culture vastly different from day 14 onwards from that of standard, making accurate determination of units impossible. However, raw cpm of T wsc day 14-28 were vastly suppressed compared to normal.

suppression of IL 2 production seen in TBH splenocyte cultures can be entirely reversed by nylon wool fractionation (Fig. 3). Nylon wool fractionation of N+V splenocytes yielded normal results. T+V nonadherent cells demonstrated greatly enhanced IL 2 production compared to TBH.

IL 3 Production by Splenocytes

The IL 3-dependent cell line FDC-P1 was used to determine the IL 3 content of Con A-stimulated splenocyte supernatants prepared at various time intervals after initial vaccine and/or tumor cell injection. Shown in Fig. 4 is a graphic representation of IL 3 levels in N, T, N+V, and T+V splenocyte cultures at several time points post tumor cell inoculation. IL 3 values for N, N+V, and T+V splenocyte cultures were similar throughout the study, however Day 21 and Day 28 TBH splenocyte cultures were severely suppressed in production of IL 3 compared to N splenocyte cultures.

Levels of IL 3 in splenocyte cultures of animals vaccinated with ppv were also examined (Fig. 5). Cultures of splenocytes from normal animals vaccinated with ppv demonstrated steady IL 3 levels throughout the study, not significantly different from normal splenocyte cultures. Animals vaccinated with both tumor and ppv maintained normal IL 3 levels until the tumor became measurable at Day 21. At this point, IL 3 levels

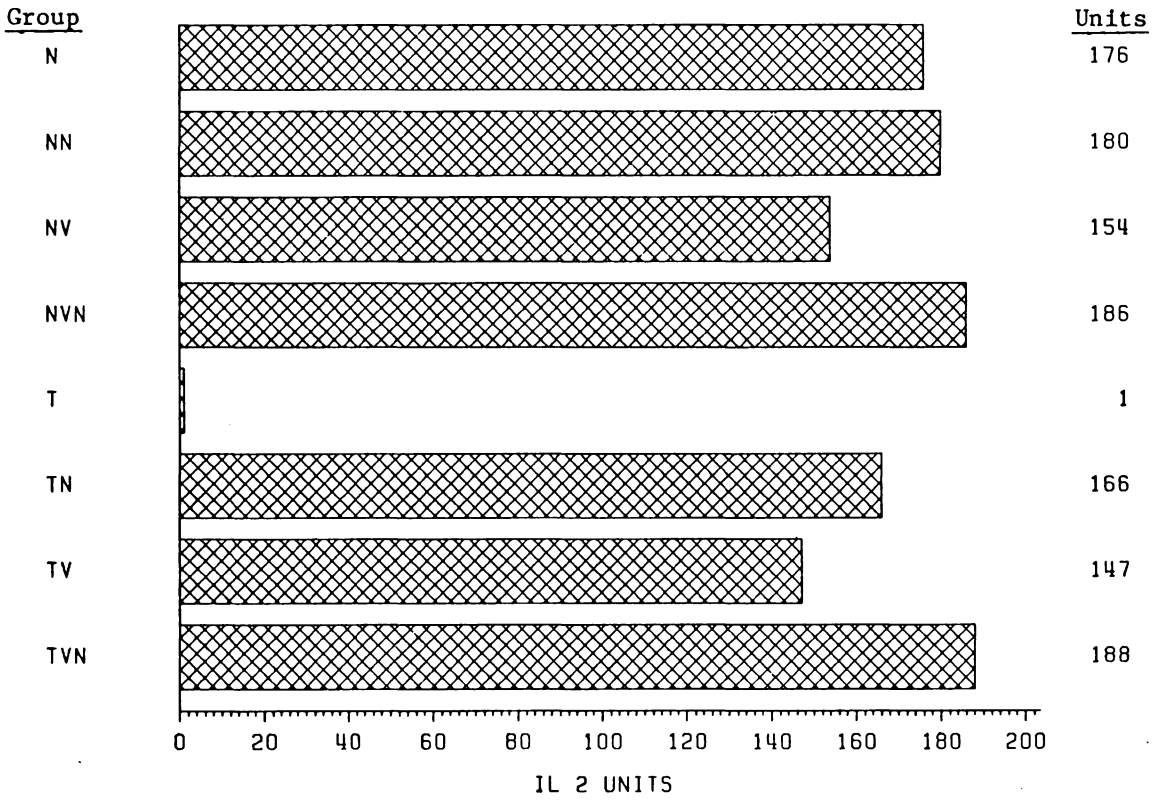


Figure 3. IL 2 production by unfractionated splenocytes and nylon wool nonadherent splenocytes from N, N+V, T, and T+V Day 28 hosts. Unfractionated splenocytes are labelled N, NV, T, TV; nonadherent (fractionated) splenocytes are labelled NN, (normal nonadherent); NVN (normal vaccinated nonadherent); TN (tumor-bearing nonadherent); and TVN (tumor cell and vaccine recipient nonadherent). Nylon wool fractionation had a minimal effect on IL 2 production by NN, NVN and TVN cultures compared to unfractionated, but greatly enhanced IL 2 production in TN cultures compared to unfractionated.

