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THE EFFECT OF PROPIONIBACTERIUM ACNES ON POLYCLONAL B CELL ACTIVATION IN MICE

By

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ABSTRACT

THE EFFECT OF PROPIONIBACTERIUM ACNES ON POLYCLONAL B CELL ACTIVATION IN MICE

By

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The potent immunomodulator Propionibacterium acnes is capable of inducing autoimmune hemolytic anemia in mice. Polyclonal B-cell hyperactivity, the only consistent abnormality reported for mouse models of the autoimmune disorder systemic lupus erythematosus, was investigated. Polyclonal B-cell activation was evaluated by detecting spontaneous increases in the production of serum antibodies and antibody secreting cells against haptens to which the animal has never been exposed. Spontaneous antibody production was evaluated using ELISA and ELISPOT techniques.

P. acnes was capable of inducing IgM polyclonal B cell responses. This polyclonal activation was characterized by increased numbers of IgM anti-hapten antibody secreting cells and serum antibody concentrations which peaked around 5 days post P. acnes administration. The characteristics of the polyclonal B-cell response induced by P. acnes was similar to the polyclonal B-cell activation reported for lipopolysaccharide.

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INTRODUCTION

Administration of killed P. acnes, an immunomodulating bacterin, consistently produces a regenerative hemolytic anemia in mice.[180,50,51] After a single dose of P. acnes, reductions in hematocrit and hemoglobin are noted by day 2, reach their lowest levels around day 14-18, and gradually return to normal by day 40.[51,180] Hemoglobin and hematocrit values decline to about 60-80% of baseline and reticulocyte counts may increase to 20%.[51] Repeated injections of P. acnes can prolong the duration and increase the severity of the anemia.[51] A wide variety of mouse strains respond with a similar hemolytic episode although the degree of the anemia differs in intensity.[51,50,180]

The pathophysiologic mechanism(s) responsible for this anemia has not been fully elucidated. P. acnes does not induce direct hemolysis in vivo or in vitro.[182] Marked splenomegaly and splenic RBC sequestration are induced by P. acnes but anemia develops in both splenectomized and intact mice indicating that hypersplenism is not directly responsible for the anemic process.[51] A positive direct antiglobulin test (Coomb's test) is detectable as early as 5 days post P. acnes challenge and indicates the hemolytic anemia is immune mediated.[291,102]

Cox proposed that the P. acnes induced immune mediated hemolytic anemia was an "innocent bystander" hemolysis resulting from anti-P. acnes antibodies interacting with P. acnes antigen adsorbed to RBCs.[51] P. acnes does adsorb to RBCs in vitro[51,80] and mice do mount an antibody response against the P. acnes organism.[334] However, in vivo evidence supporting the

presence of P. acnes immune complexes on the surface of RBCs is lacking.

The specificity of the immune response appears to be directed at the RBC and not against any foreign substance attached to the RBC membrane. Autoantibody production post P. acnes has been detected with hemolytic plaque assays using neat isologous RBCs.[182] In addition, serum from P. acnes treated mice and antibodies eluted from their RBCs are capable of producing a positive indirect antibody test (indirect Coomb's).[291] This autoimmune response does not appear to result from shared antigens between the bacteria and the erythrocyte since no cross reactivity is noted between P. acnes and mouse RBCs.[180]

Autoimmunity is a phenomena that results when there is a breakdown in tolerance toward self antigens. With P. acnes treatment, autoantibodies have been detected not only against erythrocytes but against fibrinogen products and immunoconglutin, the third component of fixed complement.[50]

Murine models of autoimmunity have been extensively studied. A wide spectrum of autoantibody production is well documented in mice suffering from the autoimmune disorder, systemic lupus erythematosus (SLE). While T cell abnormalities can accelerate the onset of lupus in mice, few if any T-cell regulatory abnormalities have been found. The one consistent abnormality in all lupus mouse models is polyclonal B-cell hyperactivity.[66] P. acnes may induce autoimmunity by producing a similar polyclonal B-cell response.

P. acnes is an immunomodulator of both the specific and non-specific immune systems. Augmented humoral immunity has been documented for both T-cell dependent and independent antigen. Activated macrophages, stimulated by P. acnes, are more effective antigen presenters and they produce an undefined soluble proliferative stimulus for B cells.[214,283]

Spontaneous production of antibodies against serum soluble antigen of malaria and babesiosis has been detected in rats along with autoantibodies as early as five days post P. acnes inoculation.[30] The rapid onset of auto antibodies and spontaneous production of antiparasitic antibodies is suggestive of a polyclonal B cell response.

Spontaneous increased production of serum antibodies and plaque forming cells against haptens to which the animal has not been previously exposed is generally accepted as a response reflecting polyclonal B-cell activation.[66] The purpose of this dissertation is to evaluate spontaneous anti-hapten antibody production in mice after a single intraperitoneal injection of 2.3 mg. of killed P. acnes. Anti-hapten antibody levels in plasma were evaluated using enzyme linked immunosorbent assays (ELISA) and anti-hapten secreting spleen cells were quantified using enzyme linked immunospot assays (ELISPOT).

LITERATURE REVIEW

Propionibacterium acnes is a gram positive anaerobic bacterium, capable of altering the immunologic capacity of animals and man. This immunomodulation causes either immunopotentiation or immunosuppression of humoral and/or cellular immune responses by producing functional changes in macrophages, B and T lymphocytes and NK cells. This review will examine the mechanisms whereby these effects are mediated. Technical variables, in particular dose, route, schedule of administration and organism strain are not cited in detail unless relevant.

Taxonomy

P. acnes was originally classified as Corynebacterium parvum and was considered to be one of several strains of "anaerobic corynebacteria" based upon morphologic criteria.[234,67] However, a number of important characteristics markedly differ between anaerobic and classical aerobic Corynebacteria. Due to differences in oxygen requirements, cell wall components, antigenicity, fermentation tests and DNA homology the "anaerobic corynebacteria" were placed into the genus Propionibacteria and reclassified into 3 species, P. acnes, P. granulosum and P. avidum.[54,201,128] Of 59 strains of bacteria identified as C.parvum, 52 were found to be P. acnes.[54] Although C. parvum does not officially exist as a taxonomic entity, the name is well entrenched in the literature and is still used synonymously for P. acnes. Both names will be used as needed in quoting published works.

Various French (Pasteur and Merieux Institutes), English (National Collection of Type Cultures) and American (Burroughs Wellcome, HEW, Temple University, Virginia Polytechnic and American Type Culture Collection) strains have been reported in the literature as C.parvum. Activities vary from strain to strain.[336] The majority of research cited in this review used C.parvum obtained from Burroughs Wellcome Co. or Merieux Institute. The strains supplied by each source, though designated C. parvum, are not identical. They differ in physical and physiological properties.[81] However for the sake of simplicity, since they posses similar antitumor and immunomodulating capabilities, distinction between the two will not be made unless relevant.[336,81]

History

Anaerobic coryneform bacteria were first described in the medical literature by Meyer in 1926.[177] He identified,as Corynebacterium parvum infectiosum, a small gram-positive anaerobic rod-shaped organism isolated form a chronic and ultimately fatal infection in a woman. Similar or identical organisms were isolated from 23 other cases of which most were puerperal infections.

Prevot and his colleagues described a series of human patients that had reticuloendothelial system (RES) disease and concurrent bacteremia with anaerobic corynebacteria.[223,237,236,235] Prevot isolated 12 different strains of anaerobic corynebacteria. He was the first to observe that injection of animals(rabbits) with certain strains of heat killed anaerobic corynebacteria produced a marked stimulation of the monocyte-macrophage system.[234] Repeated injections led to the animal's death which he referred to as "lethal stimulation of the reticuloendothelial system."

Halpern in 1963 found that one particular strain, C. Parvum 938B, was especially potent in producing reticulostimulation as measured histologically and functionally by increased carbon particle clearance from the blood of mice. He also noted marked hepatomegaly and splenomegaly.[105] Halpern later used this strain to inhibit tumor growth in mice.[101,104] His anti-tumor studies helped establish C. parvum as the predominant anaerobic coryneform species for intensive research.

The immunomodulating activities of P. acnes has also been used experimentally to protect animals from a variety of infectious agents. P. acnes bacterin has mainly been successful in establishing prophylactic protection against selected viral,[38] bacterial[3] and protozoal[173] etiologies.

P. acnes has been intensely studied since the mid 60's in hopes of utilizing its immunomodulating activities for cancer therapy. During this era, hundreds of research and clinical studies have evaluated the anti-tumor activities of heat killed or formalin-fixed suspensions of whole organisms. Unfortunately, P. acnes did not turn out to be the simple treatment panacea as hoped. It's use in human patients has been less successful than anticipated[301,169,109,345] while commonly producing a variety of undesirable side effects.[55]

P. acnes is not an exotic or rare bacteria. Propionibacteria reside as saprophytes in and on the bodies of animals and humans.[250] It is commonly isolated from acne vulgaris lesions in humans. Relatively high levels of antibody to P. acnes are commonly detected in normal mice and humans.[334] Not surprising in view of the ubiquitous nature of the Propionibacteria and the high degree of serological cross-reactivity between strains.[128]

Morphological Alterations

Hyperplasia of lymphoid organs is the most striking morphologic alteration occurring after P. acnes inoculation. Spleen weights of mice increase 3 to 7 fold[105,35] and liver weights increase 2 fold[36] after a single intravenous or intraperitoneal injection of killed organisms. The degree of splenic and hepatic hyperplasia is directly related to the dose of P. acnes but the kinetics of the response are relatively dose independent.[5] The increase in organ weight is evident within a few days of P. acnes administration, peaks in the second week, and then gradually returns to normal by 4 to 6 weeks.[105,5,186] Similar findings have been reported in rats, rabbits and guinea pigs[227,269]. Splenomegaly is not a complication of P. acnes administration in humans most likely due to the much lower dosage levels used.[344]

The route of P. acnes administration markedly affects the tissue distribution of P. acnes and the distribution of P. acnes in turn correlates with lymphoid organ hyperplasia.[188] Subcutaneous administration generally causes less splenomegaly than intravenous or intraperitoneal injections.[269] Oral administration is without effect.[256] Subcutaneous P. acnes produces lymphadenopathy of regional lymph nodes with minimal enlargement of non-regional nodes.[36,186] Lymph node hyperplasia is not noted with intravenous administration most likely due to lack of bacterial sequestration in regional nodes.[263]

Splenomegaly is mainly the result of alterations within the red pulp due to increased cellular proliferation within the spleen[35] and/or cellular trapping of bone marrow derived cells.[53] Histologically, increased numbers of histiocytes, lymphocytes, macrophages, and hematopoietic cells are observed.[35] The number of extractable nucleated cells from enlarged spleens is about twice the number extracted for normal spleens.[20,5,315]

Although the number of nucleated cells per spleen is elevated, the number of nucleated cells per mg. of spleen is less than that of untreated controls. Therefore, a significant proportion of the splenic weight increase is the result of fluid, erythrocytes and/or cells not readily extractable by the usual methods.[344]

P. acnes induced lymph node hyperplasia is the result of a striking increase in cell division of lymphocytes and macrophages[305,214,43] In addition, there is sequestration (trapping) of lymphocytes.[53]

Hepatomegaly is usually associated with mononuclear cell infiltrates[105] composed predominantly of macrophages.[187] These cells form diffuse infiltrates or distinct granulomas. The macrophage population increases as a result of local replication of macrophages within the liver and from recruitment of macrophages from the bone marrow.[317]

Macrophage Activation

P. acnes is a very powerful monocyte-macrophage activating agent [105,237] and is chemotactic for macrophages even in the absence of serum.[326] Once attracted to the site of P. acnes deposition, the macrophage is rapidly activated by direct interaction between poorly defined plasma membrane receptors and peptidoglycan molecules on the surface of the bacteria.[216,235,53]

The P. acnes activated macrophage undergoes a variety of morphologic, biochemical and functional alterations. Lysosomal enzymes levels are elevated and the capacity to generate oxygen radicals increases in the macrophage after P. acnes activation.[180] Activated macrophages possess profound direct and indirect immunomodulating activity on the specific and nonspecific immune systems resulting in either immunostimulation or immunodepression. The immunomodulating actions of the P. acnes stimulated macrophage can therefore increase either

resistance or susceptibility to viral, protozoal, bacterial and neoplastic diseases.[90,173,14,339,153]

The seemingly contradictory effects of P. acnes on the immune system is probably the result of the heterogeneity of the activated macrophage population.[153] Activated macrophages can be separated into subpopulations based on morphologic and functional characteristics with different cell subsets performing different and often antagonizing immunomodulating tasks.[153,203]

The immunomodulating action that results is determined by the predominant macrophage subset which varies in a temporal manner post P. acnes administration. Early in the response to P. acnes, small to medium sized macrophages predominant.[153] These cells are derived from newly arrived macrophages and are mainly responsible for the accessory cell activity and immunostimulatory effects induced by P. acnes.[153] As the reaction to P. acnes continues, the nature of the macrophage population changes. Large more differentiated macrophages dominant.[153] This macrophage subpopulation is responsible for immunosuppression, cytostatic and cytotoxic activities.[153,203]

P. acnes markedly stimulates the phagocytic capacity of the monocyte macrophage system. It boosts not only nonspecific phagocytosis but also enhances removal or destruction of poorly opsonized particles, such as pathogenic bacteria or parasites encountered for the first time.[324] Although the rate and efficiency of in vivo antigen clearance is increased, the initial uptake of the antigen by P. acnes activated macrophages is slower than with normal macrophages. However, the large numerical increase in phagocytic cells post P. acnes administration easily compensates for the slower endocytic activity of the individual cell.[325]

Normal and P. acnes stimulated macrophages differ not only in phagocytic activity but also in antigen processing. Antigen is degraded to a lesser extent by the P. acnes stimulated macrophage with a larger percentage of antigen remaining on the cell membrane surface.[325] This potentially intensifies the presentation of antigen to lymphocytes.[325] In fact, P. acnes is capable of restoring the depressed accessory cell function found in tumor bearing mice.[220]

Interaction of P. acnes with the macrophage cell membrane, facilitates the release of monokines and other immune messengers that can augment both antigen dependent[300] and independent immunity.[322] P. acnes potentially augments the presentation of antigen to T and B cells by inducing changes in surface membrane receptors and recognition molecules. P. acnes produces an increase in the expression of Ia surface membrane antigen on alveolar and hepatic macrophages. Alveolar macrophages also develop increased numbers of Fc receptors.[171] Fc expression is variable amongst hepatic macrophages ,being increased, decreased or unchanged post P. acnes. The differential response to stimulation of Fc receptors among hepatic macrophages suggests that subpopulations of macrophages respond in different ways to P. acnes modulation.[171]

Suppressor macrophages appear to mediate their suppressive effects by the production of prostaglandins[130] and/or the release of superoxide, oxygen radicals and hydrogen peroxide.[116] At the present time it is unclear which of these substances is the predominant mediator of suppression or whether suppression may be a composite of the effects of several mediators.

T Lymphocyte Modulation

P. acnes can either suppress[123] or augment T cell function.[196,267] Moderation of T cell function appears to be controlled by activated macrophages. The ultimate effect of P. acnes on T cell function is influenced by time and route of bacterin administration.

T lymphocytes populations detected in the spleen and lymph node from P. acnes treated mice differ from normal. Relative numbers of splenic T cells are decreased by half after a single injection of P. acnes. However, absolute numbers of splenic T cells are approximately equal to those of untreated controls.[175,36] T cells in the lymph nodes have a slight relative decrease but an absolute increase.[107] In addition, P. acnes significantly decreases T cell numbers in the thymus of mice.[74] This effect was hypothesized to result either from a direct action on the thymus or through the release of adrenal steroids as a result of stress. It is not clear how alterations in absolute or relative numbers of T cells affect overall T cell function since the specific effects on different T cell subsets have not been defined.

T cell function, under certain experimental conditions, can be suppressed by P. acnes. In vitro responses such as mixed lymphocyte reactions and phytohaemaglutin responses as well as in vivo responses such as graft vs. host reactions are suppressed after intravenous administration of P. acnes.[264,263] P. acnes activated macrophages mediate the suppression with the inhibitory effect requiring cell to cell contact.[264,143] Prostaglandin E-producing suppressor macrophages, which are capable of suppressing T lymphocyte proliferation in vitro, have been incriminated as the effector cell responsible for depression of T cell function. Suppressor macrophages are detected in the spleen of mice 7 days after P.acnes therapy.[280]

Depression of T cell mediated responses is only temporary.[181,179,143] T cell activity declines around 10-14 days post P. acnes and returns to full activity by 15-21 days.[179,143] This short term amnesiac state is characterized by the presence of primed T cells and by the absence of T effector and suppressor cell.[179] The differentiation of the primed T cells is blocked by the failure to deliver to the primed T cells a differentiation or proliferation signal.[179] The exact mechanism responsible for this failed differentiation has not been defined.

In contrast to systemic depression of T cell function, T cell function at the site of P. acnes deposition may be accentuated. Deposition of P. acnes directly into neoplasms can augment both tumor specific delayed type hypersensitivity reaction[196] and cytolytic T cells.[260] These T cell functions may be increased locally due to the trapping of immunomodulating cells at the site of P. acnes deposition.[22]

B Lymphocyte Modulation

P. acnes under proper experimental conditions has either adjuvant[123,214] or suppressive activities[316,116,130] on the humoral immune system. The dosage level of P. acnes, route of administration of either bacterin or antigen, and the time interval between P. acnes administration and antigen exposure affect the level of antibody enhancement[123,112] or the development of antibody suppression.[49,6]

Augmented antibody responses are noted with T cell dependent[20,214,208,98] and T cell independent[123,62] antigens. P. acnes does not act as an adjuvant for antigens which by themselves are good immunogens but does possess powerful adjuvant properties for relatively weak antigens.[229,227] Both IgM and IgG are affected[49] and relative binding affinities of antibodies rapidly increase as part of the adjuvant activities.[227,228]

Adjuvant activity is present with high doses of P. acnes and lacking when low doses are used.[123] The time interval between P. acnes administration and exposure to antigen is also critical to the response. An intravenous injection of P. acnes 4 days prior to intravenous immunization with type 3 pneumococcal polysaccharide, a T-independent antigen, results in elevated specific antibody levels. In contrast, the intravenous injection of P. acnes simultaneously with type 3 pneumococcal polysaccharide depresses the antibody response.[123].

Other experimenters have found that not only is the timing of P. acnes and antigen important but also the route of bacterin and antigen administration. Intravenous administration of P. acnes always augments the response to SRBCs (sheep red blood cells), a T cell dependent immunogen, whether the SRBCs are injected intraperitoneally or intravenously.[89,49] However, the effect on antibody production is variable when P. acnes is administered intraperitoneally.[49]

Elevation or suppression of specific antibody production can result when P. acnes is injected intraperitoneally. The ultimate antibody response depends on the time interval between bacterin and antigen administration and upon the route of antigen exposure. P. acnes injected intraperitoneally augments the response to intravenously administered SRBCs. Augmentation also occurs when P. acnes and SRBCs are both injected intraperitoneally on the same day.[49] However, suppression occurs when the P. acnes is administered intraperitoneally 1 to 16 days before administering SRBCs by the same route.[49] In addition, intraperitoneal P. acnes given prior to a sensitizing or to a secondary challenge with SRBC is capable of inhibiting the development and the expression of memory cells.[49]

Although splenic plaque forming cell responses to sheep erythrocytes are depressed when the sheep red cells are given intraperitoneally after a previous intraperitoneal injection of P. acnes, total antibody levels obtain near normal titers.[6] The P. acnes suppression may therefore be limited to spleen cells with the suppressive activity remaining localized to the regions of P. acnes sequestration.