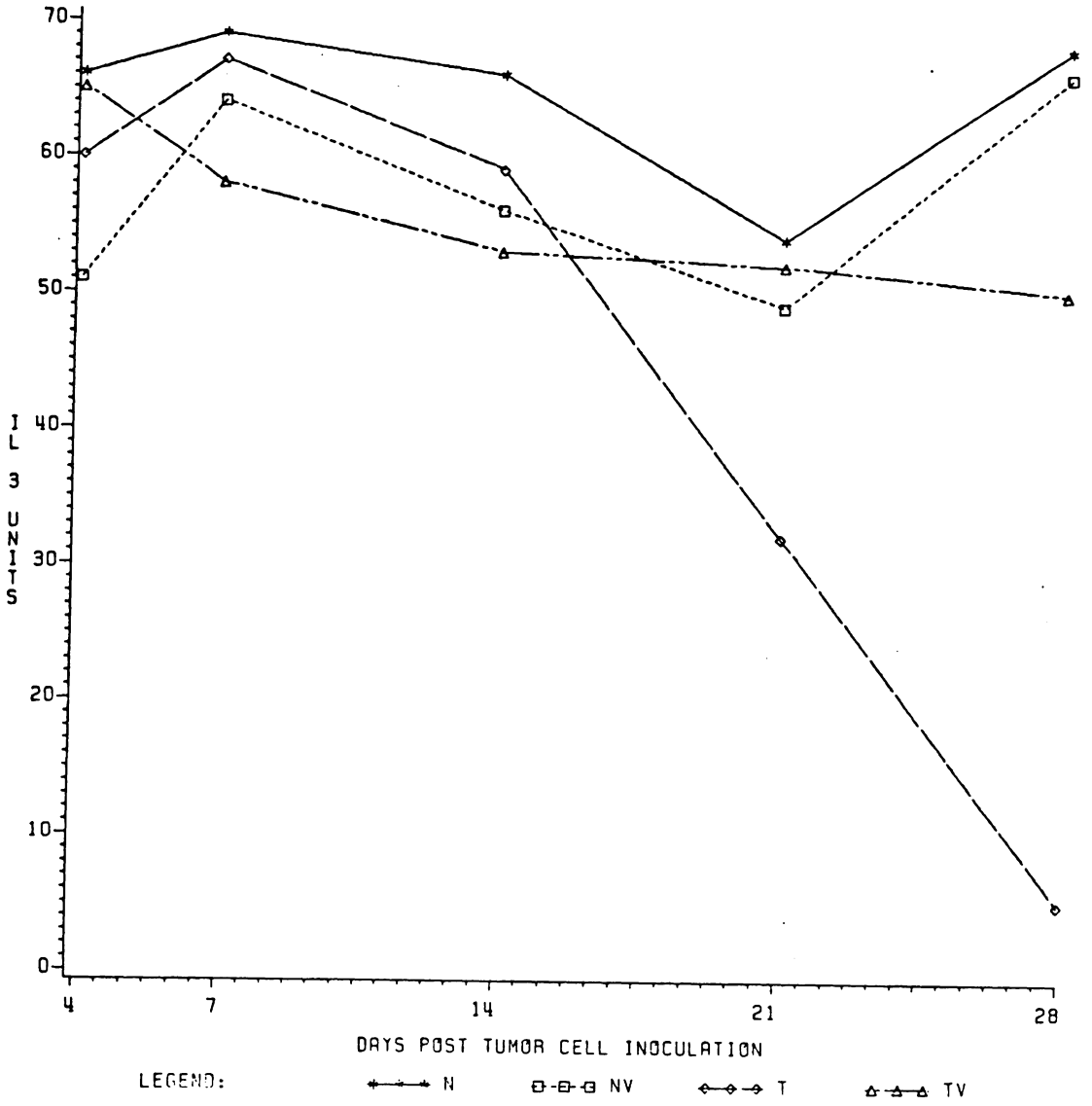


Figure 4. Kinetic study of IL 3 production by Con A-stimulated splenocytes from N, N+V, T, and T+V hosts. Supernatants were prepared at 4, 7, 14, 21, and 28 days after vaccine and/or tumor cell administration as described in Fig. 1. The parallel line assay revealed no difference (p=.05) between N, N+V, and T+V units for the duration of the study. Units from TBH (labelled 'T') cultures could not be tested for significance in the latter half of the study due to nonparallelism of the sample vs. standard.