Inhibition of antibody production is attributed to peroxidative damage to B cells by activated macrophages.[116] Macrophages do not readily degrade P. acnes after phagocytosis. This provides a continuous stimulus for chronic low level enzymatic generation of superoxide. The release of superoxide and its derivatives (hydrogen peroxide, hydroxy radical) damage nearby lymphocytes and reduce B cell responses.[116]

Suppression of B cell responses occurs only at high dosage levels. B cells appear to have a higher threshold for suppression than to T cells.[143]

NK Cell Modulation

P. acnes can either elevate[107,111,219,192,217] or suppress NK cell activity.[26,192,217] The NK cell functional changes are time, dose and route dependent.[192,217,82] In addition, the degree of NK cell activation varies between rodent strains, between strains of P. acnes and between batches of the same strain of P. acnes prepared by different methods.[82] Suppression of NK activity occurs independent of T cells.[192]

When high doses are used, splenic NK activity is elevated for 1-3 day after P.acnes treatment followed 7 days later with marked depression of NK activity.[218,259,107] NK activity remains depressed for about 2 weeks.[192] Suppression of NK activity is weak and less consistent with low dose therapy[192,218] and with some systems the suppression of NK cell responses

does not occur.[217]

Interferon Modulation

P. acnes is a potent interferon producer both in vivo and in vitro.[115] Persistent levels of interferon are detectable in serum following P. acnes treatment of mice and rats.[115] Gamma interferon is the predominant form of interferon induce by P. acnes.[219,115,323] Interferon production post P. acnes is independent of mature T cells with no difference in interferon production noted between spleen cell cultures obtained from normal mice, athymic mice and cultures treated with anti-theta antisera.[115]

While direct increases in interferon production are not documented in cultures of peritoneal exudate cells from P. acnes treated mice, interferon production is enhanced, compared to untreated controls, when lipopolysaccharide or IL-2 are added to the culture media.[219]

Interferon production in P. acnes treated mice is dependent upon the presence of macrophages, although the macrophage is not the source of interferon production.[323,115] Interferon production occurs after the activated macrophages interact with NK-like cells.[219]

Anti-viral Activity

P. acnes can enhance host resistance to viral infections under appropriate conditions. Similar to anti-tumor and anti-bacterial protection, anti-viral activity can vary depending on the genetic composition[172] and age of the host[90] as well as the dose[293], strain[144] and administration route of P. acnes.[293,144] Critical however to the P. acnes anti-viral resistance is the timing of the P. acnes relative to viral challenge.[172,142]

Prophylactic P. acnes therapy given 3-12 days prior to experimental viral challenge effectively protects mice against acute infection with Herpes simplex virus,[144,142,292] influenza virus,[172] encephalomyocarditis virus,[90,38] murine cytomegalovirus,[90] Semlinki Forest virus,[90] mouse hepatitis virus[292] and vaccinia virus.[292] Prophylactic P. acnes protection is not universal to all experimental viral challenge systems. P. acnes was not able to protect mice against 100 LD_{50} doses of mouse adapted avian influenza A virus.[27]

While P. acnes given 3-12 days prior to viral challenge is protective, P. acnes given 2 days prior to or simultaneously with the viral inoculation affords no anti-viral protection.[144,142] Several days are therefore needed for P. acnes to induce anti-viral protective mechanisms. In various acute experimental models, fulminant viral disease can develop prior to the time required for P. acnes to produce its antiviral effect and no enhanced antiviral activity is observed.[293]

Therapeutic P. acnes immunomodulation also is ineffective against most established acute viral infections.[142] However, certain experimental chronic viral systems can respond favorably to therapeutic Propionibacterium administration. Chronic mouse hepatitis virus infection, when treated for 10 consecutive days with P. granulosum 2 weeks after viral inoculation, had significant lowering of the liver virus titers, less paralysis and decreased mortality.[292]

P. acnes may exert a systemic antiviral protective action. This is evident when intraperitoneal injections of P. acnes protect mice from viral challenge induced by either intraperitoneal or respiratory routes.[90] The route of P. acnes administration, however, can influence the degree of systemic protection. The effect of administration route on systemic antiviral protection is best exemplified by the finding that subcutaneous

P. acnes produces no anti-viral activity.[293]

Intranasal P. acnes provides mice a greater magnitude of protection against influenza virus than systemic P. acnes immunomodulation.[172] This suggests that local responses may also be modulated by P. acnes and may contribute to the overall resistance to viral challenge.

The antiviral response to P. acnes is age dependent. P. acnes does not enhance resistance to viral infection in suckling mice. A maturation of host defense mechanisms appears to be required prior to protective stimulation by P. acnes.[90]

The major anti-viral mechanisms induced by P. acnes are non specific in nature and are the result of enhanced macrophage activation,[90] NK cell activation [172,10] and/or interferon production.[142,172,141] Antiviral activity can be found without changes in specific immune responses such as cytolytic T cell activity, delayed hypersensitivity reactions or anti-viral antibody.[172] In fact, in mice pretreated with intranasal P. acnes 3 days prior to intranasal influenza viral challenge, significantly less infectious virus is present in their lung tissue compared to controls as early as 24 hours post viral challenge. This protection occurs before any potential specific immune response can be initiated and at a time when lung macrophage numbers have increased 5 to 10 fold.[172] Athymic mice have similar protective effects modulated by P. acnes as their thymus intact counterparts, indicating independence of the T cell immune response in P. acnes anti-viral activity.[172]

Only strains of propionibacteria capable of activation of the monocyte-macrophage system are able to produce anti-viral activity.[144] P. acnes activated macrophages are resistent to influenza viral infection when compared to the susceptible resident macrophages in normal lungs.[172] P. acnes prevents the secondary uncoating with release of viral genomic

DNA from the viral cores into the cytoplasm of activated macrophages. Adsorption of the virus to the plasma membrane, uptake into lysosomes and primary uncoating and release of viral cores into the macrophage cytoplasm, however, was not affected. These findings are consistent with interferon induced viral resistance.[42]

P. acnes can induce interferon production in cultures of murine and human lymphocytes.[115] High levels of interferon have been observed in serum of mice 5 to 12 days after intravenous or intraperitoneal administration of propionibacteria.[115,144] Maximal anti-viral activity correlates with increased interferon levels.[172] The failure of P. acnes to protect against viral infections when given simultaneously with virus may result from the viral disease developing faster than the P. acnes induce interferon levels.[293]

NK cell activity in the lung increased 8 fold post intranasal P. acnes. NK cell activity was further enhanced post infection with influenza virus.[172] It is worth noting that virus infection alone increases NK cell activity to about the same level seen after pretreatment of mice with P. acnes followed by virus infection.

Specific anti-viral cellular or humoral immunity post P. acnes has seldom been evaluated. Serum antibody titers to influenza virus measured 10 and 21 days after viral infection have no significant differences between control mice and mice pretreated with intranasal P. acnes.[172] Insufficient data is available to document whether P. acnes consistently fails to augment anti-viral antibody production.

Antibacterial Activity

P. acnes can render animals less susceptible to a variety of lethal bacterial infections, including Brucella abortus,[103,2] Bordetella pertussis,[2] Staphylococcus aureus,[2,278,145] Staphylococcus pyogenes,[145] Listeria monocytogenes,[290,254,340] Klebsiella pneumonia,[145] Streptococcus pneumoniae[46] Pneumococci[321,322] and Salmonella enteriditus.[49] The enhanced protection is not uniform and depends on the interaction of multiple variables supplied by the host, pathogenic organism and the p. acnes induced macrophage activation. Strain differences of P. acnes, methodology of bacterin preparation, dosage, route of inoculation and timing of the P. acnes inoculation all affect the efficacy of P. acnes protection against bacterial challenge. In addition, the pathogenicity of the challenge organism and its route of administration produced variable degrees of protection.

The major protective mechanism induced by P. acnes against acute bacterial infections appears to be the result of intense stimulation of the monocyte-macrophage system.[145] The protection has a biphasic pattern with the period of maximum protection varying between studies.[145,2,49] Intraperitoneal P. acnes stimulates an early short term local and nonspecific protection with maximal activity 1 to 2 days after P. acnes administration. During this short initial protective phase, P. acnes is found in large quantities within the peritoneal cavity with minimal systemic distribution.[145] This early protective phase occurs when neutrophil numbers and NBT reduction is enhanced.[339,145] This early phase does not always coincide with macrophage numbers or systemic activation of macrophages. Activated macrophage numbers do not increase until about 4 days post P. acnes treatment.[339]

Following the initial short term protective phase, there is a period of several days during which time the initial maximal peak in systemic macrophage stimulation declines, macrophages dominate the peritoneal exudation and no augmentation of bacterial protection occurs. After a 2 to 3 week lag period, a long term secondary phase of enhanced anti-bacterial activity occurs which lasts up to 3 weeks and coincides with a second though less pronounced peak in macrophage activation. During this secondary phase, P. acnes becomes systemically redistributed with moderate amounts in the spleen, pancreas, liver, bone marrow and lymph nodes of the thoracic duct.[145]

Early phase protection is independent of T or B cell function.[145] In fact, increased phagocytic activity and enhanced resistance is apparent in T cell depleted or nude mice.[145,254] The participation of T cells and B cells in the late phase of protection, although not essential, may be considered because lymphocytes increase numerically during the secondary protective phase.[145] The contribution of B cells can be questioned since no increased antibody titers to Salmonella enteritidis was noted in P. acnes treated mice.[44]

The results of P. acnes against E. coli is variable depending on the time sequence of administration. In fact, increased susceptibility to Salmonella enteritidis[44] and Escherichia coli has been reported.[340,339] Mice treated intraperitoneally with P. acnes 1 day prior to intraperitoneal E. coli challenge have increased resistance.[339,340] The predominant leukocyte in the peritoneal cavity at this time is the polymorphonuclea (PMN)[340] leukocyte. PMNs are reported to be of primary importance in host resistance to E. coli.[299]

In contrast, mice treated with intrperitoneal P. acnes 4 days prior to E. coli challenge are more susceptible to infection.[340,339] For the initial 3 hours post bacterial challenge, E. coli is more rapidly cleared from the P. acnes pretreated mice than untreated controls. At this time, activated macrophages predominate in the peritoneal cavity. These macrophages have enhanced phagocytic activity in vivo and a high degree of intracellular killing of E. coli in vitro.[340,339] However, activated macrophages are more susceptible to the cytotoxic effects of lipopolysaccharide (LPS) or endotoxin from gram negative bacteria than are nonactivated macrophages.[15,289] Macrophage numbers in P. acnes pretreated mice greatly diminish compared to controls after 6 hours post E. coli challenge. While E. coli numbers rapidly diminish in the first 6 hours post challenge, as the macrophage population declines the E. coli population increases to lethal levels.[301]

The reason for the increased susceptibility of P. acnes treated mice to E. coli is unresolved. The decline in macrophage numbers may interfere with normal macrophage trafficking of the inflammatory process with subsequent compromise of the innate immune system. Damaged macrophages may release biologically active mediators that compromise the cardiovascular system and contribute to shock. Another possibility is that autolytic or damaged macrophages release lysosomal enzymes that damage healthy tissue.

Anti-Parasite Activity

Pretreatment with P. acnes promotes resistance in mice to Babesia sp., Plasmodium sp., Trypanosoma cruzi, Leishmania sp., Toxoplasma gondii, Schistosoma mansoni and Echinococcus multilocularis.[49,48,41] Prophylactic administration of P. acnes can also protect calves from lethal challenge

with Theileria annulata.[173] Protection appears to be non-specific and is hypothesized to be the result of activated macrophages.[49,173]

Anti-Tumor Activity

Administration of P. acnes to tumor bearing animals can, under certain conditions, inhibit tumor growth or cause complete tumor regression.[329,285,190,265] However, the efficacy of P. acnes anti-tumor activity is highly variable. In fact, enhancement of tumor growth has been reported.[22,168,283]

P. acnes bacterin does not exert any direct cytotoxic effect on neoplastic cells.[307] The antitumor activity induced by P. acnes is complex and involves a predictable sequence of interactions between neutrophils, macrophages, cytolytic T cells, suppressor T cells and interferon gamma. The antitumor activity has a tumor antigen independent and tumor antigen dependent phase.[307] Both cytostatic and cytotoxic activities have been reported.[143]

The tumor antigen independent phase develops rapidly, has a relatively short duration and involves neutrophils, activated macrophages and interferon. Between 6 and 24 hours following intralesional treatment with P. acnes, in vivo tumor cytoreduction occurs. This initial anti-tumor activity coincides with the influx of tumoricidal neutrophils into the site of P. acnes inoculation.[161,159] P. acnes is capable of directly initiating this neutrophil chemotactic response[154] and, during this phase, neutrophils actively ingest P. acnes.[161] While small tumor loads can be eliminated by neutrophils, this initial response is not sufficient to completely eradicate a large tumor load (> 10^5 tumor cells).[159]

Activated macrophages also participate in the early rejection of tumors in a non-tumor specific manner.[26,202,11,12] The activation of tumor suppressive macrophages by P. acnes occurs in both athymic[88] and T cell deprived mice [131,88,23] and therefore does not appear to be dependent upon T cells and/or their products. Nor is the activation regulated by interferon gamma.[134] While the exact P. acnes component responsible for macrophage activation has not been delineated, it appears to be associated with some firmly bound component of the bacterial cell wall.[28]

There appears to be a cooperative effect between the bacteria and the neutrophils in the induction of cytotoxic macrophages. According to Lichtenstein and Chapes, macrophage chemotaxis and activation is mediated by those neutrophils which have responded to the presence of P. acnes.[161,39] When neutrophils are exposed to P. acnes in vitro and then transferred to recipients, the number of bacteria needed to induce cytotoxic macrophages is approximately 1/500th the dose needed to induce cytotoxic macrophages by direct intraperitoneal injection.[40] The granulocytes may be serving as efficient vehicles for targeting the bacteria to the macrophages or they may modify the bacteria in a manner that enhances P. acnes stimulatory activity.[40]

With in vivo and in vitro systems, those neutrophils which have ingested P. acnes are in turn phagocytized by macrophages. Only those macrophages containing intracellular P. acnes are cytotoxic for tumor cells.[39,185] Antitumor activity is directly related to the resistance of P. acnes to degradation after phagocytosis.[252] While not necessarily essential to macrophage activation by P. acnes, the neutrophil appears to accentuate the process.

In contrast, with in vitro systems depleted of neutrophils, sustained membrane stimulation of the macrophage by extracellular P. acnes is a prerequisite for tumoricidal activity.[134] Macrophage tumoricidal activity in vitro can be maintained for several weeks provided that free microorganisms are continuously present in culture. Macrophage tumoricidal activity rapidly declines when the macrophages are washed and free P. acnes removed even though the macrophages have engulfed and were degrading numerous P. acnes organisms.[133]

The activated macrophage antitumor phase begins between 48 and 72 hours after the administration of P.acnes bacterin[155], is maximal at 5 days[221] and can persist for up to 2-3 weeks.[134,221] The activated macrophage can suppress tumor growth in a cytostatic and/or cytotoxic manner.[202,11,12,143]

The cytostatic activity is short lived and appears around 12-14 days post bacterin administration and lasts for about 1 week. Tumorostatic activity is a function of large well differentiated macrophages.[153] Although the exact mechanism is not known, the activated macrophage may interfere with or inhibit either the production of or the response to a growth or differentiation factor.[143] P. acnes might operate through the activation of interferon which has antiproliferative effects on tumor cells.[141]

P. acnes activated macrophages and Kupffer cells produce elevated levels of cytotoxic factors and elevated production of oxygen radicles. No significant correlation was found between the levels of cytotoxic factor and oxygen radical production suggesting that different subsets of macrophages may be responsible for each activity.[108] The chemical composition of the cytotoxic factors were not determined. Nonspecific tumor cytolysis by PMNs and activated macrophages is of relatively short duration, only provides minimal prolongation of survival with most tumor

systems and is not sufficient to explain the overall antitumor activity of P. acnes.[16,35]

Alternatively to the nonspecific anti-tumor macrophage activity and perhaps coincidentally with it, tumor specific immunity is enhanced by the adjuvant effect of P. acnes. Peritoneal exudate cells from mice injected intraperitoneally with P. acnes have enhanced immunostimulatory activity.[153] Immunostimulation is a function of small and medium sized macrophages which are derived from newly arrived monocytes. The monocyte modulates the immune function via a soluble mediator which does not require direct cell to cell contact.[153]

Nonspecific tumoricidal macrophage activation can also be mediated by the release of soluble factors from P. acnes sensitized T lymphocytes as part of a delayed type hypersensitivity (DTH) reaction against the bacteria.[23,305] Cell mediated DTH against P. acnes has been reported to be an important effector immune response when P. acnes is administered into tumors even after the neoplasia is well established.[305,35] Macrophages after contact with P. acnes are more efficiently activated by lymphokines. Accentuated lymphokine activation can assumably potentiate the macrophages' anti-tumor responses.[76,77] The subsequent inflammatory reaction induced by the DTH against P. acnes organisms causes tumor regression in a non-specific manner.

With experimentally induced Meth A tumors , intralesional P. acnes results in striking tumor regression only in those mice previously given an immunizing dose of P. acnes. Utilization of a P. acnes sensitivity reaction to destroy tumor cells is restricted to a period corresponding to the time of maximal active responsiveness to the immunizing dose of P. acnes. Minimal anti-Meth A tumor activity is generated at sites distal to the immunomodulator's location.[305]

Intravenous injections of P. acnes can inhibit the growth of some tumor types. Tumor inhibition is not associated with increased numbers of macrophages but with increased levels of macrophages activation.[331,330] The tumor infiltrating macrophages are derived from blood monocytes which arrive at the tumor site in a pre-activated state.[200] Intratumor macrophage activation is usually inhibited by high concentration of suppressor factors generated at the tumor site.[286] Moore surmised that P. acnes is capable of surmounting these suppressor factors by activating monocytes at sites distant to the tumor where suppressor factors are at ineffective concentrations. Thus pre-activated monocytes/macrophages may enter the neoplasm and exert their antitumor mechanisms before being inactivated by tumor suppressive factors.[200]

P. acnes significantly augments specific antitumor responses which are mediated by T lymphocytes.[267] Regressing tumors are heavily infiltrated not only with macrophages but also with lymphocytes.[188] Cytolytic T cell antitumor activity occurs about 8-9 days post P. acnes inoculation.[69,194] Intralesional P. acnes augmented approximately eight-fold the number of cytolytic T cells within lymph nodes draining experimentally induced P815 mastocytoma compared to untreated tumor bearing control mice.[129] In addition, cytolytic T cells in the spleen were simultaneously increased approximately 14-fold.[129]

Long survival times are produced by P. acnes immunomodulation of antigen specific cytolytic T lymphocytes.[16,129,69,194] These cytolytic T lymphocytes direct their antitumor arsenal against neoplastic cells displaying tumor-specific transplantation antigens on their cell surface. Likhile found that permeating injections of P. acnes into growing tumors can result in rapid rejection of both the primary tumor and distant metastases.[164] Protection is afforded against subsequent tumor challenge

of identical but not against unrelated tumor cell lines.

Immunopotentiation of antigen specific cytolytic T cells is most likely the reason that P. acnes antitumor efficacy is directly related to tumor antigenicity.[329,285,342,190,189,139] Experimental animals become immune post P. acnes to further challenge with identical and strongly immunogenic tumors. However with weakly immunogenic tumors, no systemic immunity is produced and tumor rechallenge results in neoplastic development.[238] Weakly immunogenic tumors are general less responsive to the anti-tumor properties of P. acnes presumably due to the net tumor antigen burden being insufficient to exploit the adjuvant properties of P. acnes.[342]

Recent investigations indicate that tumors are capable of inducing the development of T suppressor cells.[29] These suppressor T cells appear at the onset of palpable tumor formation and suppress interleukin 2 production.[29] IL-2 is thought to promote cytotoxic T lymphocyte precursor proliferation and gamma interferon production.[131] Intralesional P. acnes treatment appears to maintain IL-2 production levels and prevents the development of tumor-induced suppressor cells.[249]

Augmentation by P. acnes of tumor specific DTH has also been reported.[196] The tumor specific DTH appears to have an antigen specific recognition stage and a non-antigen specific effector stage.[76,77] Miyata, using a Meth A fibrosarcoma system, documented tumor specific DTH immune lymphocytes with nearly equal activity in both P. acnes treated and untreated tumor bearing mice. However, macrophages from P. acnes treated mice were functionally more active and more effective in killing tumor cells in vivo and in vitro. The macrophages from the P. acnes treated mice seemed to be more responsive to lymphokine activation. Once activated, the macrophages were nonspecific in their tumoracidal activities.[196] P. acnes does not appear to produce any enhancing

antitumor antibody response.[285] However, P. acnes increases the antibody dependent cell mediated cytotoxicity of peripheral blood mononuclear cells and peritoneal exudate cells.[14,113] Monoclonal antitumor antibody has been used in conjunction with P. acnes to take experimental advantage of this phenomena.[257]

The P. acnes anti-tumor activity is highly dependent on a variety of ill defined factors. Genotype[222], age and stress level of the host[104], nature and size of the tumor[104,22,257] route of tumor cell administration [22], route and dosage of P.acnes[185,184,285,241], strain of P. acnes used[214,178,82,17] and the time interval between administration of P.acnes and tumor challenge[285,205,133] have all been implicated in affecting P.acnes' anti-tumor activity.[190] The balance between all these variables determines the effectiveness of the antitumor response of P. acnes. These variables have resulted in a variety of experimental conditions making comparison of the literature difficult since results from various laboratories are not always concordant.

The route of P. acnes administration can determine the ultimate anti-tumor protective response.[333,332] Direct contact between the immunostimulant and the tumor cells favors effective immunomodulation by P. acnes. Intralesional injection of P. acnes has produced regression of tumors in the mouse[267,165,196], rat[163], dog[170] and human.[185] Tumor regression has also been achieved when P. acnes is administered by intracavitary routes to treat tumors on serosal or mucosal surfaces.[174,14,319] While intraperitoneal P. acnes resulted in complete to partial regression in ovarian cancer in women, administration of P. acnes intravenously or subcutaneously did not augment the responses to chemotherapy in controlled randomized trials.[314] Likewise, subcutaneous grafts in mice of a mammary carcinoma or lymphosarcoma appear resistant to intravenously injected P. acnes.

However, a protective effect is achieved when P. acnes and tumor cells are given by the same route, either intraperitoneally or intravenously. Intravenous and subcutaneous P. acnes can promote tumor growth of highly antigenic tumors possibly by trapping immunomodulating cells at the site of P. acnes sequestration away from the tumor site.[22] Effective immunotherapy therefore requires administration of P. acnes directly into the neoplasm or into the compartment containing the tumor cells.[14,184,285]

The antitumor effects of P. acnes is dose dependent.[241] The lowest dose which produces the maximal antitumor response is regarded as optimal. Supraoptimal doses produce no higher level of antitumor activity and often diminish the desired antitumor effect.[241,93,186] High dosages of P. acnes enhances phagocytic function while producing little stimulation of cytotoxic T cells.[16] Lower dosages of P. acnes produces prolonged immunity associated mainly with cytotoxic T lymphocytes and to lesser degrees with NK cells.[16] Disappointing results have been reported in various human studies using relatively high dosages of P. acnes administered intravenously[301,169] or subcutaneously[109] while encouraging results have been reported with smaller dosages of P. acnes administered directly into the neoplasm.[16,185,158,14] Both the route of P. acnes administration and the therapeutic dosage probably contributed to the lack of antitumor efficacy in many clinical trials.

P. acnes prophylactic activity can vary from one tumor system to another. Potent prophylaxis is obtained against nonisogeneic Ehrlich ascites and isogeneic AKR leukemia tumor systems. However, negligible suppression is present against isogeneic YC8 lymphoma cell lines.[253] The difference in the response between isogeneic and nonisogeneic tumor cells is most likely directly related to P. acnes ability to augment better the specific immune defenses against strongly antigenic tumors.[285]

The tumor burden affects the results achieved with P. acnes. Excessively high challenge doses of tumor cells can erase the potential protective activity of P. acnes.[329,104,266] The most marked therapeutic activity has been achieved against small tumor burdens in hosts that have maintained immunocompetence.[13] The optimal tumor load for maximizing P. acnes antitumor effects occurs when the neoplastic cell numbers are sufficient to produce 100% tumor mortality in control groups.[253]

P. acnes has less anti-tumor activity in aged mice than young adults.[26,342] In spite of the relatively weaker response to P. acnes, prolonged survival times are produced in aged mice. Repeated intraperitoneal injections of P. acnes are required to induce temporary tumor regression. The altered response is proposed to have resulted from a suppressed immune system in aged mice which have only 10-20% of the humoral and cellular immune activity found in young adults.[341]

P. acnes combined with chemotherapy, irradiation or hyperthermia can in certain experimental situations augment the effectiveness of either therapy alone.[26,307] To exert this synergistic effect, P. acnes could not be administered indiscriminately as the results were dependent upon the timing of P. acnes administration post tumor implantation.

Acute psychological stress, as produced by social interactions or electrical shock, can reduce the antitumor actions of P. acnes in mice.[304] Psychologic conditions can induce the release of endogenous corticosteroids. Stress can reduce the specific antitumor immune response by enhancing suppressor T-cell function[210,152] and suppression of cytolytic T-cell activity.[152] Reduction of the anti-tumor immune response by suppressor T-cells can allow active growth of immunogenic tumors. In addition, glucocorticosteroids may potentially inhibit the anti-tumor activities of P. acnes presumably by stabilizing the macrophage cellular

membranes which prevents the subsequent exocytosis of lysosomal enzymes.[268]

Diethylstilbestrol, another steroid hormone, inhibits P. acnes induced antitumor activity in vitro and in vivo by decreasing the ability of P. acnes to activate antitumor macrophages.[1]

P. acnes may actually interfere with effective anti-tumor immunity. This immunosuppressive effect is mediated by prostaglandin E producing suppressor macrophages which inhibit T-cell immune responses.[266,279] These suppressor macrophages are derived from the bone marrow and their appearance has a temporal relationship to the P. acnes administration.[279] Time course studies indicate the emergence of macrophages capable of suppressing T-cell function about 7 days post P. acnes challenge.[285] This suppressive macrophage subpopulation is composed of large morphologically complex macrophages with accentuated phagocytic capabilities.[203,153]

Terpenning described a macrophage produced soluble suppressor factor that was antigen dependent and prohibited in a genetically unrestricted manner the ability of pre cytotoxic lymphocytes to differentiate into mature functional effector cells.[300] The macrophage may therefore be able to suppress T-cell function in both an antigen dependent and independent fashion.

Nature of the Immunomodulating Factor of P. acnes

A number of compounds of bacterial and nonbacterial origin have been found to modulate the immune system. These compounds have been notably diverse in both substance and function thereby complicating attempts to discover any unifying mechanism for immunomodulating activity.[318] One potential problem in deciphering the activity of P. acnes is due to the complexity of the organism. P. acnes contains multiple determinants that independently affect different subsets of cells. Each subset has

different functions some of which may be beneficial and some harmful to the host.[162,160]

Reticulostimulatory and immunomodulating activities are not unique to P. acnes. Although little systematic testing has been done with gram positive organisms, anti-tumor effects have been reported for certain strains of Lactobacillus, Bifidobacteria, Streptococcus, and Listeria.[132,147,258,176] Only strains of Bifidobacteria have reported reticulostimulatory activity as measured by splenomegaly.[147]

Immunomodulation is not unique to gram positive organisms. Certain gram negative bacteria most notably strains of Bordetella pertussis, E. coli and Salmonella typhimurium, have reticulostimulatory and anti-tumor activities. Acid fast Mycobacterium bovis, Bacillus Calmette-Guerin (BCG) strain, posses antitumor, reticulostimulatory and immunomodulating capabilities.[343,231,193] Muramyl dipeptidase, the active component of BCG; lipid A, the active component of E.coli, S. typhimurium and B. pertussis and pertussis toxin, an additional active component of B. pertussis; are not present in P. acnes.[294,148,8]

The reticulostimulatory activity of P. acnes is associated with some firmly bound component of the cell wall with peptidoglycan or a peptidoglycan-polysaccharide complex being essential for activity.[235,53,240,4,248,128,55,239,17] Mechanical disruption of P. acnes results in loss of activity directly proportional to decreased particle size.[4,53,28] Recombination of disrupted cell walls and protoplasm does not restore reticulostimulatory or antitumor activities.[28,35] Bacterial cells remaining after extraction with a variety of neutral solvents (ie. chloroform, methanol) had lost little if any reticulostimulatory or other biologic activity.[4,53,28,35]

Various poorly defined substances with reported biological activity have been extracted from P. acnes. A phenol/water extracted glycopeptide had anti-tumor and reticulostimulatory activity albeit less than whole P. acnes.[4] In addition, lipid extracts have been described with reticulostimulating and chemotactic properties.[79,80,255,222] Phospholipid extracts produce macrophage activation, increase phagocytic and bactericidal capacity of the liver and spleen, activate NK cells and result in interferon production.[79,80,53] However, the characteristic hepato-splenomegaly induced by whole P. acnes is not produced and residue remaining after extraction retains antitumor activity that is as effective as whole cells.[171] One author was unable to confirm any macrophage activation from lipid or water soluble extracts using the techniques of the authors previously cited.[28]

P. acnes appears to have multiple determinants that independently affect different subsets of cells with different biological function.[162,160] Biochemically derived fractions of P. acnes are capable of producing more selective effects on host cells. A pyridine extract is capable of eliciting antitumor PMN activity but is ineffective in producing macrophage activation. This pyridine extract is not able to provide the same degree of antitumor activity as the whole P. acnes organism. The extraction process has depleted the bacteria of some critical determinants necessary for successful immunotherapy.[156]

The controversy over the active component of P. acnes has not been resolved. The various biologic properties ascribed to P.acnes may not necessarily reside in a single molecule or molecular complex. P.acnes , like B. pertussis, could possess multiple active factors as suggested by the reported activity of both water soluble and lipid extracts. It seems that the bacteria need to be presented to the host in the intact form in

order for all of the P. acnes immunomodulating capabilities to be produced.

Complications

Transient pyrexia, nausea, emesis, headache, hypertension, diaphoresis and malaise are common following intravenous, intralesional, intraperitoneal or subcutaneous administration of P. acnes in humans.[14,114] Abdominal pain is an additional problem associated with intraperitoneal injections.[14] Local granulomas commonly develop after intradermal injections at the high end of the dosage scale.[265] Serum bilirubin and SGOT levels tend to be mildly elevated in many patients.[114,330] Splenomegaly is not noted but the dosage in people is considerably less than that commonly used in animal experiments.

Thromboembolic complications have been documented in man and mice following intravenous administration of P. acnes.[195] P. acnes stimulates macrophages to produce a procoagulant tissue-factor-like substance which requires factor VII for its expression. This strong procoagulant activity is therefore capable of activating the extrinsic pathway of blood coagulation.[195]

P.acnes bacterin consistently produces hemolytic anemia in mice. This phenomena has been reported in 12 different mice strains[102,182,211,51,291] and in rats.[321,50] The anemia is detectable between 3 and 5 days after P. acnes administration, peaks between days 14 to 18 and has resolved by day 40.[182,51] Concurrent with the anemia is a reticulocytosis that is consistent with the hemolytic nature of the condition.[182,291] Hemoglobin values decrease by 2 to 4 grams/dl and reticulocyte values range from 4 to 15%.[51] The degree of anemia and reticulocytosis vary with the mouse strain and dosage of P. acnes. The pathogenesis of P. acnes induced anemia has not

been fully elicited although several theories have been proposed.

In vitro erythrophagocytosis by splenic macrophages is augmented after administration of P. acnes.[180] Nussensweig first proposed that the anemia resulted from accelerated RBC destruction secondary to P. acnes induced hyperactivity of the splenic monocyte-macrophage system.[182] The pathogenesis was likened to the hemolytic anemia produced by zymosan activation of the monocyte-macrophage system. Zymosan increases erythrocyte phagocytosis by the spleen in the absence of any detectable anti-RBC antibodies.[97] The pathogenicity of hypersplenism has been subsequently refuted since splenectomized and non-splenectomized mice develop hemolytic anemia of similar magnitudes and durations.[51]

RBC autoantibodies have been detected in mice concurrent with the P. acnes induced hemolytic anemia.[102,214] A positive direct Coomb's test occurs as early as 5 days post P. acnes injection and is still present on post treatment day 25.[214] Spleen cells producing antibody against isologous red cells, as measured by the hemolytic plaque assay, are detectable 2 days after P. acnes administration. Anti-RBC plaque activity peaks by 5-7 days post treatment and gradually declines to control levels over the next 7 - 10 days.[182]

Autoantibodies, however, have not been consistently found in all mice treated with P. acnes.[102,51] Although P. acnes produces anemia consistently in C57/Bl and NZB mice, only some animals of both strains developed anti-RBC antibodies as detected by the direct antiglobulin test. The response is more marked in the NZB strain possibly reflecting an acceleration of their genetic predisposition to the development of spontaneous autoimmune hemolytic anemia.[102] In contrast to other reports, McCraken was unable to detect the presence of RBC autoantibodies in 8 different mouse strains treated with P. acnes.[182] It is not known whether

the differences in RBC auto-antibody detection resulted from variables in technique, Coomb's reagent or individual mouse responses.

Cox was able to detect in P. acnes treated rats the presence of auto-antibodies against fibrinogen products and immunoconglutinin.[30] He surmised that auto-antibodies to these serum proteins produced immune complexes that nonspecifically adhered to erythrocytes. These immune complexes, he theorized, opsonized RBCs and resulted in their subsequent removal by the activated monocyte-macrophage system. Positive direct anti-globulin tests reported in P. acnes treated mice were therefore not detecting anti-RBC antibody but immune complexes adsorbed to the RBC surface. Although plausible, no direct evidence was presented to substantiate the presence of immune complexes on the RBC surface or this pathogenic mechanism.

The detection of auto-antibodies against RBCs and soluble serum proteins suggests that P. acnes is capable of activating clones of lymphocytes having activity against self. All individuals possess the capabilities of producing antibodies against self. This potentially harmful anti-self activity is normally suppressed to prevent auto-destruction. P. acnes appears to produce a malfunction in the suppressive regulatory controls over auto-reactive lymphocyte and to manifest auto-immune disease.

EXPERIMENT I

Indirect Enzyme-Linked Immunosorbent Assay

Indirect enzyme linked immunosorbent assay, ELISA, is an immunodiagnostic procedure used to qualitatively or quantitatively detect specific antibody activity in biologic fluids.[309,311,230,191,151,31] ELISA methodology is adaptable to small diagnostic laboratories due to technical simplicity, stable reagents and relatively inexpensive equipment requirements.[309,311,151,7] The indirect ELISA uses polyvinyl or polystyrene microtiter wells, tubes, beads or other suitable materials as a solid phase platform upon which all subsequent antigen antibody interactions are immobilized and enzymatically visualized.[243] Indirect ELISA sensitivities are in the ng/ml range, equaling or surpassing in some procedures the sensitivity of radioimmunoassays and immunofluorescent techniques.[311,288,75,71,72,223]

In order to detect specific antibody activity by the indirect ELISA, all appropriate immunoactivity must be immobilized on an insoluble support.[337] This support, called the solid phase, is sensitized by passively absorbing or cross-linking to its surface an appropriate antigen.[74,310,151] The antigen sensitized solid phase serves as a stage upon which all subsequent specific immunoactivity will become immobilized (Figure 1a). Once the solid phase is antigen sensitized, any potential nonspecific protein binding sites on the solid phase are blocked by the addition of a protein that is immunologically inert in the assay system.[31] Ideally, the resulting solid phase has immunoreactivity limited to the

antigen coating the ELISA test surface.

When the test sample is added, any antibodies capable of complexing with the sensitizing antigen become, via their specific antigen bridges, specifically bound to the solid phase (Figure 1b). Nonreacting antibodies, which can potentially interfere with test results, remain free in solution and are eliminated from the test surface by gentle washings.[151,310,75,328] The solid phase therefore extracts only antigen specific antibodies from the test sample.

Antibodies bound to the solid phase are detected immuno-enzymatically via an enzyme labelled indicator antibody. The enzyme label is attached to the indicator antibody in a manner retaining both immunologic and enzymatic activity.[206,99,327] The immunologic activity of the indicator antibody is directed against the solid phase bound antibody derived from the test sample. When the enzyme labelled indicator antibody complexes with the test sample antibody, the indicator antibody and its enzyme label become in turn bound to the solid phase (Figure 1c). Nonreacting indicator antibodies are removed by gentle washings.[309,311,151,337,261]

Solid phase bound enzyme activity is detected by the addition of a chromogenic substrate. The intensity of the resulting enzymatically produced color change is directly proportional to the amount of test sample antibody complexed to the antigen on the sensitized solid phase.[309,151] Quantification of antibodies in test samples is accomplished by comparison with standard antisera.[310]

ELISA METHODOLOGY

a. Solid Phase Is Antigen Sensitized

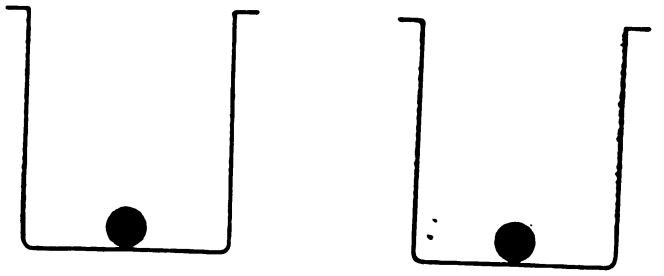
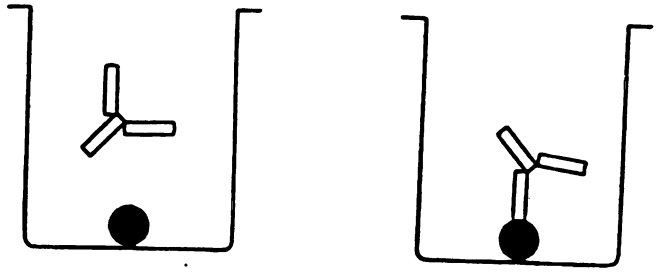


Plate Washed

b. Test Sample Added

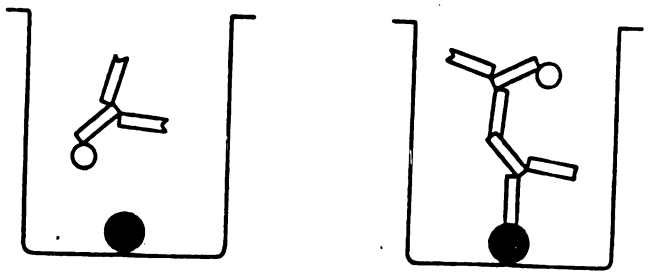


No Antigen Specific Antibody Present

Antigen Specific Antibody Present

Plate Washed

c. Enzymes Labelled Antibody Added

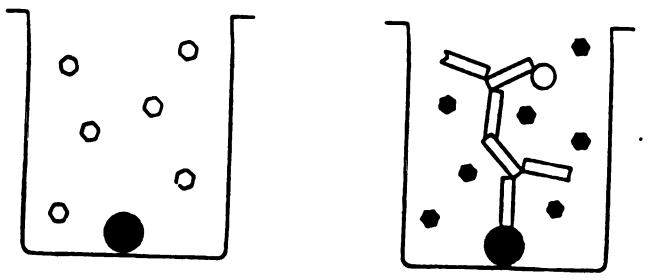


Conjugate is Not Immobilised On Solid Phase

Conjugate Is Fixed To Immobilised Antibody

Plate Washed

d. Chromogenic Substrate Added



No Substrate Hydrolysis Indicates Lack Of Antigen Specific Antibody In Test Sample

Substrate Hydrolysis Is Proportional To Antigen Specific Antibody In Test Sample

Figure Experiment 1.0

Objectives

The objective of this experiment was to devise specific and precise indirect ELISA methodologies to detect mouse serum IgM and IgG antibodies against the haptens fluorescein isothiocyanate (FITC) and 2,4,6-trinitrobenzene sulfonic acid (TNP). Anti-hapten methodologies have been previously described and have sensitivities of less than 1 ng/ml.[310] Although the theoretical concept of indirect ELISA testing are relatively simple, numerous variables in reagents, materials and technical expertise can effect test results.[309,328] Therefore a methodical systematic approach was utilized to evaluate each test component and to establish optimal testing conditions.