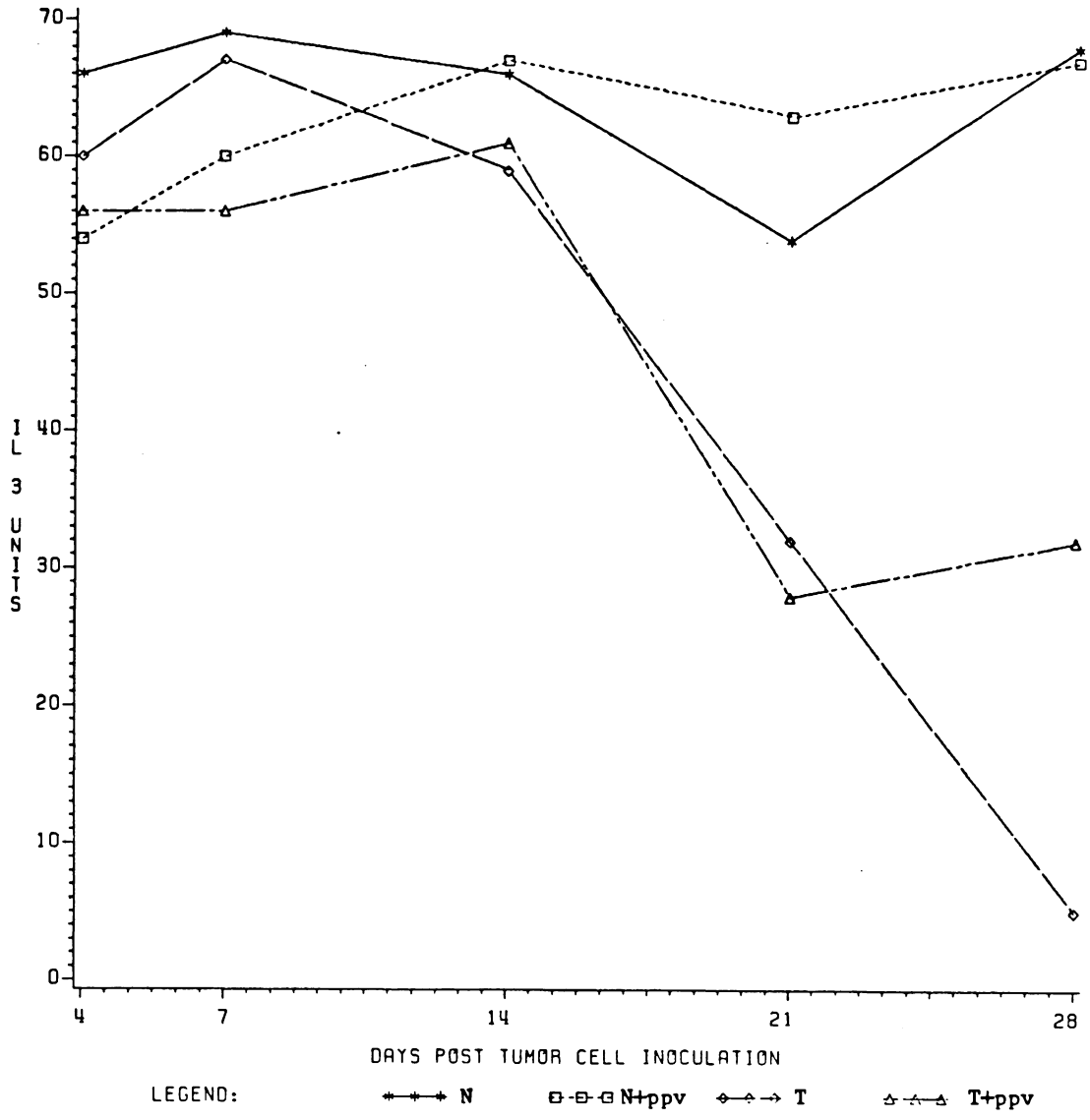


Figure 5. Kinetic study of IL 3 production by Con A-stimulated splenocytes from N, N+ppv, T and T+ppv hosts. Splenocyte cultures were prepared at Days 4, 7, 14, 21, and 28 as described in Fig. 2. Log phase partially protective (ppv) vaccine was used. No significant difference between N and N+ppv units was noted (p=.05). T and T+ppv units could not be tested for significance in the latter half of the study due to nonparallelism of sample vs. standard. A mixture of splenocytes from both T+ppv injected animals which developed tumor and T+ppv animals which did not develop tumor were used to prepare T+ppv cultures depicted here.

began dropping in a manner similar to unvaccinated TBH. As was the case with IL 2, IL 3 production by T+ppv splenocytes were above TBH counterpart levels if the splenocyte culture was prepared from pooled splenocytes from both T+ppv mice bearing tumor and T+ppv mice protected by the partially protective vaccine. However, if T+ppv Day 28 splenocytes from T+ppv tumor bearers were assayed alone, IL 3 production was comparable to TBH splenocyte cultures. Conversely, Day 28 T+ppv splenocytes from T+ppv-treated animals with no tumor were assayed for IL 3 production, levels of IL 3 comparable to those produced by N+ppv animals were observed.

IL 3 Production after Indomethacin Treatment

Addition of indomethacin to Con A-stimulated splenocyte cultures did not significantly enhance IL 3 production by N, N+V, T+V, or N+ppv splenocytes (Table 7). Furthermore, indomethacin treatment did not significantly enhance T or T+ppv splenocyte IL 3 production through Day 14 of the study. Day 21 and Day 28 T and T+ppv IL 3 values could not be evaluated using Finney's Parallel line assay. The slope of these preparations is very different from that of the WEHI standard preparation, probably due to various factors produced during tumor growth. This condition of non-parallelism makes comparison between standard and test preparations statistically invalid.

Table 7. IL 3 activity of splenocyte cultures treated with indomethacin[a]

	Group	IL 3 Activity (units/ml)[b] Con A only[c]	Con A + Indomethacin[d]
Day 4	N	66	66[e]
	T	60	50
	N+V[f]	51	51
	T+V	65	66
	N+ppv[g]	54	48
	T+ppv	56	50
Day 28	N	68	74
	T	15	22
	N+V	66	63
	T+V	50	47
	N+ppv	67	69
	T+ppv	8	15

[a] IL 3 activity was measured via FDC-P1 assay.

[b] Units of IL 3 were calculated by probit analysis.

[c] Two ug/ml of Con A added per 10^7 splenocytes at beginning of 24 hr incubation period.

[d] 10^{-7} M indomethacin

[e] Indomethacin treatment did not significantly ($p=0.05$) affect IL 3 production by N, N+V, or T+V splenocytes. Units of Day 28 T and T+ppv splenocyte supernatants could not be tested for significance due to nonparallelism of sample vs. standard.