Materials and Methods

Haptens: Fluorescein isothiocyanate (FITC) isomer I and 2,4,6-trinitrobenzene sulfonic acid (TNP) were purchased from Sigma Chemical, St. Louis, Missouri.

Protein-Hapten Conjugates: Egg albumin-TNP and egg albumin-FITC conjugates were used to immunize mice for anti-hapten control sera production. Fetal calf sera-TNP (FCS-TNP) and fetal calf sera-FITC (FCS-FITC) conjugates were used to sensitize the ELISA solid phase.

TNP conjugates were produced according to the technique of Rittenburg.[246] Twenty-four mgs. of TNP were dissolved in 2 mls. of 0.1 M phosphate buffer, pH 7.1. One hundred mgs. of egg albumin or FCS protein were added and constantly stirred during a 1 hour incubation at room temperature. The solution was dialyzed against 0.1 M PBS, pH 7.2, until no free TNP was detectable in the dialysate by spectrophotometric scanning. The resulting egg albumin-TNP or FCS-TNP conjugates were aliquoted and stored at -20° C.

FITC conjugates were produced according to the technique of Press.[232] Ten mg. of FITC isomer I were combined with 75 mg. of either egg albumin or FCS protein in 1 ml. of 0.238 M sodium bicarbonate. The solution was incubated at 20° C for 24 hours. The solution was then dialyzed against 0.1 M PBS pH 7.2 until the dialysate had no visible fluorescence under U.V. light. The resulting egg albumin-FITC and FCS-FITC conjugates were aliquoted and stored at -20°.

Since TNP and FITC are light sensitive, all reaction steps and subsequent anti-hapten test procedures were done with subdued light or in the dark.

Mice: BalbCxC57 male and female mice, ranging in age from 6 weeks to 1 year were utilized. Mice were bred, housed and cared for by the Laboratory Care Services in the Clinical Center at Michigan State University under standardized conditions that conform to ALAAS conditions.

Anti-Hapten Control Sera: Egg albumin-TNP and egg albumin-FITC were diluted 2 to 1 with aluminum hydroxide adjuvant to give a final protein concentration of 10 mg./ml and 7 mg/ml, respectively. The immunization dosages were based upon the protein concentration of the egg albumin-hapten conjugate. Mice were immunized intraperitoneally with either 1.5 mg. (1.5 mls.) of egg albumin-TNP or 1.4 mg. (1.5 mls.) of egg albumin-FITC. Mice injected intraperitoneally with 0.15 mls. of 0.1 M PBS, pH 7.2, served as negative controls.

Nine days post treatment, mice were bled via the ophthalmic venous plexus while under ether anesthesia.[33] After 20 minutes at room temperature, the clotted blood was centrifuged for 10 minutes at 1000 g's.[110] The serum was harvested, aliquoted and stored at -20° C until analyzed.

Peroxidase Conjugated Antisera: Horseradish peroxidase conjugated goat anti-mouse IgG and IgM affinity purified antisera was commercially purchased (Cooper Biochemical Inc., Malvern, Pa.). The specificity of each antiserum was assessed by both double immunodiffusion[32] and immunoelectrophoresis[37] against whole mouse sera and FCS. Antisera producing only a single line of identity against mouse sera and having no activity against FCS were utilized.

Chromogen: 3,3' 5,5'-tetramethylbenzidine hydrochloride (TMB) was purchased commercially (Sigma Chemical Co. St.Louis, Mo.). TMB is one of the most sensitive chromogens for the detection of low enzyme concentrations and has the added advantage of being noncarcinogenic.[137,308,24,87,118]

Polystyrene Microtiter Plates: Polystyrene, 96 well, round bottom microtiter plates (#2587 Corning Glass Works, Corning, N.Y.) were used as the solid phase. Plates were tested by lot, using a technique modified from Voller et al.,[311] for acceptability with ELISA methodology. Three plates per lot received 200 ul per well of a 1:100 dilution of pooled mouse sera in 0.05 M carbonate buffer, pH 9.6. After incubating overnight a 4° C, the plates were washed 3 times in 0.1 M PBS, pH 7.2, containing 0.05% Tween 20. Two hundred ul. of horseradish peroxidase labelled goat anti-mouse IgG antisera; diluted 1:200 in 0.1 M PBS, pH 7.2, containing 0.05% Tween 20 and 1% FCS; was added to each well. Following a 2 hour incubation at room temperature, the plates were washed 3 times with 0.1 M PBS, pH 7.2, containing 0.5% Tween 20. Two hundred microliters of the chromogen substrate were added to each well of the microtiter plate. The chromogen substrate consisted of 0.626 M 3'3 5'5 tetramethylbenzadine in borate buffer, pH 5, containing 0.003% hydrogen peroxide. After 15 minutes at room temperature the enzymatic reaction was stopped by the

addition of 100 ul of 1 N HCL. Absorbance of the resulting yellow colored reaction for each well was determined at 360 nm. Only lots of plates with a between well coefficient of variation of less than ± 10% were acceptable for ELISA methodologies.

Data Reporting Format: All raw absorbance data was converted to the percent of positive control method for ELISA reporting.[63] The percent of positive control value was calculated by dividing the test sample absorbance by the positive control absorbance and multiplying by 100%.[100] This reporting format has interpretive simplicity, requires only a single serum dilution and reduces the rigorous quality control of substrate incubation time since absorbance ratios remain constant within limits throughout substrate incubation.[63,100]

A positive to negative ratio was used when comparing sample results for levels of nonspecific background noise. The percentage positive control value for positive control mice were compared to that of negative control sera. Background noise was considered to be within acceptable limits if this comparision had a ratio of 5:1 or greater.[311]

Blocking of Nonspecific Protein Uptake: Nonspecific binding of immunoreagents to the solid phase is usually prevented when Tween 20 and/or protein are included in diluents and washes. These precautions were not sufficiently effective for the anti-TNP or anti-FITC ELISA protocols used in this laboratory.

FITC-FCS and TNP-FCS diluted in either 0.1 M carbonate buffer, pH 9.6, or 0.1 M carbonate buffer containing 0.2% FCS, pH 9.6, were compared for suitability in sensitizing the solid phase of the anti-hapten ELISAs. All sensitizing reagents remained within the test well during an overnight incubation at 4° C. Standard ELISA protocol was subsequently followed and the results compared for sensitivity and specificity.

Determination of Optimal Test Conditions for anti-TNP and anti-FITC Indirect ELISA: The anti-hapten indirect ELISA is a modification of the basic indirect ELISA procedure pioneered by Enguall and Perman.[310,74]

All steps were done under subdued light or in the dark to prevent inactivation of TNP or FITC. FCS was added to all reagents having incubation stages to reduce potential background activity from nonspecifiec protein uptake. Optimal test specifications for the solid phase sensitizing antigen, test sera and peroxidase conjugated antibodies were determined by methodical stepwise titrations.

Optimal solid-phase sensitizing conditions were determined for each FCS-hapten conjugate. FCS-TNP and FCS-FITC were diluted in 0.05 M carbonate buffer containing 0.2% FCS, pH 9.6, to a final FCS-hapten protein concentration of 360, 72, 14 or 2.8 ug./ml. One hundred ul. of the appropriately diluted FCS-hapten conjugate were added to individual microtiter wells. Microtiter wells receiving 100 ul. of 0.05 M carbonate buffer containing 0.2% FCS served as negative controls to monitor for undesirable background activity.

FCS-hapten conjugates were passively absorbed to the solid phase during an overnight incubation a 4°C. Unbound antigen was removed by gently washing the wells 4 times with 0.1 M PBS, pH 7.2, containing 0.5% Tween 20.

To prevent nonspecific protein uptake, unbound protein binding sites on the solid phase were blocked by adding to each well 200 ul. of 1% FCS in 0.1 M PBS, pH 7.2. Plates were incubated for 3 hours at room temperature and subsequently flicked to remove the blocking reagents but not washed.

After blocking, the solid-phase produced by each sensitizing antigen concentration was chessboard titrated against sera from normal mice and mice immunized against egg albumin-TNP or egg albumin-FITC. All mice sera were diluted in 0.1 M PBS ,pH 7.2, containing 0.5% Tween 20 and 0.5% FCS. Sera dilutions of 1:100, 1:200 and 1:400 were evaluated. Diluent without mouse sera served as a controls to monitor nonspecific background noise. Microtiter plates were incubated for 2 hours at room temperature followed by 3 washes with 0.1 M PBS, pH 7.2, containing 0.5% Tween.

The indicator antibodies used for subsequent visualization of the anti-hapten immune complexes were goat anti-mouse IgG or IgM antibodies conjugated with horseradish peroxidase.[137] The indicator antibodies were diluted in 0.1 M PBS, pH 7.2, containing 0.5% Tween 20 and 0.5% FCS. Dilutions of 1:100, 1:200 and 1:400 were used to evaluated the optimal working conditions for anti-mouse IgM indicator antibodies. Anti-IgG indicator antibodies were assessed for optimal activity using dilutions of 1:250, 1:500 and 1:1000. One hundred ul. of appropriately diluted peroxidase conjugated antibodies were incubated in the microtiter wells for 2 hours at room temperature. Unreacted indicator antibody was removed by 4 washes with 0.1 M PBS, pH 7.2, containing 0.5% Tween 20.

The soluble chromogenic substrate 3-3', 5-5' tetramethylbenzadine (TMB) was used to visualize the antigen-antibody complexes bound to the microtiter wells. Fifteen mg. of TMB were added to 100 mls. of deionized water containing 0.70 gms. of borax and 0.36 gms. of succinic acid. The mixture was stirred vigorously for 2 hours at room temperature then filtered through a #1 Whatman filter to remove any undissolved TMB. Immediately prior to use, 2 mls of 0.03% hydrogen peroxide was added to 10 mls. of the filtered TMB solution and gently vortexed. One hundred ul. of the hydrogen peroxide "activated" chromogen was placed in each well and

allowed to interact with any solid-phase bound enzymes. Enzymatic activity was stopped after 15 minutes at room temperature by the addition of 100 ul. of 1 N HCl.

The absorbance from each well was determined at 360 nm. using an ELISA plate reader. The ElISA reader was blanked against background noise control test wells that received all reagents except mouse sera. Samples were run in duplicate and the results averaged. Minimal requirements for test acceptability was an absorbance reading greater than 0.50 and a minimum positive to negative ration of 5:1.[311] The concentration of antigen, test sera and indicator antibody producing the highest absorbance value with the least amount of background activity was determined to be optimal.

Precision: Once optimal concentrations of all reactants were established, within run and between run precision were determined. Mouse IgM and IgG anti-TNP and anti-FITC antibodies were semiquantitated on 3 separate runs. Egg albumin-TNP and egg albumin-FITC immunized mouse and normal control mouse sera were analyzed. Five duplicate test wells for each mouse were evaluated on each run. A predetermined positive control sera for each hapten served as a reference standard to which all other sera were compared. Comparisons were made using the percent positive ratio method which expressed test results as a percentage of the standard reference sera's absorbance value. Intra and inter assay precision were determined from the coefficients of variation (CV)[137,18,63] calculated from the positive sera's test results.[63]

Specificity: The specificities of the anti-hapten ELISA tests were evaluated by an inhibition assay.[100] Mouse sera from egg-albumin-TNP and egg albumin-FITC immunized mice and sera from normal control mice were optimally diluted for their appropriate assays in 0.1 M PBS, pH 7.2.

Free TNP or FITC were added to different aliquots of each test sera. The final free hapten concentration was 50 ug/ml. The free haptens were incubated for 30 minutes at room temperature with test sera to specifically inhibit appropriate antibody activity. Both neat and hapten inhibited sera were evaluated using the indirect anti-hapten antibody ELISA protocols determined previously. Absorbance values of non hapten inhibited sera were compared to hapten incubated sera and the results expressed as percentage inhibition.

RESULTS

Specific Antibody Activity Against FCS: No significant background IgM or IgG anti-FCS activity was detected by ELISA in sera from control, egg albumin-FITC immunized or egg albumin-TNP immunized mice (Figure Experiment 1.1).

	% Concentration of FCS Used to Sensitize ELISA Solid Phase				
	0	0.008	0.04	0.2	1.0
IgM Anti-FCS	0.006 +/- 0.001	0.007 +/- 0.002	0.000 +/- 0.002	0.001 +/- 0.002	0.005 +/- 0.002
IgG Anti-FCS	0.004 +/- 0.003	0.004 +/- 0.002	-0.001 +/- 0.003	0.004 +/- 0.001	-0.003 +/- 0.002

Figure Experiment 1.1
Mean Absorbance for All Mouse Sera Tested

FCS sensitized wells tested with PBS in lieu of mouse sera had similar baseline readings and variability to those tested with mouse sera (data not shown). The lack of antibody activity against FCS permitted the use of FCS as a blocking reagent to prevent nonspecific protein binding to the solid phase of the ELISA test wells. In addition, FCS was a suitable

protein to use as a hapten carrier for sensitizing the solid phase.

Blocking with FCS to Reduce Nonspecific Protein Uptake by the Solid Phase: Prevention of nonspecific solid phase binding by test samples and/or reagents was augmented by adding 0.2% FCS to the 0.05 M carbonate buffer used to dilute the solid phase sensitizing antigen. This assay modification was required in addition to the more conventional approach of adding protein and/or Tween 20 to all immunoreagents subsequent to the solid phase sensitization step.

Use of a blocker protein during the solid phase sensitization step is not utilized with most conventional ELISA procedures. However, without this blocker modification the anti-FITC ELISA had extensive undesirable background noise. FITC wells sensitized without the addition of the 0.2% FCS blocker had respective negative control sera absorbance readings for the IgM and IgG anti-FITC assays that were 60.1% ± 3.6% and 55.5% ± 10.5% of the readings obtained from positive control sera. IgM anti-FITC activity for negative control sera was reduced to 0.0% and IgG anti-FITC activity was reduced to 5.8% ± 0.8% when the solid phase sensitizing reagents contained 0.2% FCS (Figure Experiment 1.2 & 1.3).

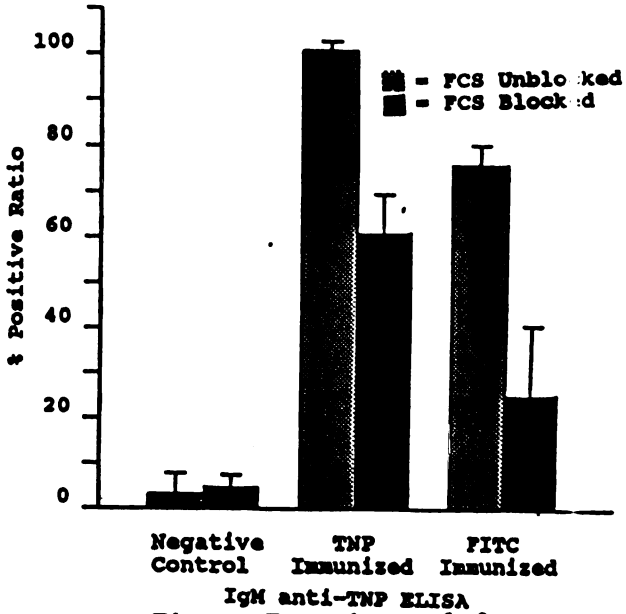


Figure Experiment 1.2

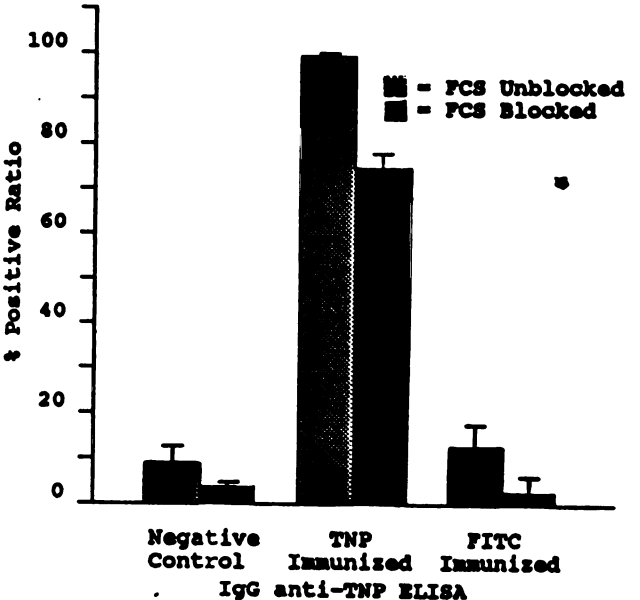


Figure Experiment 1.3

The 60.1% reduction in negative control sera activity was similar to the 68.7% reduction in positive control sera activity for the IgM anti-FITC assay after FCS blocking. With the IgG anti-FITC assay, negative control sera activity was reduced 49.8% and positive control sera activity declined by 35.0% post FCS blockage. The similar reductions in assay results after FCS blockage suggests that nonspecific protein binding to the solid phase was artificially elevating the absorbance readings for all sera samples tested for anti-FITC activity.

Regardless of the omission or addition of 0.2% FCS to the sensitizing buffer, IgM and IgG anti-TNP activity for negative controls were less than 10% of the positive control values (Figure Experiment 1.4 and 1.5).

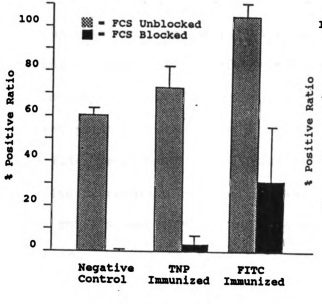


Figure Experiment 1.4

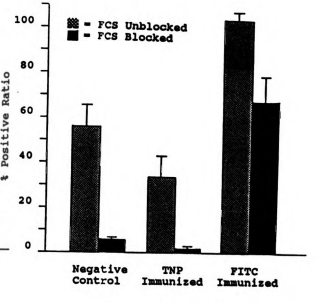


Figure Experiment 1.5

The 40.6% reduction in IgM anti-TNP activity and the 23.8% reduction in IgG anti-TNP activity for positive control sera post FCS blockage exceeded the potential background noise for the negative control. This suggests that FCS blockage had minimal effect on anti-TNP specificity but reduced anti-TNP assay sensitivity. However, post FCS blockage absorbance readings remained above 0.050 absorbance units which was the predetermined lowest acceptable limit for the ELISA assays. In order to maintain consistency between the anti-FITC and anti-TNP ELISA protocols, 0.2% FCS was incorporated into the solid phase sensitizing solutions for all subsequent anti-hapten ELISA protocols.

Titration of Immunoreactants: The optimal working conditions for the indirect anti-hapten ELISAs were determined by a methodical stepwise comparison of various concentrations of solid phase sensitizing hapten, test sera and peroxidase labelled antibody. The optimal concentration for all immunoreactants was defined as that concentration resulting in an absorbance value greater than 0.050 and producing the greatest positive to negative ratio. For each variable being evaluated, the highest absorbance reading obtained from the positive control sera was determined to be the percent positive control reference point to which all other test readings were compared. The positive negative ratio was determined by comparing the percent positive ratio value from TNP immunized mice with that obtained from FITC immunized mice. To maintain adequate specificity, a minimum positive to negative ratio of 1:5 was required.

TNP-FCS and FITC-FCS protein concentration of 3, 14, 72 and 360 ug/ml of coating buffer were evaluated for effectiveness in sensitizing the solid phase of their respective ELISAs.

For the IgM and IgG anti-TNP ELISAs the optimal sensitizing concentration was 72 ug of TNP-FCS protein/ml of sensitizing buffer (Figure Experiment 1.6 and 1.7). At this antigen concentration the IgM and IgG anti-TNP ELISA had respective readings of 0.063 and 0.099 with maximal positive to negative ratios of 5:1 and 17:1.

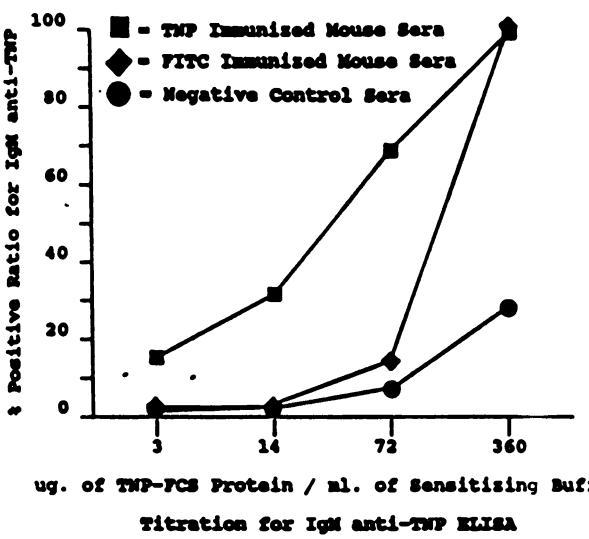


Figure Experiment 1.6

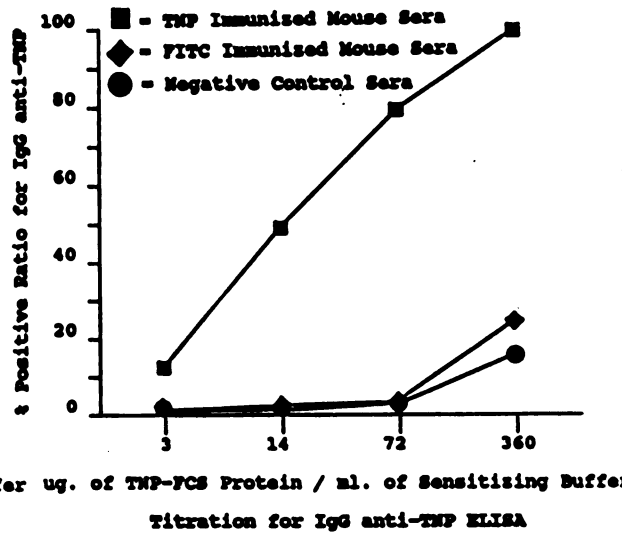


Figure Experiment 1.7

The IgM anti-FITC ELISA's optimal sensitizing concentration was 14 ug. of FITC-FCS protein/ml of sensitizing buffer (Figure Experiment 1.7). At this antigen concentration the IgM ELISA had an absorbance reading of 0.104 and a maximal positive to negative ratio of 10:1. The IgG anti-FITC ELISA had an optimal sensitizing concentration of 3 ug. of FITC-FCS protein/ml of sensitizing buffer (Figure Experiment 1.8). At this antigen concentration the IgG anti-FITC ELISA had an absorbance reading of 0.103 and a maximal positive to negative ratio greater than 100:1.

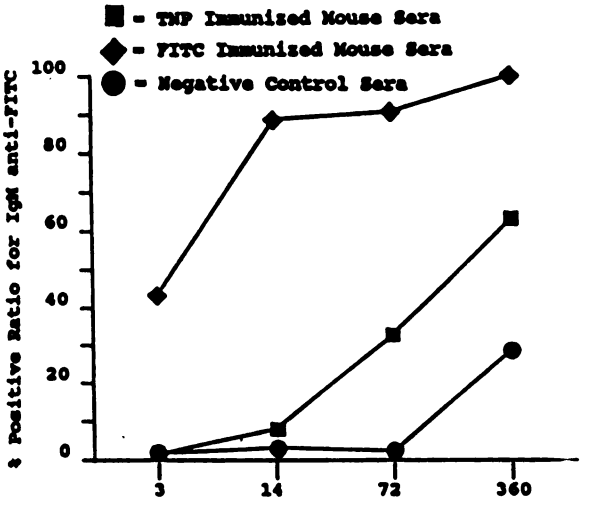


Figure Experiment 1.8

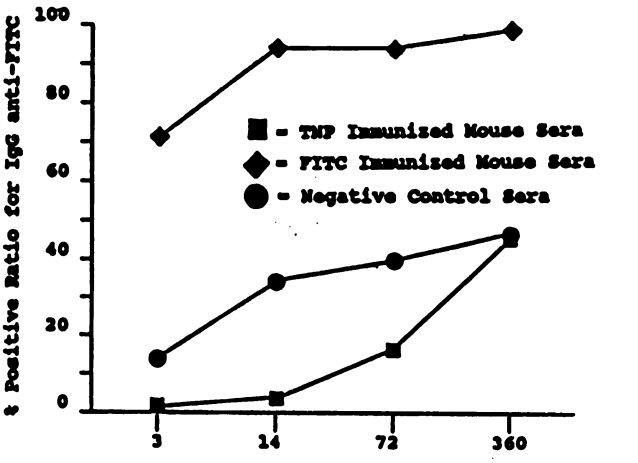


Figure Experiment 1.9

Sera dilutions of 1:100, 1:200 and 1:400 were evaluated to determine the optimal sample needed to detect anti-hapten antibody concentrations. Because of the number of tests being run and the limited volume of sera available from an individual mouse, the 1:100 dilution was determined to be the maximal concentration that could be used without exhausting the available sera supply.

Optimal sera dilutions for the IgM and IgG anti-TNP ELISA were 1:100 and 1:200 respectively (Figure Experiment 1.10 and 1.11). For the IgM anti-TNP ELISA, the positive control sera, diluted 1:100, had an absorbance reading of 0.081 and a positive to negative ratio of 6:1. The 1:200 dilution of the positive control sera, had an IgG anti-TNP absorbance value of 0.097 and a positive to negative ratio of 7:1.

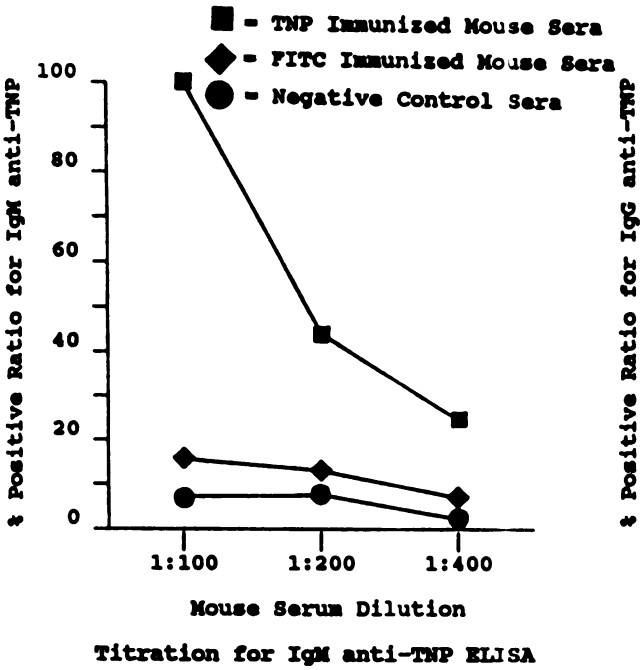


Figure Experiment 1.10

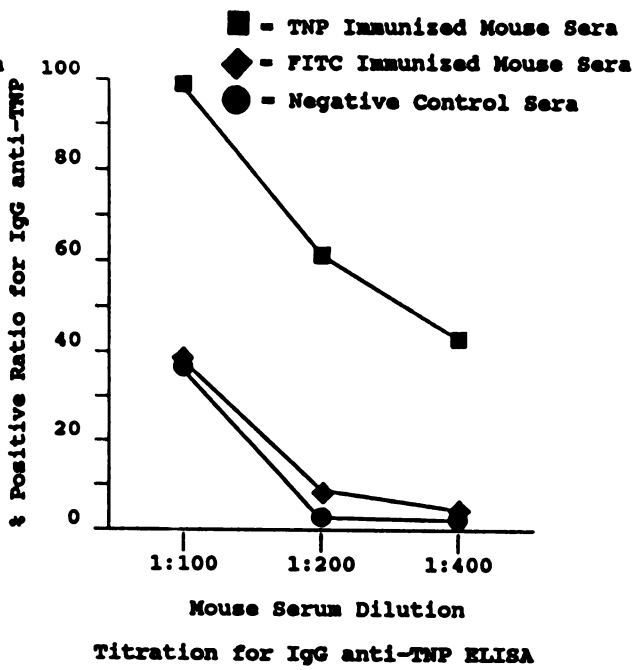


Figure Experiment 1.11

The IgM and IgG anti-FITC ELISAs also had optimal sera dilutions of 1:100 and 1:200 (Figure Experiment 1.12 and 1.13). The 1:100 dilution of the positive control sera had an IgM anti-FITC absorbance reading of 0.127 and a positive to negative ratio of 18:1. The positive control sera diluted 1:200 resulted in an IgG anti-FITC absorbance of 0.152 and a positive to negative ration of 51:1.

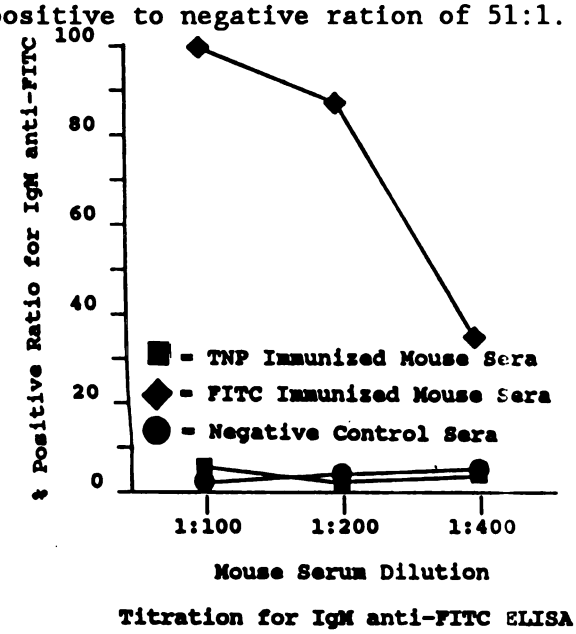


Figure Experiment 1.12

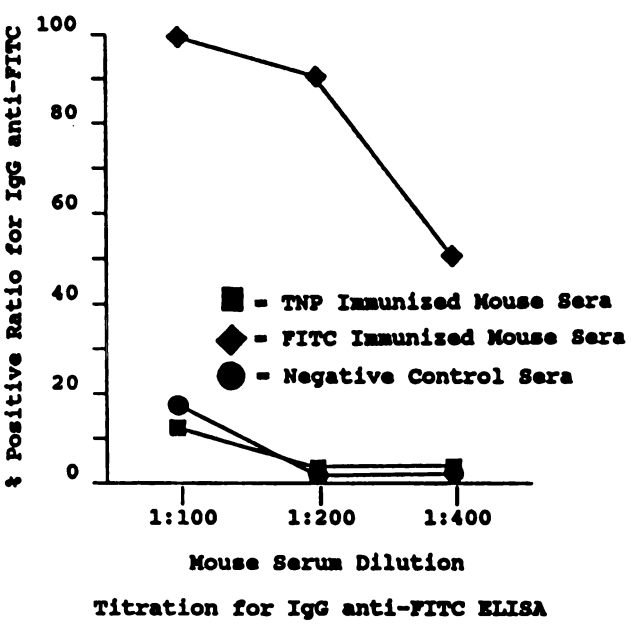


Figure Experiment 1.13

Optimal working concentration of the indicator antibody were determined by evaluating 1:100, 1:200 and 1:400 dilutions of peroxidase conjugated anti-mouse IgM and 1:250, 1:500 and 1:1000 dilutions of peroxidase conjugated anti-mouse IgG.

The optimal dilution of the peroxidase conjugated anti-IgM mouse antisera was 1:400 for the IgM anti-TNP ELISA (Figure Experiment 1.13). With this concentration the absorbance reading was 0.054 and the positive to negative ratio was 14:1. The peroxidase conjugated anti-mouse IgG antisera had an optimal dilution of 1:500 for the IgG anti-TNP ELISA (Figure Experiment 1.14). This dilution resulted in an IgG anti-TNP absorbance of 0.092 and a positive to negative ratio of 7:1.

For the IgM anti-FITC ELISA, the optimal peroxidase conjugated anti-mouse IgM dilution was 1:200 (Figure Experiment 1.15). This concentration resulted in an IgM anti-FITC absorbance value of 0.080 and a positive to negative ratio of 9:1. A 1:500 dilution of peroxidase conjugated anti-mouse IgG produced an IgG anti-FITC absorbance of 0.120 and a positive to negative ratio of greater than 100:1 (Figure Experiment 1.16).

Precision: IgM and IgG anti-TNP and anti-FITC antibody levels were determined on sera collected 9 days post immunization with either egg albumin-TNP, egg albumin-FITC or PBS. Two mice from each treatment group were tested. Three consecutive daily runs were evaluated (Figure Experiment 1.17).

Means and standard deviations were determined within and between runs for all samples. Coefficients of variation were calculated for all positive test controls. Comparisons were made between precision information calculated from absorbance readings and data converted to percent positive ratios.

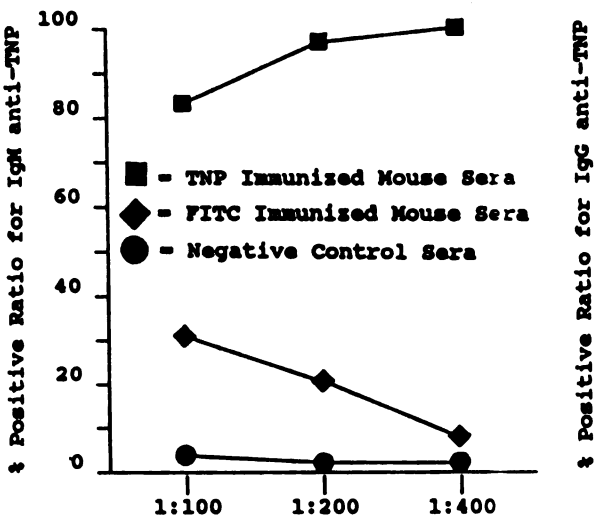


Figure Experiment 1.14

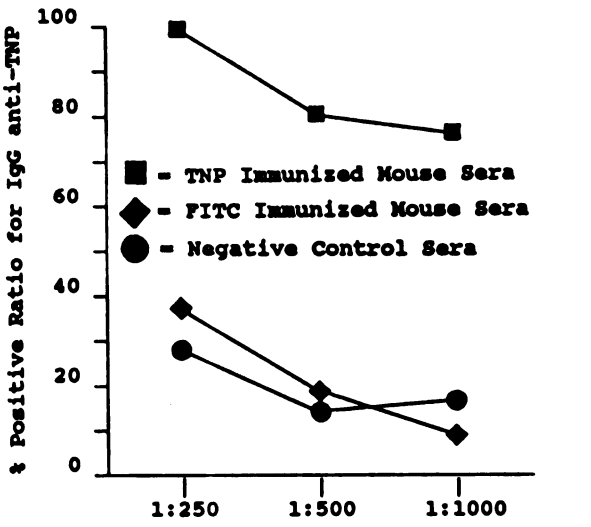


Figure Experiment 1.15

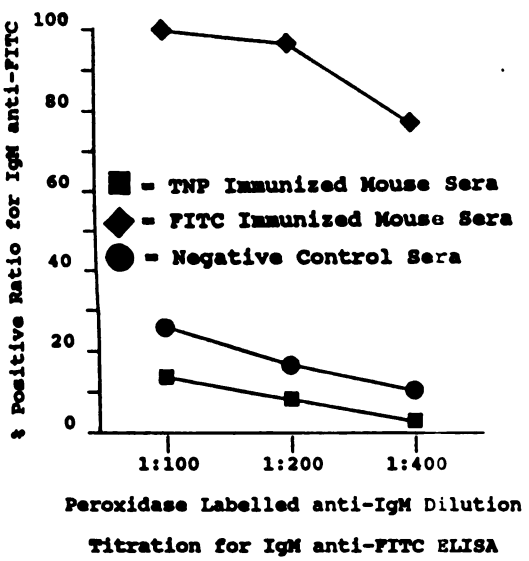


Figure Experiment 1.16

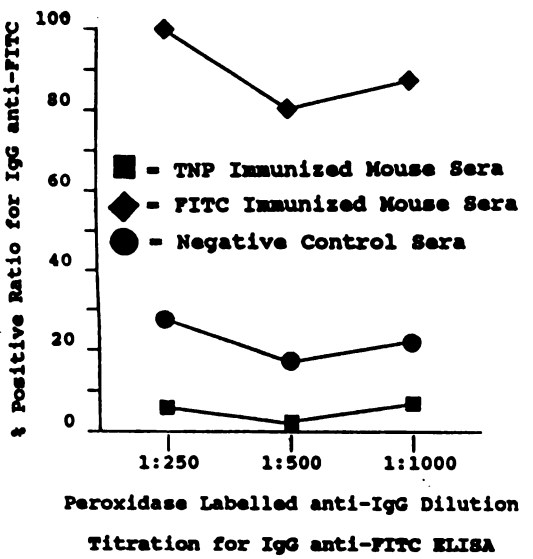


Figure Experiment 1.17

The percent positive ratio reporting methodology expresses test results as a percentage of the absorbance value obtained from a standard positive reference sera (percent positive ratio = absorbance of test sample/absorbance of reference sample x 100%). Use of percent positive ratio data reduced the between run coefficient of variation for positive control sera by 65%. This reduction was statistically significant (p=.005) by the t test for comparison of sample means. No significant change for within run variability was found. All laboratory data and discussions will use percent positive ratios unless otherwise specified.

The within run standard deviation (Figure Experiment 1.18) of the means for all positive control sera (n=8) and for all test samples (n=72, data not shown) were respectively 6.3% ± 3.0% and 5.9% ± 4.4%. The equivalent value in absorbance units was 0.006 ± 0.003. Absorbance variability between blank ELISA wells containing only PBS was 0.002 ± 0.004. Using the t test for comparison of sample means, no statistical difference (p=.001) in absorbance variance was found between PBS blank and test wells. The absorbance variability for the 4 anti-hapten ELISA tests is comparable to assay variances reported in other ELISA systems.[140,241,139]

		CV for Absorbance Values					CV for % Positive Ratio Values				
	Positive Control	Run 1	Run 2	Run 3	Within Run	Between Run	Run 1	Run 2	Run 3	Within Run	Between Run
IgM Anti-TNP	TNP Immunized A	10.2	8.6	12.8	10.5	11.8	10.2	8.7	13.1	10.7	4.6
	TNP Immunized B	14.5	7.7	11.4	11.2	17.6	13.9	7.6	10.8	10.8	18.6
IgG Anti-TNP	TNP Immunized A	8.7	2.3	2.5	4.5	7.4	8.7	2.2	2.5	4.4	3.1
	TNP Immunized B	4.8	4.2	7.9	5.6	12.3	4.8	3.3	7.9	5.3	6.0
IgM Anti-FITC	TNP Immunized A	9.1	3.9	7.3	6.8	10.7	9.5	4.2	7.1	6.9	3.4
	TNP Immunized B	6.9	5.8	5.2	6.0	8.0	7.1	5.9	5.4	6.1	6.3
IgG Anti-FITC	TNP Immunized A	2.3	3.4	6.1	3.9	8.2	2.1	2.8	6.1	3.7	5.3
	TNP Immunized B	3.4	2.4	2.6	2.8	7.1	2.7	3.6	2.6	3.0	7.2
		Mean			6.4	10.4	Mean			6.3	6.8
		SD of Means			+/-3.0	+/-3.5	SD of Means			+/-3.0	+/-5.0

Figure Experiment 1.18
Standard Deviation for Positive Control Sera

The percent positive ratio, between runs, standard deviation of the means for all positive control sera (n=8, Figure Experiment 1.17) and for all test sera (n=72, data not shown) were respectively 6.8 $\pm$ 5.0 and 6.7 $\pm$ 5.1%. The equivalence in absorbance units was 0.008 $\pm$ 0.005. A statistical difference between PBS blank well and between runs means was found by the t test for comparison of sample means. The increased variability between runs is expected due to minor fluctuations in day to day reagent composition, environmental conditions and user fatigue. Both

the means and range of the CV for the anti-hapten ELISAs were comparable to other reported ELISA protocols.[251,140,139]

The positive control sera between runs coefficient of variation ranged from 3.1% to 18.6%. Tests with CV of less than 10% are conventionally considered acceptable. The IgM anti-TNP ELISA had the only CV greater than 10%. The positive control sera used for the IgM anti-TNP precision run produced the lowest absorbance values (means for the 2 controls were 0.051 and 0.068) compared to the other anti-hapten ELISA protocols. The inherent variability of spectrophotometric readings have a greater influence on the CV when absorbance readings are within or below this range. However, the variability in the absolute absorbance readings for this test were equivalent to the other anti-hapten ELISA protocols and therefore of equal precision.