[f] Splenocytes from animals injected on Day 0 with 500 ug stationary phase P. acnes vaccine and/or 10^4 tumor cells.

[g] N+ppv and T+ppv animals injected on Day 0 with 500 ug log phase P. acnes vaccine and/or 10^4 tumor cells.

IL 3 Production After Nylon Wool Fractionation

Nylon wool fractionation of splenocytes was found to reverse the suppression of IL 3 production seen in TBH splenocyte cultures. Nonadherent TBH cells produced amounts of IL 3 comparable to nonadherent N cultures. Nonadherent N+V and T+V cell cultures exhibited comparable IL 3 levels, as did nonadherent N+V and N cultures (Fig. 6). Thus, it appeared a nylon wool adherent suppressor cell was present in TBH splenocytes only. In addition, TBH splenocytes were suppressive to IL 3 production when admixed with N splenocytes. However, the addition of TBH nonadherent cells to N splenocytes was not suppressive (Fig. 7).

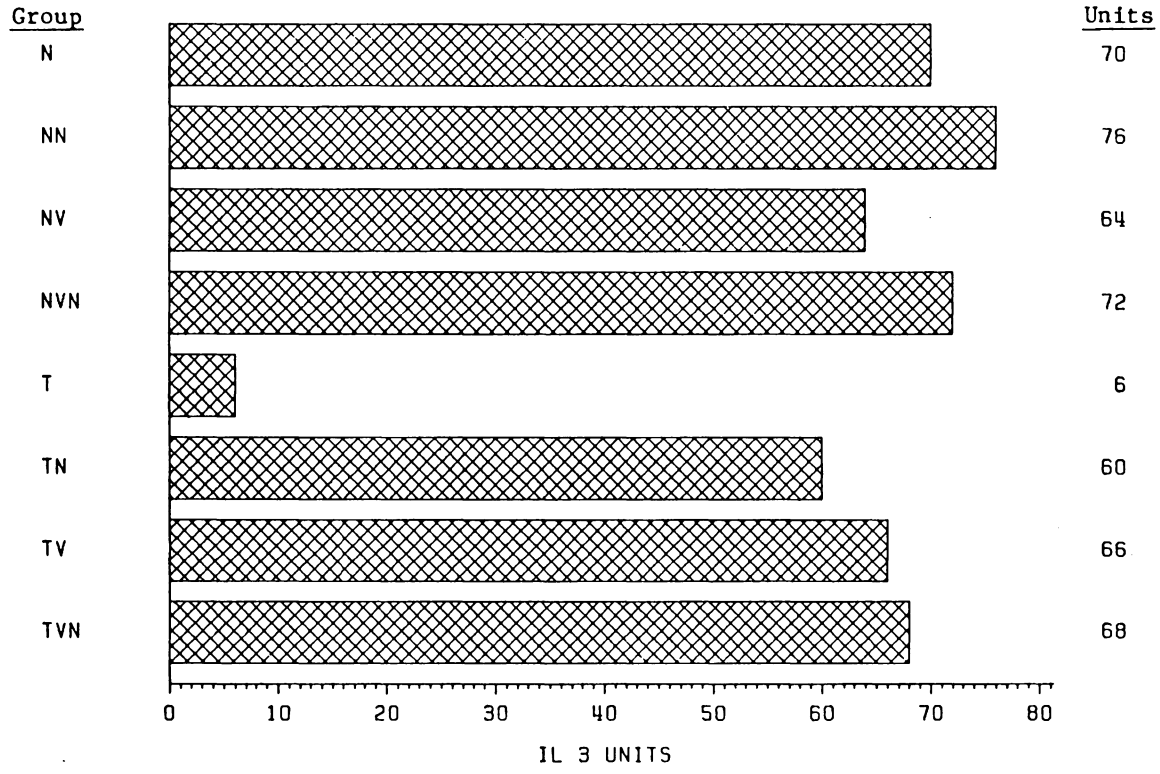


Figure 6. IL 3 production by unfractionated splenocytes and nylon wool nonadherent splenocytes from N, N+V, T, and T+V Day 28 hosts. Unfractionated splenocytes are labelled N, NV, T, TV; nonadherent (fractionated) splenocytes are labelled NN, (normal nonadherent); NVN (normal vaccinated nonadherent); TN (tumor-bearing nonadherent); and TVN (tumor cell and vaccine recipient nonadherent). Nylon wool fractionation had a minimal effect on IL 3 production by NN, NVN, and TVN cultures compared to unfractionated, but greatly enhanced IL 3 production in TN cultures compared to unfractionated.