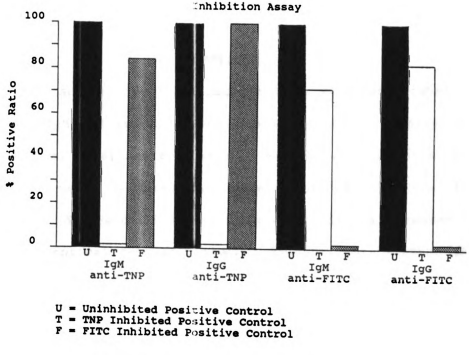


Figure Experiment 1.19

Specificity: Inhibition assays were used to evaluate the specificities of each anti-hapten ELISA procedure. The percent positive ratios of uninhibited, TNP inhibited and FITC inhibited positive control sera were compared. Inhibition was accomplished by incubating excess TNP-FCS or FITC-FCS with positive control sera for 30 minutes prior to ELISA testing.

Only that antigen specific for the antibody being evaluated was capable of completely blocking ELISA activity. TNP maximally inhibited the IgM and IgG anti-TNP ELISAs. Both assays lost 100% of their activities. TNP inhibited IgM and IgG anti-FITC ELISAs by only 28.2 and 17.5% respectively. FITC produced 100% inhibition of both the IgM and IgG anti-FITC ELISAs. The IgG anti-TNP assay was uninhibited by FITC while IgM anti-TNP ELISA activity declined by only 14.5%. The decrease in antibody activity produced by unrelated antigen was similar to that reported by other workers and is the result of nonspecific inhibition produced by high concentrations of proteins.[100]

Discussion

Accurate and precise ELISA methodologies were established for the detection of IgM and IgG mouse antibodies against the haptens TNP and FITC. Methodical trial and evaluation, the only acceptable verification method,[137] was used to determine optimal working dilutions for all immunoreagents. Standard ELISA techniques were modified as needed for the production of optimal test conditions. Each reagent, whether obtained from a commercial source or prepared in-house, and each test system had its own unique assay requirements. The sensitivity, specificity and reproducibility of ELISAs are dependant mainly upon the insoluble phase used to immobilize antigen, concentration of test sera analyzed and enzyme

conjugate employed.[7] Optimal working concentrations were determined for hapten-FCS solid phase sensitizing reagents, mouse test sera and peroxidase conjugated antisera. Optimal working concentrations are summarized in the Figure Experiment 1:20.

	IgM anti-TNP	IgG anti-TNP	IgM anti-FITC	IgG anti-FITC
Hapten-FCS Concentration	72 ug of TNP-FCS protein per ml	72 ug of TNP-FCS protein per ml	14 ug FITC-FCS protein per ml	3 ug FITC-FCS protein per ml
Mouse Sera Dilution	1:100	1:200	1:100	1:200
Peroxidase Conjugated Anti-mouse Antisera	1:400	1:500	1:200	1:500

Figure Experiment 1.20
Optimal Working concentration

Test results were reported using the percent positive ratio which expresses test results as a percentage of a standard reference sera's absorbance value. This method required only a single serum dilution and is easily understood. In addition, since absorbance ratios tend to remain constant over time, slight variations in intra- and inter-runs' incubation times and temperatures intra- and inter-runs produce less variability in reported data and minimizes CVs.[63,100]

Optimal reagent concentration produced the maximal percent positive ratio between anti-TNP and anti-FITC control sera. The minimum acceptable percent positive ratio was 5:1[311] and the minimal absorbance reading for the positive control was 0.050 absorbance units.[18] These test conditions precluded the minimal background noise to signal ratio while maintaining sensitivity and precision.[137]

Polystyrene plastic microtiter plates are routinely used as the solid-phase for ELISAs due to their unique properties of optical clarity, high binding capacity, rigidity and reproducible production characteristics.[243] However, plastics vary greatly between manufactures and between production lots in their capacity to efficiently adsorb proteins onto their surfaces.[135,138,136,7] All microtiter plates used for the anti-hapten ELISAs were therefore derived from the same manufacturer's production lot to reduce adsorption variabilities. A random sampling of plates from this lot showed no significant difference in their ability to adsorb protein as evaluated by passive adsorption of mouse IgG to the solid-phase surface (data not shown).

A common problem in ELISA is non-specific or specific, undesirable, binding to the solid phase by different sera or reagents. Less than 25% of the solid-phase surface is covered by antigen protein after sensitization.[31,212] Nonantigen sensitized regions can potentially adsorb test sera proteins or labelled antibody in a non specific manner during subsequent incubations. This can be reduced by the addition of various proteins, with or without Tween 20, to the buffers used as diluents.[137,312,7] This blocking protein must be immunologically inert for the ELISA being used. If test sera have antibodies directed against the blocking protein, an increase in background noise to signal will be produced and sensitivity and specificity are reduced.

FCS was found to be a suitable blocking reagent for the prevention of non specific protein uptake by the solid phases of all anti-hapten ELISAs. FCS was initially evaluated as the blocking protein for the anti-hapten ELISAs because it is an assay requirement in subsequent ELISPOT assays used to evaluate anti-hapten antibody production in vitro. Simplicity and consistency dictated the need to utilize the same basic

reagents in both test systems.

No detectable innate anti-FCS antibody activity was noted in normal control mice, or mice immunized with TNP-egg albumin or FITC-egg albumin. FCS was therefore a suitable reagent to employ as a hapten carrier for solid phase sensitization and as a blocking reagent for the prevention of non specific binding to the solid phase.

All antigens used to sensitize the solid phase were diluted in 0.05 M carbonate buffer, pH 9.6, containing 0.2% FCS. The supplementation of extra protein during the sensitizing phase markedly reduced nonspecific protein uptake of subsequent immunoreagents. The resultant reduction in background increased the specificity of all anti-hapten ELISA protocols.

The reason why additional protein was required in the sensitizing buffer is not clear. Protein, such as BSA, is commonly added to sensitizing buffers used in coating ELISA plates with monoclonal antibodies and results in an increase in sensitivity and specificity of assay results.[127] Additional protein enhanced the amount of monoclonal antibody on the plate surface and protected it from denaturation. Adsorption of reagent is independent of competing proteins provided that the total amount is below the saturating concentration for the solid phase.[34] Free binding sites can therefore be blocked by adding a nonreacting protein to the sensitizing reagent which minimizes the background signal.[243] It is possible that one or both of these mechanisms contributed to the protocol requirements for the anti-hapten assays.

The importance of the interaction between the antigen and the solid phase cannot be overemphasized.[135] Protein adsorption to plastic is believed to occur through electrostatic, Van der Waals, hydrogen bonding and most importantly hydrophobic interactions. Each protein has a different binding constant.[21,44] The protein concentration required to most

adequately sensitize the solid phase also differs with variances in assay conditions and assay requirements.[135,34] These observations seem to hold true for the anti-hapten ELISAs developed in this laboratory.

The optimal FITC-FCS concentrations required to sensitize the solid phase of the IgM and IgG anti-FITC ELISA were respectively 14 and 3 ug. of hapten-FCS protein per ml. These concentrations were similar to the 1 to 10 ug. of antigen per ml reported as optimal in other reported ELISA test systems. Both IgM and IgG anti-TNP ELISAs required 72 ug. of TNP-FCS protein per ml. to adequately sensitize the solid phase. The reason for the increased regent concentration for the sensitizing phase of the anti-TNP assays was not examined. High concentrations of sensitizing antigen, up to 100 ug/ml, have been required for some ELISA test protocols.[215,127] Possible reasons include alteration of the hydrophobicity of the FCS after TNP conjugation, a lower density of TNP conjugation to the FCS or steric hinderance of antibody binding due to the location of TNP attachment on the FCS. High antigen concentrations on the solid phase produce the most sensitive assays.[338] Since the noise to signal ratio was within acceptable limits and the test precision was acceptable, the higher antigen concentration was acceptable for the anti-TNP assays.

Sera dilutions of 1:100 were optimal for IgM anti-TNP and anti-FITC ELISAs. Both IgG anti-hapten assays had optimal sera dilutions of 1:200. These sera dilutions were similar to those used in other ELISA assays in this laboratory. The lower dilution level for IgM test protocols most likely reflects lower IgM serum concentrations in comparison to IgG.

Peroxidase conjugated anti-mouse IgM and IgG had working dilutions comparable to other ELISA systems in the laboratory. The commercially prepared anti-IgM reagents in this laboratory's experience are generally less potent than anti-IgG. This was exemplified by more dilute working

concentration of the anti-mouse IgG peroxidase conjugated antisera.

The relative variability between the different anti-hapten ELISAs, as measured by variation in absorbance units, was similar for all anti-hapten protocols. The average within run variability was 0.006 ± 0.003 and the average between run variability was 0.008 ± 0.005 absorbance units. This degree of variability is inherent to the ELISA system due to optical variations in the bottom of test wells, variable internal reflections from the sides of the wells, meniscal distortion of the transmitted light beam, electrical and mechanical imperfections in the instrumentation, pipetting errors, variations in incubation times and temperatures and user error.[18,328,140,251] This inherant error is trivial over most of the absorbance range becoming significant only at absorbance values below 0.050. The level of precision obtained in the 4 anti-hapten ELISAs is comparable to other reported ELISA systems.[251,140,139]

Coefficients of variation for IgG anti-TNP, IgM anti-FITC and IgG anti-FITC ELISAs were less than 10% for within runs and between runs. Only the IgM anti-TNP ELISA had occasional within runs and between runs CV ranging above 10%. The absorbance values for the IgM anti-TNP precision runs ranged from 0.049 to 0.078. These were the lowest readings for any of the anti-hapten tests. The lower the absorbance value the greater influence the inherent variability will have on the CV. The average within runs standard deviation for the anti-TNP ELISA was 0.006 ± 0.001 absorbance units. Between runs standard deviations averaged 0.015 ± 0.004 absorbance units. Although the CV for the anti-TNP ELISA was occasionally greater than 10%, the average standard deviation of the IgM anti-TNP test means was comparable to that for other reported IgM ELISA protocols.[226,224] Therefore the IgM anti-TNP ELISA was considered to have acceptable precision for the purpose of this study. In order to maintain as high a

precision level as possible, all ELISA testing for subsequent experiments was done on a single run.

All anti-hapten ELISAs had a high degree of specificity as indicated by inhibition assays.Incubation of TNP with test sera inhibited all of the IgM and IgG anti-TNP ELISA activity. TNP reduced IgM and IgG anti-FITC activity by only 28.2 and 17.5%. Incubation of FITC with test sera resulted in 100% inhibition of IgM and IgG anti-FITC activity. FITC did not inhibit the IgG anti-TNP activity and only reduced the IgM anti-TNP results by 14.5%.

The ELISA principles provided a very satisfactory basis for the development of a clinically useful test for the detection of IgM and IgG anti-TNP and anti-FITC antibodies. These protocols were practical, simple, efficient, rapid, and reproducible. The specificity and sensitivity were considered acceptable for the measurement of potential spontaneous anti-hapten antibody production resulting from polyclonal B cell activation.

The Solid-Phase Enzyme-Linked Immunospot Assay

The solid-phase enzyme-linked immunospot assay (ELISPOT) is an immunodiagnostic test for enumeration of specific immunoglobulin secreting cells (ISC).[57,297] The ELISPOT methodology is based upon the well established principles of ELISA which account for the synonyms, ELISA-plaque and ELISA-spot assay.[270,119,84]

The ELISPOT procedure is technically simple. ISCs, isolated from lymph nodes, bone marrow, spleen or blood are incubated on antigen-coated polystyrene culture dishes.[57,58,121] The antigen sensitized culture dishes serve as the solid phase upon which all subsequent immunoactivity is localized and eventually visualized. Antibodies produced by ISCs, if specific for bound antigen, bind to the sensitized solid phase within the immediate microenvironment of the ISC.[270,57,198] Any antibody produced but not specifically recognizing the solid-phase bound antigen will remain in solution and be removed by gentle washings. These localized, solid-phase bound zones of antibody are visualized after application of an enzyme-labelled species specific anti-immunoglobulin and an appropriate enzyme substrate. The enzyme substrate is contained within an agarose gel which confines any resultant insoluble colored enzyme product within the immediate area of antibody deposition.[270,57,198] The resulting colored circular zone or spot of enzyme activity corresponds with a single ISC having specificity for the solid-phase sensitizing antigen.[57,198,199,270] The resulting immunospots can be quantified to determine the specific cellular antibody producing activity of the cell population being studied.

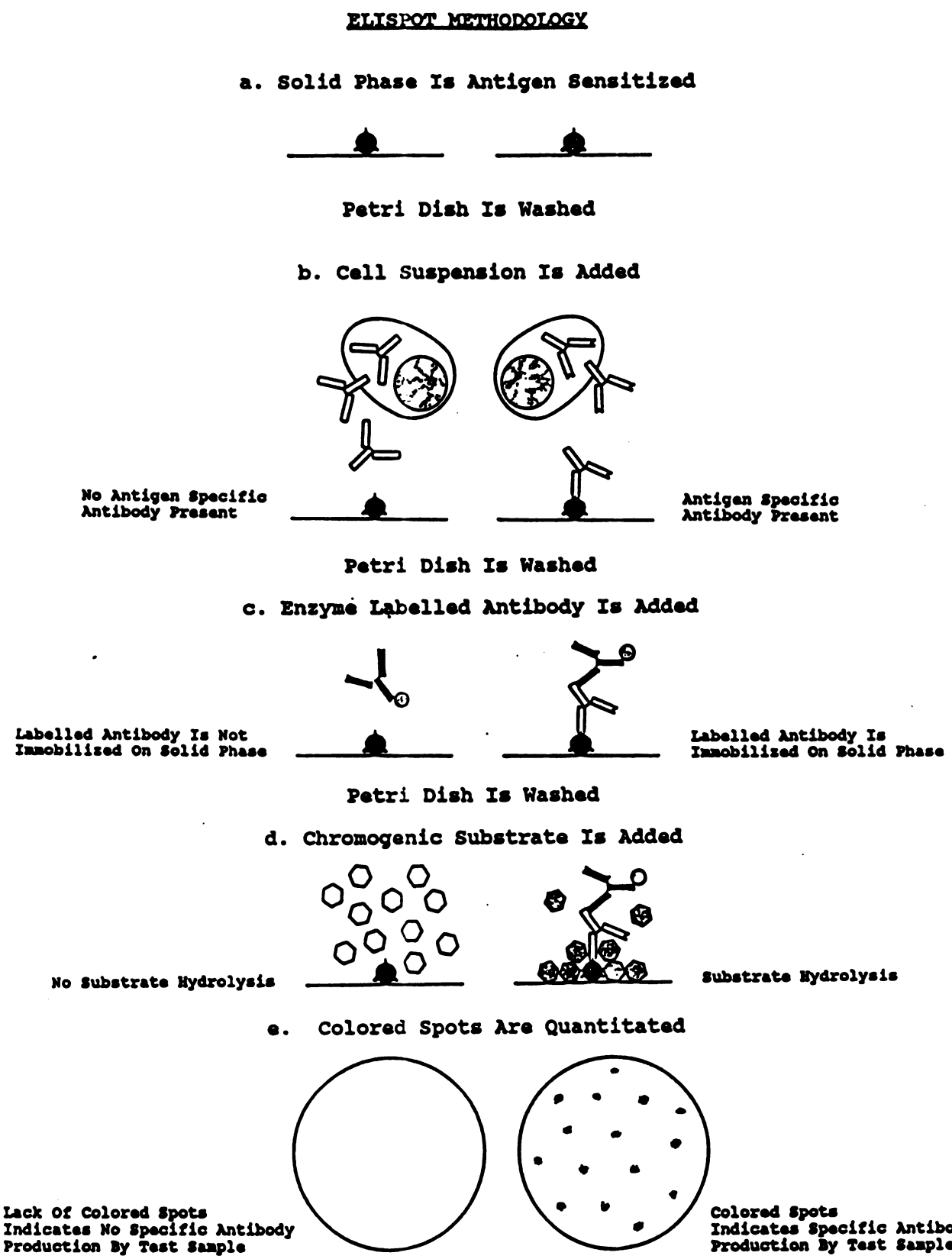


Figure Experiment 2.0

ELISPOT has been employed using soluble and cellular antigens to detect specific antibody production in the mouse, rat and human.[204,57,60,58,273,119,122,84,272,260] ELISPOT has been repeatedly found to have equivalent or greater sensitivity than conventional direct or indirect hemolytic plaque-forming cell (HPFC) assays.[57,306]

Historically, HPFC assays have been widely used as indicator systems to analyze antibody secretion and regulation.[125] HPFC assays are specific quantitative immunoassays used to detect ISCs in a semi-solid medium via complement dependent lysis of either neat or antigen coupled cellular targets.[126,91,247,125,198] However, the applicability of the procedure has met with technical and theoretical problems mainly inherent in the use of the hemolysis reaction as the indicator system.[59]

Plaque assays have proven difficult to use in some systems. Especially troublesome are those involving responses to cellular antigen which are not readily lysed (ie. nucleated cells and human RBCs) or responses involving "incomplete" antibodies (ie. mouse anti-erythrocyte) which require careful selection of complement and amplifying antisera.[198,166,167,209] Constraints are often placed upon the choice of antigens used experimentally with HPFC assays due to instability of some conjugated targets or difficulties associated with conjugating carrier proteins to RBCs in a manner which maintains reproducible susceptibility to subsequent lysis.[146,183,30,84]

Reproducibility of HPFC assay results vary widely. Between run coefficient of variation may be as high as 86%.[225,91,84] Differences in age and source of target RBCs as well as variation in the RBC surface density of conjugated antigen can alter susceptibility of the target cell lysis between test runs.[125,91,225,122] Plaque assays also have impaired reproducibility due to the variability in daily preparation of target cells.

The ability of HPFC assays to accurately detect specific antibody activity of a given isotype, allotype or idiotype is questionable.[227] The direct HPFC technique detects mainly but not exclusively IgM ISCs. Uncertainty exists about the nature of additional antibody isotypes which contribute to the direct hemolytic plaque formation.[270,335] Indirect hemolytic plaque techniques, utilizing species specific anti-immunoglobulin, permit detection of IgG and IgA ISCs.[198,68,276] Indirect HPFC assay results however are difficult to interpret due to the background noise inherent with the procedure.

ELISPOT avoids many pitfalls of the hemolytic plaque assay.[119,270,60] Solid-phases can be prepared in advance and stored until needed alleviating the day to day variability inherent in the preparation of HPFC assay target cells [346] Specific antibody class or subclass ISC quantitation can be achieved with selection of appropriately purified antisera.[271] ELISPOT detects ISCs by direct observation of immune complexes and is therefore independent of secondary antibody characteristics such as complement fixation or immunoprecipitation. Other procedures directly detecting antigen-antibody interaction (ie. ELISA, Radio Immuno Assay) are generally more sensitive than those procedures using secondary antibody characteristics for detection (ie. Immunoprecipitation). In addition, secondary antibody characteristics are more prone to the effects of antigen-antibody ratios and subclass variability. Theoretically these same principles may attest to the superior sensitivity and precision of ELISPOT compared to HPFC assays.

The sensitivity of the ELISPOT assay has been shown to be equal or superior to the HPFC assay.[274,119,270] The ELISPOT is particularly more sensitive in detecting IgM antibodies. This difference in sensitivity is possibly due to the heterogeneity in complement fixation inherent with IgM

or due to the inability of the hemolytic plaque assay to detect antibodies of low affinity.[274]

Although ELISPOT is technically simple, ideal test conditions are determined only after careful selection of the solid phase and systematic chessboard titration of all immunoreactants. The solid phase and its optimal sensitization is critical to the subsequent sensitivity of ELISPOT.[270,297,119] The ideal solid phase is non-wettable polystyrene dishes.[270] Hydrophilic tissue culture treated surfaces tend to bind antigen less avidly and are not suitable for ELISPOT protocols.[122] Round petri-dishes are adequate[270,198] but swirling of cells during transport may result in uneven distribution.[273] Square well plates produce a more uniform cell dispersion and although preferred are not always available.[273] Recently, nitrocellulose membranes have been employed as solid-phase supports instead of polystyrene.[56,197] This modification resulted in reduced concentrations of coating material and was reported to be at least 5 times more sensitive in ISC detection.[56]

Efficacy of ELISPOT ISC quantitation varies with the concentration and molecular form of the antigen.[122] Low molecular weight antigens (ie. ovalbumin, bovine serum albumin) or hapten carriers require high concentrations (mgs/ml) to achieve acceptable solid phases sensitization.[121,270,271] This is in marked contrast to the lower concentrations (ugs/ml) used with standard ELISA techniques. The high molecular weight polymers present in protein antigens are responsible for sensitizing the solid-phase of ELISPOT assays.[122] These polymers vary in concentration between different batches of antigen depending on commercial source and/or variations in processing.[120] This variable degree of native protein aggregation is most likely responsible for the variability in the concentration of similar antigens required to optimally sensitize the

solid phase.[122] The reason for this phenomena is currently not known. Polymerization of low molecular weight antigens or pretreatment of the solid phase with glutaraldehyde can increase antigen binding efficiency approximately 50%.[121,122,273,119]

Complex high molecular weight antigens (ie. Ascaris suum extract, thyroglobulin) do not have detectable between batch difference in solid phase binding. Optimal sensitizing concentrations on a w/v basis are less than those required for low molecular weight antigens. In addition, polymerization or glutaraldehyde solid phase pretreatment is not required for efficient antigen up-take.[119]

Efficacy of anti-hapten (ie. DNP) ISC detection was influenced by the degree of hapten conjugation to the carrier protein.[122] Highly substituted carriers were found superior. When lacking any carrier protein, hapten bound to glutaraldehyde pretreated plastic provided an excellent solid phase for ELISA but was unsatisfactory for ELISPOT. Hapten sensitized solid phases resulted in detection of only 10% of the immunospots observed with a BSA-DNP solid phase.

Additional reports attest to the differences in optimal sensitizing conditions between ELISA and ELISPOT procedures. Urea denatured ovalbumin has advantages over the native protein in ELISA[45] but produces negligible immunospot activity.[122] In contrast, while polymerized ovalbumin is superior with ELISPOT this is not the case with ELISA.[122] The discrepancy between optimal sensitizing conditions remains unexplained.

A variety of tissues including spleen, lymph nodes, bone marrow, respiratory, myeloma and peripheral blood have been used as ISC sources for ELISPOT.[273,271,270,119,272,204,57,297] Isolated cells are suspended in PBS or growth media containing 10% FCS.[270,57] The high concentration of FCS or equivalent protein is essential to prevent cell clumping and nonspecific protein

binding to the solid phase.[270] The number of cells employed varies with researcher preference. As few as 165,000 spleen cells/ml gives rise to a substantial number of immunospots.[57] Cell densities greater than 3 x 10^7/ml sometimes resulted in spot confluence.[297]

The volume of media used to culture the cells is an important parameter. The minimum volume of culture media required to cover the bottom surface of the culture plate results in maximal sensitivity.[57] The number and intensity of ISC spots decreased as culture media volume increased.[57] Duration of cell incubation on the solid phase also effects sensitivity. Immunospot numbers, found after 30 minutes incubation, increased in nearly linear fashion to reach maximum counts by 2 to 4 hours.[57,270] Prolongation of incubation past 4 hours produced larger diameter spots with occasional confluence and an undesirable darker background reaction.[57]

ELISPOT protocols have routinely used 37° C as the standard cell incubation temperature. While suitable for IgG and IgM ISC detection, IgE isotype was optimally detected when cultured at 32° C.[273] This phenomena was surmised to have resulted from IgE-antigen complexes being partially susceptible to dissociation at higher temperatures.

The developing enzyme labelled antisera used in standard ELISA systems can be employed with ELISPOT.[270,198] Specific antibody classes, isotypes, allotypes or idiotypes can be enumerated depending upon the specificity of the enzyme labelled antisera. Affinity purified antibodies are optimal since they are most easily standardized and produce minimal or no undesirable specific antibody cross reactivity between antibody classes.[271,119] The concentration of enzyme tagged antibody is determined by chessboard titration and may differ from that used with ELISA.

Incubation times and temperatures used for the enzyme labelled antisera vary from overnight at 4° C to 2 hours at 37° C.[270,84] No effect on ELISPOT sensitivity or specificity has been reported for temperature or time factors. The use of 4° C overnight incubation with PAP techniques does however permit the use of more dilute antisera. Expensive antisera is thereby conserved and background noise reduced.

Horseradish peroxidase or alkaline phosphatase are the 2 enzymes generally employed with ELISPOT. Selection of enzyme conjugate appears to depend on investigator preference. No systematic comparison between enzymes has been reported. Enzyme labelled antisera against common laboratory species and human antibodies are commercially available or easily prepared.

Chromogenic substrates are specific for the enzyme conjugated to the developing antibody. The resulting product of enzymatic activity must be insoluble in order to remain at the site of solid phase bound antibody. Paraphenylenediamine or diamino benzidine are commonly used with horseradish peroxidase.[57] These substrates are potential cancer causing agents and must be used with caution.[313] Alkaline phosphatase procedures use 5-bromo-4-chlor-3-indolyl phosphate p-toluidine salt (5 -BCIP) which has no known biohazardous activity.[270]

The number, intensity, definition and size of immunospots are augmented with time after administration of chromogenic substrate.[84] Detectable immunospots increased 20 to 250% after a 16 hours incubation with the chromogenic substrate compared to 2 hours incubation time. The time enhancement was more pronounced for IgM than IgG ISCs. IgM was assumed to appear more slowly due to low affinity antibodies and lower IgM antibody concentrations. According to classical enzyme kinetics, the concentration of substrate transformed by a very low concentration of

enzyme is proportional to reaction time. Consequently, a rather long time would be required for low enzyme levels to yield a visible deposition of product.[84]

The speed, intensity and number of immunospots formed can be enhanced by coupling a redox reaction to the 5-BCIP enzymatic reaction catalyzed by alkaline phosphatase.[84] From 2 to 10 times more ISC antibody foci were noted when nitro tetrazolium salt was added to the detection system. During the conversion of indoxyl (present in 5-BCIP) to indigo by alkaline phosphatase, hydrogen ions are produced. These ions reduce nitro blue tetrazolium salt and result in precipitation of the corresponding formazan with intensification of spot formation.[21,84] Comparison of redox coupled 5-BCIP sensitivity to horseradish peroxidase reactions have not been made.

Immunospots can be enumerated macroscopically[57,270] but greater precision and accuracy are achieved with the use of 100x magnification.[277] Immunospots are brown, circular and well individualized granular foci. Occasional preparations have small "false spots" which appear to be due to nonspecific enzyme product absorption onto contaminating particulates within the gel.[119] These "false spots" are located above the agarose/solid phase interface where true immunospots are formed. Localization of spot formation within the gel is discernable only under magnification.[274,119,273] In addition, these false spots are denser and lack granularity.[59] Filtration of substrate and agarose prior to use reduces these nonspecific artifacts.[273] Other "false spots" occur at the agarose/solid phase interface and may represent highly activated B cells or macrophages.[86] These latter artifacts are dense and irregular in contrast to the typical circular and granular ELISPOT.[57,270]

Foci of enzymatic activity vary in darkness and range from 0.1 to 1.0 mm in diameter which is 100 times the average size of a lymphocyte.[57,122] Covert differences in coating density of the solid phase antigen does not appear to contribute to variation in spot size.[119] Variability of ISC foci is noted with all antibody isotypes tested.[57] IgM foci are generally smaller then IgG foci. This may reflect the slower diffusion rate for the larger IgM molecule.

Discrepancy in foci size may also reflect variability in antibody affinity. IgG immunospots, although retaining variability, are skewed toward larger sizes in secondary antibody responses.[122] A similar finding is noted during secondary immune responses evaluated with the conventional HPFC technique. Increased hemolytic plaque size was hypothesized to be the result of temporal increases in antibody affinity.[61,122] Differences in rate of antibody formation however can not be excluded. High affinity antibody is associated with larger plaques and low affinity antibody with smaller plaques.[274] These same phenomena may also result in variations in immunospot size and density.[213,122]

The ELISPOT is a versatile immunoenzymatic methodology which provides a useful alternative to conventional hemolytic plaque assays for investigating antibody production at the cellular level. ELISPOT is more sensitive and precise than the hemolytic plaque assays traditionally used to evaluate ISCs. Therefore, lower levels of antibody production can be detected with smaller deviations from baseline being significant. In addition, specific antibody isotype identification makes ELISPOT more versatile in characterizing immune reactions than standard HPFC assays. ELISPOT technology is new and the methodology is undergoing change as researchers gain familiarity with the test. The potential for this unique test is yet to be fully realized.

EXPERIMENT II

Objectives

The objectives of Experiment II were to establish an accurate, sensitive and precise solid-phase enzyme-linked immunospot assay (ELISPOT) for the quantization of IgM and IgG immunoglobulin secreting cells having specific antibody activity against either fluorescein isothiocyanate (FITC) or trinitrophenol (TNP). These anti-hapten ELISPOT assays will be used to evaluate the potential spontaneous anti-FITC and anti-TNP antibody production in mouse spleen cell cultures from untreated controls and P. acnes treated mice.

Materials and Methods

Haptens: FITC isomer I and 2,4,6-trinitrobenzene sulfonic acid were purchased from Sigma Chemical, St. Louis Missouri.

Protein-Hapten Conjugates: FITC and TNP haptens were conjugated to either egg albumin or fetal calf sera (FCS) as previously described in Experiment I under ELISA materials and methods.

Polymerization of FCS-FITC: Polymerized FCS-FITC was prepared via glutaraldehyde cross linking.[122] Briefly, 2.5% glutaraldehyde in 0.1 M PBS, pH 7.5, was added dropwise at 4° C to FCS-FITC in 0.1 M PBS pH 7.5 to a final albumin to glutaraldehyde molar ratio of 25:1. The reaction was terminated after 4 hours at 4° C by the addition of glycine to a final albumin to glycine weight ratio of 50:1. The resulting product was dialyzed against 0.1 M PBS, pH 7.5, aliquoted and stored at -20° C.

Culture Plates: Round, 60x15 mm, non tissue culture treated plates (Falcon 1007) were used. Nontissue culture treated dishes have superior ability to bind protein compared to those which are tissue culture treated.[242,243]

Enzyme Conjugated Antisera: Goat anti-mouse IgM and IgG peroxidase conjugated antisera were purchased from Cappel Worthington, Malvern Pa. Antisera were evaluated for specificity via standard double immunodiffusion and immunoelectrophoretic techniques.[52,37]

Mice: Balb-c x C57 male and female mice ranging from 6 to 12 weeks of age were utilized. Mice were bred and housed in the Laboratory Animal Care Service facilities at Michigan State University under standard conditions. Purina mouse chow and water were provided ad libitum.

Immunization of Anti-hapten Controls: Positive control mice were immunized intraperitoneally with either 1 mg. egg albumin-FITC or egg albumin-TNP in aluminum hydroxide adjuvant.[275] Negative control mice received an equal volume of sterile physiologic saline intraperitoneally.

Spleen Cell Preparation: Five days post immunization, mice were euthanized via cervical dislocation under light ether anesthesia. Spleens were aseptically removed and their cells dislodged by gentle forceps teasing into ice-cold RPMI, containing 10% FCS.[204] Cell clumps were broken apart by passing the cell suspension through a 25 gauge needle.[273] Isolated cells were pelleted at 200g for 10 minutes.[84] After decanting the supernatant, the cells were subjected to a 10 second distilled water osmotic shock to lyse erythrocytes. Isotonicity was restored with double osmoality PBS pH 7.2. FCS was added to a final concentration of 10% to reduce clumping of cells during repelleting at 200g for 10 minutes.[273,297] Spleen cells were resuspended in RPMI (Roswell Park Memorial Institute culture media) containing 10% FCS to a concentration of 3 x 10^6 viable

cells per ml. as determined by trypan blue exclusion and manual hemocytometer counts.

Determination of Optimal Test Conditions: The anti-TNP and anti-FITC ELISPOT techniques were a modification of the basic ELISPOT procedure described by Czerkinsky et al.[57]

All steps were done under subdued light or in the dark to prevent inactivation of light sensitive FITC and TNP. Falcon 1007, 60x15 mm round petri dishes were deemed suitable for solid phase sensitization by trial and evaluation. Working concentrations of solid phase sensitizing antigens and of peroxidase conjugated marker antibody were established by methodical chess board titrations.

The solid phase was sensitized with either FITC-FCS or TNP-FCS diluted in 0.1 M carbonate buffer pH 9.6. Protein concentrations of 2, 1, 0.5 and 0.25 mg/ml were evaluated for each FCS-hapten conjugate. Negative control plates were sensitized with unadulterated FCS at a protein concentration of 2 mg/ml. Antigens were passively bound to the solid phase during an overnight incubation at 4° C. Unbound antigen was removed during 3 washes with 0.1 M PBS pH 7.2.

To prevent nonspecific protein binding to the solid phase, any remaining unreacted binding sites were blocked with 3 mls of 5% FCS in 0.1 M PBS, pH 7.2, incubated on the plates for 1 hour at 37° C in a humid chamber. Plates were flicked but not washed to remove excess buffer just prior to the next step.

Two million spleen cells in 0.66 mls of RPMI containing 10% FCS were incubated on the sensitized solid phase for 5 hours at 37° C in an atmosphere containing 100% humidity and 10% CO_2. During this incubation phase, antibodies produced by splenic plasma cells specifically bound, if their activity was appropriate, to the FCS-hapten sensitized solid phase.

Immediately upon removal from the incubator, dishes were chilled on ice for 10 minutes to stop additional antibody production. Unbound antibody was removed by 2 washes with ice cold 0.1 M PBS buffer, pH 7.2.

Three mls. of peroxidase conjugated goat anti-mouse IgG or IgM antisera diluted in cold 0.1 M PBS buffer pH 7.2 containing 0.05% Tween 20 and 1% FCS was incubated on the solid phase overnight at 4° C. Antisera not bound to the solid phase was removed with 3 washes of cold 0.1 M PBS buffer pH 7.2. A 1:100, 1:500, and 1:1000 dilution of each developing antisera was titrated against FCS sensitized negative control dishes and against each FCS-hapten concentration used to coat the test dishes.

The chromogen substrate used to visualize the solid phase bound enzyme activity was prepared fresh just prior to use. A 0.5% agarose solution in 0.1M PBS, pH 7.2, was brought to a boil and subsequently cooled to 46° C in a water bath. Fifty mg. of 3'5' diaminobenzidene (DAB) was dissolved in 10 mls. of PBS pH 7.2, filtered through a 0.22 um filter and warmed to 46° C.[273] The DAB solution was added to the agarose to a final 0.1% concentration. Hydrogen peroxide was added to the substrate-agarose mixture to a final concentration of 0.01%. The substrate was protected from the light at all times due to its photosensitivity.

After substrate preparation, ELISPOT plates were flicked to remove the final wash and immediately layered with 1 ml of chromogen substrate. Specific immunospot activity was permitted to develope during an overnight incubation at room temperature. Immunospots were quantitated under 40x magnification using a dissecting microscope. An immunospot was defined as a brownish granular circular stain located at the solid-phase/agarose interface.[270] Any color activity detected within the agarose layer was determined to be artifactual and was disregarded.[273,119] Anti-hapten immunospot activity values were corrected for any background immunospot

formation as detected on FCS sensitized control plates. Immunoreactant concentrations producing the maximal number of immunospots without any nonspecific background staining were determined optimal and used for quality control studies.

Comparison of ELISPOT Buffer Systems: The effect on the IgM and IgG anti-FITC ELISPOT assay of 0.5M Tris, 0.1M phosphate and 0.1M PBS buffers were compared for efficacy in the ELISPOT assay. Each buffer was compared at a pH of 7.2 and 8.0. Optimal immunoreactant concentrations of FCS-FITC and peroxidase conjugated IgM and IgG where used. The ELISPOT procedure was as previously described except the various buffers were substituted for the plate wash solution and for the peroxidase conjugated antibody diluent. All variants were run simultaneously and in duplicate using mouse spleen cells harvested 5 days post immunization with egg albumin-FITC in aluminum hydroxide adjuvant.

Comparison of Glutaraldehyde Polymerized and Nonpolymerized FCS-FITC Sensitizing Antigen: Glutaraldehyde polymerized FITC-FCS was used to sensitize ELISPOT solid phase dishes using protein concentrations of 92, 46, 23 and 11.5 ug/ml. Non polymerized FITC-FCS solid phase, sensitized with equivalent protein concentrations, were run in parallel. The remainder of the ELISPOT protocol utilized optimal immunoreactant concentrations and 0.5 M Tris buffer, pH 7.2 as previously described.

Within Run Precision: Within run precision was determined using optimal assay condition and spleen cells from hapten immunized and control mice. The coefficient of variation (CV) for each ELISPOT protocol was calculated from the immunospot results of 4 duplicate test plates .

Specificity: Anti-hapten specificity of FCS-TNP and FCS-FITC solid phases were evaluated by comparing the number of immunospots produced by appropriately immunized mice versus mice immunized with the alternative

hapten.[274] In addition, the number of immunospots formed against the hapten carrier protein,FCS, were determined.[119] Between group differences were analyzed using a multifactorial ANOVA.

Results

Determination of optimal test conditions for mouse anti-FITC and anti-TNP ELISPOT: Methodical chess board titrations were used to determine the optimal working concentrations of the solid phase sensitizing antigens, TNP-FCS and FITC-FCS, and of the peroxidase labelled anti-mouse IgM and IgG developing antibodies. Each specific anti-hapten antibody secreting cell produced a distinct brown granular circular focus at the solid-phase/agarose interface which ranged in size from 0.1 to 0.5 mm in diameter (Figure Experiment 2.1). Immunospots were quantitated under 40x magnification.

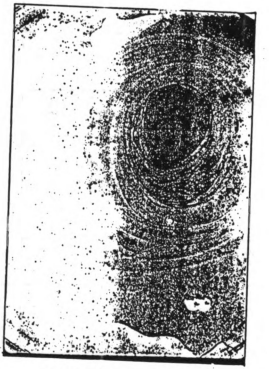


Figure Experiment 2.1
Anti-hapton Immunospots

The total number of immunospots detected for each concentration of sensitizing antigen (Figure Experiment 2.2, columns) were compared to the total immunospots detectable with each antisera dilution (Figure Experiment 2.2, rows). Row and column values were corrected for any background immunospot activity detected on the FCS sensitized negative control plates. Maximal ELISPOT sensitivity was used to determine optimal immunoreactant working concentrations.