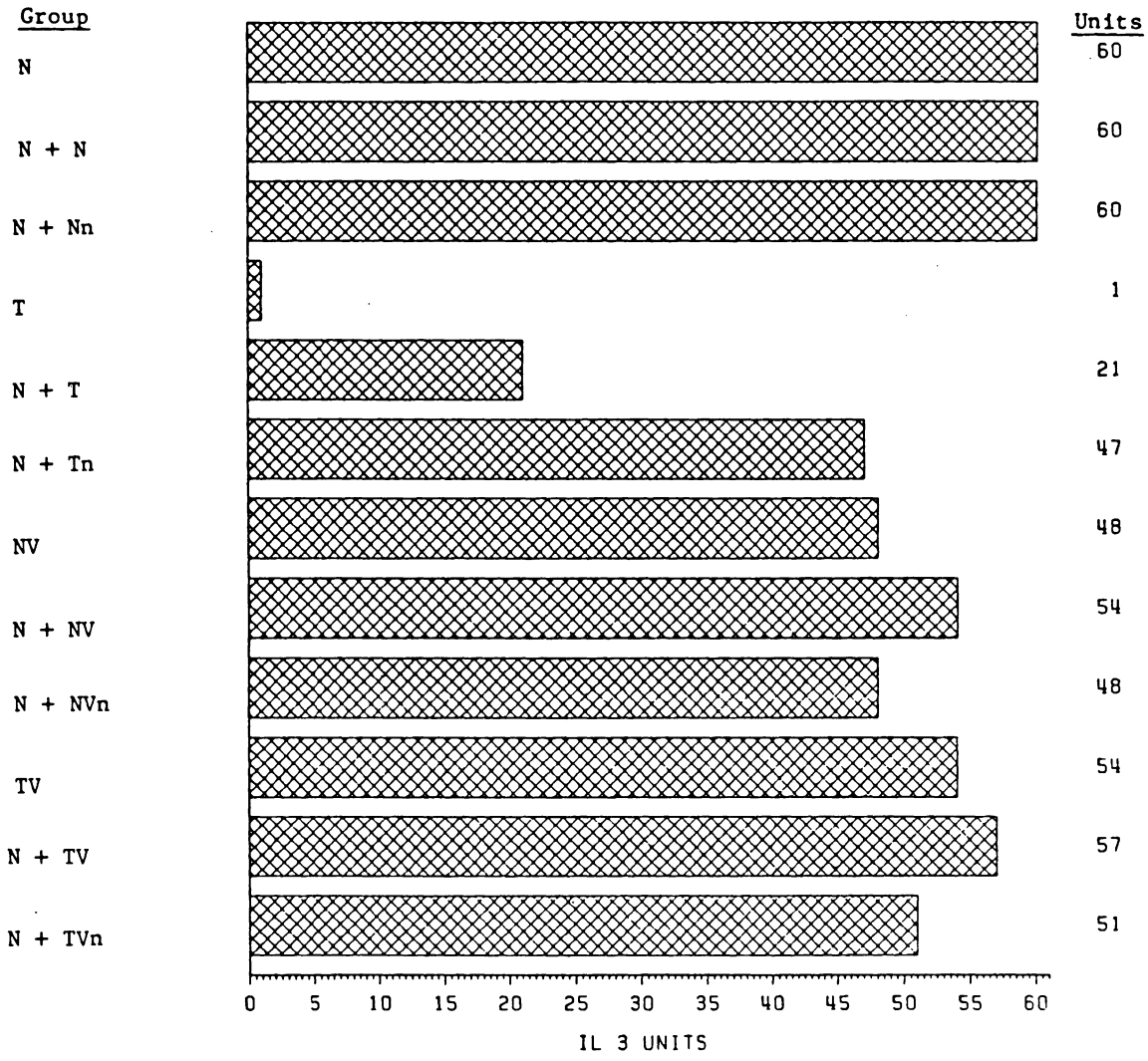


Figure 7. IL 3 production by admixture of N splenocytes with either N, T, N+V or T+V splenocytes or nonadherent cells. Normal unfractionated splenocytes (N) were admixed with unfractionated normal splenocytes (N + N), nonadherent normal (N + Nn), unfractionated TBH (N + T), nonadherent TBH (N + Tn), unfractionated normal vaccinated (N + NV), nonadherent normal vaccinated (N+ NVn), tumor and vaccine recipient unfractionated (N+ TV) or tumor and vaccine recipient nonadherent splenocytes (N + TVn). IL 3 production by unfractionated T, N+V labelled (NV) and T+V (labelled TV) splenocytes alone is also shown. Only the addition of unfractionated splenocytes from TBH was suppressive to IL 3 production (see group 'N + T'). TBH splenocytes, once fractionated, were not suppressive to IL 3 production when added to normal splenocytes (see group 'N + Tn).

DISCUSSION

In this investigation, evidence congruent with reports that local P. acnes anti-tumor action is T cell-mediated was presented. I have examined several parameters reported to be directly involved in the Interleukin cascade resulting in effective CTL formation, such as proliferative ability, IL 2 production and IL 3 production. In addition, I have examined other parameters indirectly involved, such as Ts cell formation and PGE production.

Preliminary experiments suggested P. acnes anti-tumor action was mediated by an active process in the host, however vaccination did not cause splenomegaly in the mice, indicating splenomegaly was not a good index for judging the ability of the vaccine to evoke an anti-tumor response in the host. In a procedure such as the one used in this study employing an admixture of tumor cells and vaccine, the possibility exists that the vaccine was cytotoxic to the tumor cells upon admixture, thus resulting in lack of tumor formation. My observations indicated, however, that an active anti-tumor process was triggered in the host as a result of vaccination. The most tangible argument to support this assertion was my personal observation that injection of an admixed preparation of tumor and P. acnes into young mice (10-12 week-old) resulted in complete protection, but injection of the same admixture

into older (15-20 week-old) mice resulted in tumor formation in a majority of the older mice (data not shown). study. This indicated an active process in the young mice prevented tumor formation. *In vitro* and *in vivo* evidence reported by others strengthens this assertion. Admixture of *P.. acnes* and tumor cells *in vitro* does not reduce tumor cell viability (123, 166). Other studies have demonstrated lack of cytotoxicity *in vivo* (71). *P. acnes* admixed with neoplastic cells and injected into silica-treated animals results in tumor formation in all animals, whereas injection of an admixture of *P. acnes* and tumor cells into normal animals resulted in complete protection against tumor (71). Collectively, these data indicated *P. acnes* is not directly cytotoxic to tumor cells. Instead, there appears to be an active ongoing process preventing tumor formation in animals vaccinated locally with *P. acnes*. Elucidating parts of this process was the subject of this study and is further discussed below.

From the work of others, it appeared CTL arising via the Interleukin cascade might be responsible for *P. acnes*-mediated anti-tumor activity (10, 174, 191, 192). One essential event in the sequence of events leading to the development of CTL is proliferation of various cellular populations. Work in our laboratory has shown debilitation of the cell-mediated immune response during tumor growth parallels the inability of T cells to proliferate in response to mitogen (17), soluble antigen (34, 35), and alloantigen (27, 37). *In vitro* cellular

proliferation is inhibited in TBH splenocyte cultures by tumor-induced Ts cells and soluble factors given off by these suppressor cells (17, 41). These soluble factors are capable of recruiting other cells to become suppressive. In this study, significant suppression was seen in mitogen-stimulated TBH splenocyte cultures. However, tumor cell recipients which were vaccinated with stationary P. acnes did not develop tumor and showed no decrease in blastogenesis in response to mitogen. This suggested Ts cells were not formed in vaccinated animals.

The work of others has indicated that cessation of CTL activity against tumor cells may be due to the perturbation or decline of IL 2 production (130). Our previous work has shown IL 2 production declines sharply as tumor development and growth proceeds, indicating that the maintenance of normal IL 2 levels may be involved in the prevention of tumor growth (14, 17). In view of this data, I have examined production of IL 2 in splenocyte cultures of vaccinated animals and found these levels were in the normal range. This implied whatever factor(s) was responsible for suppression of IL 2 production by TBH splenocytes was not present in splenocyte cultures of vaccinated animals. Our previous work indicates a mildly nylon wool adherent Ts cell is responsible for curtailing IL 2 production in TBH (14, 17). Others have also implicated a Ts-mediated suppression of IL 2 production (67). Nylon wool separation of splenocytes in this study demonstrated a mildly nylon wool adherent cell, presumably a Ts cell as indicated by our previous

studies, was responsible for suppression of IL 2 production in TBH. This suppression was reversible by removal of the adherent cell fraction indicating TBH splenocytes were capable of producing normal amounts of IL 2, but prevented from doing so by suppressor cells. This finding is in complete agreement with our previous reports (14, 17).

Upon examination of IL 2 levels in normal and vaccinated host splenocyte cultures, I found no suppression of IL 2 production, which suggested functional suppressor cells were not present. These findings suggest a suppressor cell prevents IL 2 production in TBH but vaccination prevents the appearance of such suppressor cells. This contention is supported by evidence from others working in slightly different models. In an analogous situation, workers in the area of tolerance report that tolerance is the result of antigen overload and the subsequent generation of Ts cells, which in many ways is similar to a cancerous situation. In such a system, P. acnes treatment suppressed the appearance of functional Ts cells (103) but had no effect on the function of effector T cells (102). Furthermore, evidence indicates P. acnes may mediate this activity through interferon(s) (104). Several groups have shown P. acnes induces gamma interferon production in vitro (76, 103, 139), and some groups have reported it induces alpha and beta interferons (42, 139) . It is possible that P. acnes-induced interferon production functions in vivo in two modalities resulting in tumor prevention. These include: i) the prevention of Ts cell-mediated shut

down of the Interleukin cascade as suggested by Knop's work (102, 103, 104); and ii) interaction of gamma interferon with IL 2 to produce effective CTL. Gamma interferon has been reported by several groups to be necessary for CTL induction (44, 48, 93).

This study also examined the effect of P. acnes on IL 3 levels. Previous work in our laboratory demonstrated IL 3 production decreases with tumor growth (15, 16). IL 3 is reported to have many functions (78) and is generally thought to act at an early step during cellular differentiation (82, 84), including inaugurating the T cell maturational process and feeding cells into the Interleukin cascade to become Th cells (79, 80, 81). It is possible P. acnes may affect IL 3 levels. My studies demonstrated normal IL 3 levels in vaccinated mice, while significant depression of IL 3 production was noted in TBH splenocyte cultures. Thus, it appears vaccination maintains normal IL 3 production in T+V mice, as was the case with IL 2. Further examination of the apparent normality of vaccinated host splenocytes compared to TBH splenocytes by nylon wool fractionation implied a nylon wool-adherent suppressor cell was responsible for a majority of suppression of IL 3 production seen in TBH, and such suppressor cells apparently do not develop in vaccinated animals. Suppression of IL 3 production in TBH was reversible by removal of the adherent cell fraction, suggesting TBH splenocytes are capable of IL 3 production, but prevented from doing so by an overriding signal from a suppressor cell. Whether this suppressor

cell is a macrophage or a T cell remains to be determined, but it is possible the Ts cells previously shown by us to prevent IL 2 production also prevents IL 3 production. Both IL 2 and IL 3 are produced by Th cells, and it is easy to envision a Ts cell-derived factor which prevents Th cells from manufacturing both IL 2 and IL 3.

Nylon wool separation is a notoriously crude method for separating cell populations, and should be used only as a preliminary method to fractionate cell populations. When it is desirable to ascertain the precise composition of a population, studies employing specific antisera to macrophage and T cell populations are helpful. Studies using specific antisera would be useful in determining the lineage of the suppressor cell found in this study, as well as characterizing cell populations found in vaccinated mice versus normal mice or TBH.

TBH spleens are known to contain a large number of macrophages which manufacture many substances including the potent immunoregulator PGE (56), which could be mediating the suppression of IL 2 and IL 3 levels in TBH splenocyte cultures. Therefore, I examined the effect of the PGE synthetase inhibitor, indomethacin (53), on IL 2 and IL 3 production. Indomethacin treatment of normal host splenocyte cultures and those of vaccinated animals resulted in greatly enhanced IL 2 production, presumably due to lack of PGE. These findings suggested that in a normal animal PGE was important in maintaining homeostasis of IL 2

levels. This finding is supported by several studies which have reported PGE controls IL 2 production in both murine (73, 188) and human systems (21, 151, 170, 183). PGE-mediated control of IL 2 production is found in non-cancerous systems and apparently plays a role in maintaining normal homeostasis (56, 151).

It is feasible that PGE may mediate much of the tumor-induced suppression of the immune system directly via PGE, however this did not appear to be the case in this study. The addition of indomethacin to TBH splenocytes only appreciably enhanced IL 2 production early after the first appearance of tumor and suppression (Day 14). After tumor-induced suppression was established (Days 21 and 28), indomethacin did little to reverse suppression, indicating macrophage-derived PGE possibly played a role in early tumor-induced suppression, but that in late tumor-induced suppression, the role of PGE is minimal. In the human system, it has been reported that macrophage-derived PGE induces the formation of Ts cells, which inhibit IL 2 production (22). In addition, reports suggest PGE may act on IL 2 production directly (183, 188). Therefore, it is possible the initial suppression of IL 2 production in TBH seen at the appearance of tumor was due to PGE affecting IL 2 production directly by acting on the Th cell in some manner, and that suppression of IL 2 production seen as tumor growth proceeds was due to PGE-induced Ts cells which act on the Th cell, suppressing IL 2 production. In normal and vaccinated animals it

appeared PGE played a homeostatic role. Indomethacin treatment had little effect on IL 3 production by splenocyte cultures from any of the treatment groups, indicating suppression of IL 3 is not directly mediated by PGE, and the direct role of PGE in normal IL 3 production is minimal.

Collectively, this data supports the assertion made by others that local P. acnes-mediated anti-tumor activity is T cell-dependent (10, 173, 174, 191). Reports indicate the debilitation of the immune response is due to a decline in the functional capacity of TBH T lymphocytes (87, 143). Much of this lack of function is thought to be due to the development of Ts cells (38). This study suggested that in locally P. acnes injected animals, Ts cells did not form, resulting in maintenance of IL 2 and IL 3 levels. Others have proposed that normal IL 2, and possibly IL 3 levels are essential in the development of CTL in normal animals (127, 181). Thus, it appeared P. acnes may function by preventing Ts cell development, thereby allowing maintenance of normal IL 2 and IL 3 levels, which permitted tumor-specific CTL formation. This proposed mode of action is in agreement with finding by others that local P. acnes action is mediated by specific CTL (10, 173, 174, 191, 192).

Compilation of the results of this study with the work of others results in the following model depicting both normal and P. acnes

cell-mediated response as well as the inhibition of the immune response seen in TBH (Fig. 8). In this model, the Th cell can be viewed as the "hub" or pivotal point. Whether this Th cell receives a positive or negative immunoregulatory signal determines whether the host will exert an immune response or exhibit hyporeactivity.

In a normal animal, Th cells receive both the signal of IL 1 (133) from macrophages and also PGE2 (56) from macrophages, possibly two different subpopulations. These signals appear to have antagonistic affects which are balanced in a normal animal. IL 1 promotes IL 2 production by Th cells (133) while PGE2 controls IL 2 production by Th cells (183). Thus, in a normal animal homeostasis may be maintained by a delicate balance between IL 1 and PGE2. In response to alloantigen stimulation, the normal animal produces IL 2 which in turn regulates gamma interferon production (48, 93, 96, 101) and acts on a pre-CTL population (48, 97, 101). This pre-CTL population, in conjunction with IL 2 and gamma interferon, eventually matures into specific CTL (48, 101).

However, in the TBH system, the tumor cell has been reported to release a factor which inhibits IL 1 production (103). Once IL 1 production is inhibited, the balance between PGE and IL 1 may be disturbed. This may result in overproduction of PGE and PGE-mediated inhibition of IL 2 production, as well as PGE-mediated induction of Ts

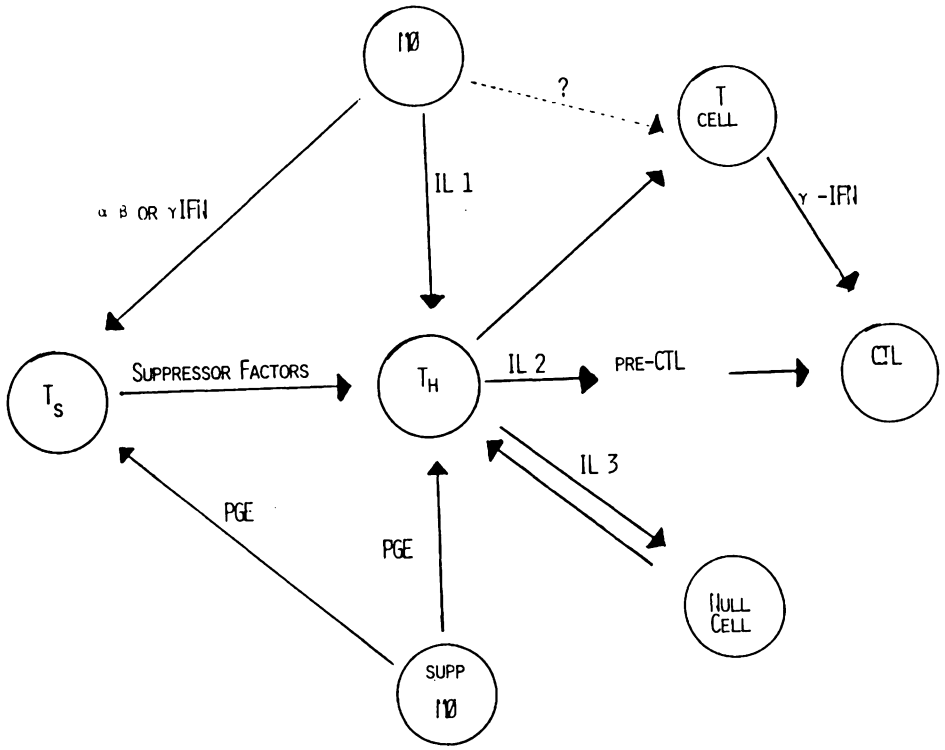


Figure 8. Proposed model of local P. acnes-mediated antitumor action. See text for explanation.

cells. Work in our laboratory has found TBH macrophages produce twice as much PGE as normal host macrophages (33). In addition, several groups have demonstrated PGE inhibits IL 2 production (151, 183, 188). PGE has also been reported to induce Ts cell formation (22). Both of these phenomena further suppress the immune response. My work suggested PGE-mediated suppression appears first, followed by Ts cell formation. Others have proposed similar biphasic suppression, the first phase consisting of suppressor macrophage and Ts cell induction, followed by the second phase, production of suppressor factors (52, 58, 147). Therefore, it is possible that tumor-induced Ts cell formation occurs via PGE, but it also possible that other tumor-derived factors induce Ts cells or that the tumor cells themselves are responsible for suppression (6, 63).

The action of P. acnes in this system appears to be multifaceted. One group has demonstrated tumor-derived suppressor factor inhibits IL 1 production by peritoneal macrophages but not by P. acnes-activated macrophages (103). Thus, it appears P. acnes-activated macrophages resist the initial perturbance of IL 1 by tumor-derived factor. P. acnes has been shown to stimulate IL 1 production in vitro (189), so it may be the negative effect of tumor-derived IL 1 suppressor factor is simply balanced by P. acnes stimulation of IL 1 production. My study suggested P. acnes also prevented suppressor cell induction, thus eliminating the second avenue of tumor-mediated disturbance of the

Interleukin cascade. Others have reported P. acnes inhibits Ts cell formation, apparently through stimulated macrophages which may inhibit Ts cell formation via interferon(s) (120). P. acnes-activated macrophages have not only been reported to prevent Ts cell induction, they have also been shown to absorb suppressor cell activity (195).

In summary, it appears P. acnes anti-tumor action may be due to several interrelated factors and ultimately mediated by T cells which arise via the Interleukin cascade. This study demonstrated IL 2 and IL 3 levels were maintained in P. acnes-treated mice. Others have shown P. acnes stimulates gamma interferon production as well as IL 1 production (139, 189). All of these factors are known to be important in successful CTL generation via the Interleukin cascade. P. acnes not only promotes CTL development, it also prevents Ts cell formation as indicated by this study and others (102, 103, 104). P. acnes-activated macrophages are reported to be responsible for this activity (120). P. acnes-activated macrophages are also responsible for IL 1 production. Thus, it appears local P. acnes anti-tumor action may arise from the cooperation of both macrophages and T cells, with the T cell being the final effector cell.

While this model seems plausible, it results from the concatenation of my work and the work of several groups studying various tumor models and tumor systems, as well as varying doses and routes of administration

of P. acnes, and therefore must be viewed with caution. Extrapolation of such results to obtain a preliminary working model is helpful but could be potentially misleading. Therefore, to confirm this model, it would be necessary to examine several of the components of this model in the specific context of one system such as the murine fibrosarcoma system employed in this study. This proposed investigation would entail: i) measurement of lymphokine and cytokine levels other than IL 2 and IL 3, such as IL 1 and alpha, beta and gamma interferon, as well as direct measurement of PGE levels; ii) characterization of cell populations present in P. acnes-treated animals using specific antisera; and iii) examination of the effect of P. acnes-activated macrophages addition to TBH splenocyte cultures. In addition, since P. acnes-activated macrophages have been reported to interfere with Ts cell formation via interferon(s) (104), interferon levels in such admixed cultures should also be measured.

Data from these proposed experiments would yield a better understanding of the mechanisms of P. acnes-mediated anti-tumor action. Hopefully, such knowledge may ultimately result in therapeutic applicability of various lymphokines, permitting external modulation of the TBH response to the host's advantage.

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