		FCS Control	TNP-FCS mg/ml			
			2.0	1.0	0.5	0.25
Anti-IgM ug/ml	20	2	83	97	113	33
	4	0	112	117	100	93
	2	0	99	92	83	78

		FCS Control	TNP-FCS mg/ml			
			2.0	1.0	0.5	0.25
Anti-IgG ug/ml	20	0	35	32	31	60
	4	0	87	67	51	76
	2	0	62	70	108	57
	0.4	0	74	57	47	41

		FCS Control	FITC-FCS mg/ml			
			2.0	1.0	0.5	0.25
Anti-IgM ug/ml	20	1	10	33	27	9
	4	0	34	37	59	66
	2	0	40	62	68	46

		FCS Control	FITC-FCS mg/ml			
			2.0	1.0	0.5	0.25
Anti-IgG ug/ml	20	1	0	5	3	5
	4	2	14	2	0	10
	2	0	0	0	0	0

Figure Experiment 2.2
Immunospots per 2 x 10^6 Spleen Cells
*Immunospots values corrected for anti-FCS background

Respective, optimal solid phase sensitizing concentrations for IgM and IgG anti-TNP ELISPOTs were 1.0 mg. and 0.5 mg. of haptenated-FCS per ml of coating buffer. IgM and IgG anti-FITC ELISPOTs were most sensitive with respective haptenated-FCS concentrations of 0.5 mg. and 2.0 mg. per ml of coating buffer.

Anti-IgM developing antisera had maximal activity at a concentration of 4 ug./ml (1:500 dilution) for the anti-TNP ELISPOT and at a concentration of 2 ug./ml (1:1000 dilution) for the anti-FITC ELISPOT. However, the 4 ug./ml goat anti-mouse IgM produced considerable nonspecific background precipitate which interfered with the immunospot quantification. It was therefore considered beneficial to utilize the peroxidase conjugated anti-IgM at a 2 ug./ml concentration in order to reduce undesirable background, to maintain consistency between both IgM anti-hapten procedures and to conserve expensive reagents. The sacrifice in sensitivity at the 1:1000 dilution was felt to be inconsequential in overall detection of IgM anti-TNP trends.

Maximal sensitivity for the IgG anti-TNP assay was achieved with 2 ug./ml (1:1000 dilution) of goat anti-mouse IgG developing antisera. Nonspecific background precipitate was minimal and individual immunospots were well demarcated and easily detectable. With the IgG anti-FITC ELISPOT, peroxidase conjugated anti-mouse IgG had maximal activity at 4 ug./ml. Background precipitation was of moderated intensity making immunospot differentiation difficult. Due to the lack of interference from background precipitation, 2 ug./ml. of IgG peroxidase antibody was determined to be optimal provided that the assay sensitivity could be improved. This antibody concentration would also maintain consistency between the IgG anti-TNP and anti-FITC protocols.

Effect of 0.05 M Tris, 0.1 M Phosphate and 0.1 M PBS Buffers on anti-FITC ELISPOT Activity: Tris, phosphate and PBS buffer systems were evaluated for their potential effect on the sensitivity of the anti-FITC ELISPOT. The optimal buffer solution for the IgM and IgG anti-FITC ELISPOT assay was 0.05 M Tris, pH 7.2 (Figure Experiment 2.3). When compared at pH of 7.2, the number of IgM anti-FITC immunospots with Tris buffer was 2.5 and 3.0 times greater when respectively compared to phosphate or PBS. Use of Tris at a pH of 7.2 resulted in 2.8 times the number of IgM immunospots than Tris at a pH of 8. An alkaline pH did not appear to alter the sensitivity of the PBS or phosphate buffer systems. Acid pH buffer systems where not evaluated since acid solutions are commonly used to elute antibodies from antigens.

Buffer	pH	IgM anti-FITC	IgG anti-FITC
.05M Tris	7.2	280	36
	8.0	100	20
.1M PBS	7.2	92	8
	8.0	80	12
.1M Phosphate	7.2	112	8
	8.0	116	8

Figure Experiment 2.3
Immunospots per 2 x 10^6 Splelen Cells

The optimal buffer for the IgG anti-FITC ELISPOT was also 0.05 M Tris, pH 7.2 (Figure Experiment 2.3). At a pH of 7.2, the number of IgG anti-FITC immunospots with the Tris buffer was 4.5 times greater that with phosphate buffer or PBS. The Tris buffer resulted in 1.8 times the number of IgG anti-FITC immunospots at a pH of 7.2 than at a pH of 8.0. Minimal to no changes in immunospot numbers were noted with alterations in pH of phosphate or PBS buffering systems.

Effect of Glutaraldehyde Polymerization on the Sensitization of the Solid Phase: Because of the low number of detectable IgG anti-FITC immunospots when native FCS was used as the hapten carrier, an increase in assay sensitivity was desired. Holt reported that polymerization of antigens may improve the sensitivity of certain ELISPOT procedures.[59,61] Polymerized FCS-FITC and unadultered FCS-FITC were therefore compared as solid phase sensitizing reagents for ELISPOT.

Glutaraldehyde polymerized FITC-FCS and unadulterated FITC-FCS were used to sensitize the solid phase of the IgG and IgM anti-FITC ELISPOT. Each sensitizing reagent was compared using doubling dilutions from 1 to 0.13 mg/ml of FCS-protein. All other immunoreagents were used at the optimal concentrations determined previously.

Polymerization of the FITC-FCS resulted in a 2.3 fold average increase for IgM and a 5.5 fold average increase for IgG immunospot formation when equal FITC-FCS concentrations were compared (Figure Experiment 2.4). Maximal IgG immunospot formation occurred with a solid-phase sensitizing concentration of 0.13 mg./ml of polymerized FITC-FCS.

	FITC-FCS mg/ml							
	1.0		0.5		0.25		0.13	
	P	U	P	U	P	U	P	U
IgM Immunospots	34	14	36	20	32	12	16	8
IgG Immunospots	20	0	14	6	14	4	36	6

P = Polymerized U = Unpolymerized

Figure Experiment 2.4
Immunospots per 2 x 10^6 Spleen Cells

For the IgM anti-FITC ELISPOT minimal differences in sensitivity were noted between the various solid phase sensitizing concentrations of polymerized FITC-FCS. In order to conserve reagent, optimal coating concentration for the IgM anti-FITC assay was determined to be 0.25 mg./ml. Polymerization of FITC-FCS resulted in a more sensitive assay and was used to prepare all subsequent anti-FITC ELISPOT assays.

Within-run Precision: Within-run coefficient of variation was calculated from the results of 4 duplicate plates for each IgM and IgG anti-hapten ELISPOT assay. Optimal immunoreactant concentrations and appropriately immunized mice were utilized. Within run CVs ranged from 6.1 to 14.1% (Figure Experiment 2.5).

	anti-TNP ELISPOT		anti-FITC ELISPOT	
	ISC*	CV	ISC*	CV
IgM	31 +/- 4	13.4%	148 +/- 9	6.1%
IgG	92 +/- 13	14.1%	117 +/- 14	12.0%

*Average per 4 replicates

Figure Experiment 2.5
ISC per 2 x 10^6 Spleen Cells

Specificity: The specificities for the anti-TNP and anti-FITC ELISPOT procedures were evaluated using the same spleen cells harvested for the within-run precision protocols. Isolated spleen cells from TNP immunized mice were incubated on FITC-FCS and FCS only sensitized solid phases. Likewise, spleen cells from FITC immunized mice were placed on TNP-FCS and neat FCS sensitized plates. Other than the use of inappropriate spleen cells on the solid phase, test protocols were identical to the simultaneously performed with-in run precession assays.

Results were compared to those obtained when appropriately immunized spleen cells were cultured on the corresponding solid phase.

	anti-TNP		anti-FITC	
	IgM	IgG	IgM	IgG
FITC Immunized	6	0	148	117
TNP Immunized	31	92	4	2

Figure Experiment 2.6
Immunospots per 2 x 10^6 Spleen Cells

The anti-TNP and anti-FITC ELISPOT procedures were highly specific in detection of anti- hapten specific antibody producing cells. Mice immunized with TNP had no detectable IgG anti-FITC and minimal IgM anti-FITC ELISPOT activity. FITC immunized mice had no detectable IgG anti-TNP and minimal IgM anti-TNP immunospot formation. Background anti-FCS activity was not noted on this run. Anti-FCS immunospots of IgG or IgM specificity have only been noted occasionally on previous evaluations. Maximal anti-FCS activity noted on previous runs has been 2 immunospots per 10^6 spleen cells.

Discussion

The ELISPOT assay was modified to enumerate both IgM and IgG anti-TNP and anti-FITC immunoglobulin secreting cells. Optimal test conditions were established and standardized to achieve an acceptable level of sensitivity and precision.

An optimal solid phase is central to the success of the ELISPOT assay.[274] Selection of the polystyrene solid phase was found critical to the anti-hapten test outcome. Only non-wettable, non-tissue culture treated polystyrene dishes were suitable for sensitization (data not

shown). This phenomena has been reported with other ELISPOT procedures.[122,270] It is unclear whether the physical properties of the plastic surface, coating antigen or a combination of both are responsible for this phenomena.

The ELISPOT was developed from the basic ELISA concept.[198,270] Although the two techniques are similar, they often require different optimal concentrations of immunoreactants. This difference was most apparent when the IgG anti-TNP ELISPOT and ELISA protocols are compared. Concentrations of unadulterated TNP-FCS needed to coat the ELISPOT solid phase were approximately 14 times greater (1 mg./ml. vs. 72 ug./ml.) than that required to sensitize ELISA plates. In addition, free FITC produced acceptable ELISA activity but resulted in no detectable ELISPOT activity (data not shown). The requirement for higher concentrations of sensitizing antigen with ELISPOT compared to ELISA has been previously reported.[122,45] The physical properties of the sensitizing antigen appear to affect its ability to effectively sensitive the ELISPOT solid phase.

The 3-dimensional configuration of the solid phase bound antigen and resultant threshold deposition of enzymatic activity may explain the lack of immunospot activity noted when FITC or TNP haptens were used without a protein carrier to sensitize the solid phase (data not shown). The hapten DNP is similarly unsatisfactory in the ELISPOT assay unless it is conjugated to a carrier protein. Holt found that a solid phase sensitized with DNP alone had only 10% of the sensitivity of an ELISPOT procedure utilizing DNP conjugated to BSA.[122] In contrast, FITC and TNP haptens have been used effectively in this laboratory to directly sensitize the solid phase of ELISA assays. Large multideterminant antigens are not required with conventional ELISA techniques where deposition of localized pigment would be undesirable.[122]

In addition, the differences in coating concentrations may be partially explained by differences in physical characteristics between the polystyrene used to manufacture ELISPOT culture dishes and ELISA microtiter plates. Differences in plastics between manufacturers and even between manufacturer's lots can affect solid phase sensitization.[312]

Glutaraldehyde polymerization of FITC-FCS antigen improved the sensitivity of both the IgM and IgG anti-FITC immunospot formation by 1.8 to 10 times depending on the initial coating concentration employed. The degree of polymerization of low molecular weight antigens (ie. albumin) is directly related to the sensitivity of the ELISPOT procedure with some test systems.[122,274] Polymerized ovalbumin was superior to native ovalbumin as a solid phase for the ELISPOT assay while this was not the case with the conventional ELISA technique.[122]

For immunospots to be visible, a high density of antigenic binding sites must be geographically accessible to the immunoglobulin secreting cells.[57] Antigen polymerization concentrates the antigenic binding sites within a given area and thereby permits localized deposition of secreted antibody and subsequent consolidation of the marker antibody's enzymatic activity. The resulting 3-dimensional array of enzymatic activity exceeds the threshold required for visual determination with the ELISPOT assay.[122] This same phenomena most likely affected the FITC haptenic binding sites located on the small carrier proteins (ie. albumin, etc.) which comprise FCS.

The effects of polymerization of TNP-FCS on the sensitivity of anti-TNP immunospot formation were not evaluated.

The buffer system used to wash the ELISPOT plates and to dilute the developing antisera profoundly affected the immunospot results. When Tris was used for washing procedures and as a diluent for developing antisera,

a 3-4.5 fold increase in both IgM and IgG immunospot formation occurred compared to PBS or phosphate buffer.

Although electrostatic binding is potentially the most potent force between antigen and antibody, its significance is unclear in biologic fluids containing high salt concentrations. High salt concentrations may neutralize or shield electrically charged regions on antigen and antibody molecules. An attempt to maximize the benefit of electrostatic forces was achieved in vitroo by replacing the highly ionized inorganic salt buffer PBS used routinely in ELISPOT assays with the organic buffer Tris which contains fewer ionized molecules.

Tris buffer resulted in detection of greater numbers of small pale immunospots. Low affinity antibodies are speculated to be responsible for small pale immunospots noted in standard ELISPOT protocols.[213,122,274] Increased electrostatic interactions resulting from reduced interference by buffer ions possibly augments the weaker stabilizing energies (0.5 to 5 kcal/mole) of the other noncovalent binding forces and resulted in more potent interactions between low affinity antibodies and antigen. Therefore, dissociation of immune complexes during ELISPOT processing would be decreased and test sensitivity would correspondingly increase.

A Tris buffer with pH 8 tended to reduce ELISPOT sensitivity. Alterations in pH could change the tertiary structure of antigen and/or antibody.[262] The three dimensional complementarity normally allows antigen and antibody to approach very closely and thereby maximizes the noncovalent binding forces between the two entities.[303] Any pH induced alteration in tertiary structure of the antigen or antibody binding sites could adversely affect immune complex formation and the subsequent immunospot formation.

Extremes in pH could also alter the antigen-antibody electrostatic (ionic) interaction between negatively charged aspartic or glutamic acids on one immunoreactant molecule and the positively charged lysine, arginine and histidine side chains on the other.[303] Electrostatic binding potential has the highest stabilization energy (5-10 kcal/mole) of all the noncovalent antigen-antibody binding forces.[85] Changes in the surface charges of antigen or antibody could therefore result in loss of detectable imunospots due to instability, decreased formation and increased dissociation of immune complexes.

Anti-TNP and anti-FITC ELISPOT within run precision ranged from 6 to 16% as measured by the coefficient of variation. This compares favorably with previously reported values.[346,270,57] The CV between replicate dishes generally does not exceed 20% unless the number of detectable immunospots is less than 20.[59] ELISPOT's coefficient of variation has been shown by direct comparison to be superior to the traditional hemolytic plaque assay's coefficient of variation which can range from 11 to 31%.[57]

The anti-hapten ELISPOT techniques had adequate sensitivity to detect IgG and IgM anti-TNP or anti-FITC immunospot activity during a primary immune response when immunoglobulin secreting cells would be minimal in number. Under optimal testing conditions during the quality control run, the number of immunospots per 2 x 10^6 spleen cells detected 5 days post immunization average 31 $\pm$ 4 for IgM anti-TNP, 92 $\pm$ 14 for IgG anti-TNP, 148 $\pm$ 9 for IgM anti-FITC and 117 $\pm$ 14 for IgG anti-FITC assays.

Moskophidis, using a peroxidase enzyme system similar to this report, was able to detect immunospots 5 days post viral challenge. He reported 41 to 266 IgM and 0 to 300 IgG anti-lymphocytic choriomeningitis (LCM) virus immunospots per 2 x 10^6 spleen cells.[204] A single haptenic determinant such as TNP or FITC would be expected to stimulate fewer

antibody clones than the antigenically complex LCM virus. Therefore, compared to the 5 day LCM response, the number of anti-hapten immunospots are within a reasonable range.

Specificity of anti-TNP and anti-FITC immunospot production was high. No cross reactivity between the two assay was detected. Exclusion of splenocytes from the assay (data not shown) resulted in no immunospot production. Lack of immunospot formation on FCS sensitized plates attested to the specificity of the assay systems and indicated that the immunospots which formed on hapten-FCS solid phases were directed against the hapten and not the carrier protein.

The objectives of establishing a sensitive, specific and precise assay for quantization of anti-TNP and anti-FITC immunospots were met. In addition, the ELISPOT procedure was economical and afforded the opportunity to process large numbers of samples routinely with a degree of ease approaching that of the conventional ELISA from which it was derived.

EXPERIMENT III

Objectives

The objective of this experiment was to evaluate the ability of P. acnes to induce spontaneous antibody production against the haptens TNP and FITC.

Materials and Methods

P. acnes: P. acnes was purchased from Burroughs Welcome; Research Triangle, North Carolina. The commercial product was a formalin killed whole bacterin suspension preserved with 0.01% thimerosal.

Mice: Female, 6 to 8 week old, C57B16 mice were purchased from Jackson Laboratories; Bar Harbor, Maine. Housing and care were provided by Laboratory Animal Care Services at Michigan State University; East Lansing, Michigan. Purina mouse chow and water were available ad libitum. Mice were ear notched for identification upon arrival. A one week acclimation period preceded initiation of the experimental protocol.

Experimental Groups: Ten mice were assigned via random numbers tables to each of 3 experimental groups, I, II and III. Five mice from each group received 0.33 mls intraperitoneally of the P.acnes suspension. The remainder of the mice served as controls and were administered 0.33 mls. intraperitoneally of sterile PBS. Both treated and control mice were housed together in the same cage.

Group I, Group II and Group III were euthanized respectively on post treatment day 5, 10 and 15. Mice were terminally exsanguinated through the orbital plexus while under light ether anesthesia. Blood was allowed

to clot for 30 minutes at room temperature and 2 hours at 4°C. Sera for the anti-TNP and anti-FITC ELISA were harvested and frozen at -20 C. Spleens were aseptically removed and placed into ice cold RPMI and processed immediately for the anti-TNP and anti-FITC ELISPOT.

IgM and IgG anti-TNP and anti-FITC ELISA: ELISA protocols utilized the optimal test conditions determined in Experiment I. All anti-hapten antibody levels were determined during a single run in order to reduce any between run variability. Samples for each antibody class and for each hapten were determined in duplicate and averaged after subtracting any background reading from the negative control wells. Variability between plates was reduced by comparing each test result to the absorbance value of the appropriate positive control with the results being expressed as a percentage of the positive control.

IgM and IgG anti-TNP and anti-FITC ELISPOT: ELISPOT protocols utilized the optimal test conditions determined in Experiment II.

All anti-hapten ISC determinations were simultaneously determined for each treatment group to reduce between run variability. Each sample was run in duplicate and the results averaged after subtraction of any background ISC present on the negative control plates.

IgM and IgG ELISPOT: Total numbers of IgG and IgM secreting cells were determined simultaneously with the anti-hapten tests. This ensured that the spleen cells were viable antibody producers and provided a means of estimating overall antibody producing activity within the spleen.

IgM and IgG ISC activities were quantified using a modification of the IgM and IgG ELISPOT described by Holt[119] and Tarkowski.[295] Briefly, Falcon petri dishes were sensitized by overnight incubation with commercially prepared (Cappell) goat anti-mouse IgM or IgG antisera diluted to 1:100 in 0.1 M carbonate buffer, pH 9.5. After rinsing the plates 3 times with

PBS, pH 7.2, non specific protein binding sites were blocked by a 1 hour incubation at 37° C with 5% FCS in sterile PBS, pH 7.2. Just prior to use the dishes were flicked to remove excess buffer. Viable cell numbers were determined by trypan blue exclusion and manual hemocytometer counts. Two x 10^4 nucleated spleen cells in 0.66 mls. of RPMI containing 10% FCS were added to each dish. Cell cultures were incubated for 5 hours at 37° C and 100% humidity followed by 2 washes with ice cold Tris buffer pH 7.2. Peroxidase conjugated goat anti-mouse IgM or IgG; diluted 1:100 in Tris buffer, pH 7.2, containing 0.05% Tween 20 and 0.5% FCS; was incubated on the dishes overnight at 4° C followed by 3 washed with Tris buffer, pH 7.2. One ml of 0.1% diaminobenzidine in PBS,pH 7.2, containing 0.5% agarose and 0.01% hydrogen peroxide was added to each dish. The chromogen reaction was allowed to develope in the dark for 18 hours. The resulting immunospots were quantified under 10X magnification.

Statistics: Data were summarized into contingency tables prior to analysis. Parametric data were analyzed by the F test for analysis of variance with blocking. The level of significance was set at 0.05.

Results

Intraperitoneal administration of P. acnes resulted in a significant ($p=0.01$) increase in the number of nucleated splenocytes throughout the experiment's duration. Spleen cell numbers from treated mice were maximal on day 5 and averaged 1.6 times control values. By day 15 spleen cell numbers from treated mice had declined averaging only 1.3 times control levels. Alterations in spleen cell numbers were similar to those previously reported in the literature[20,315] and correlated with the splenomegaly noted grossly.

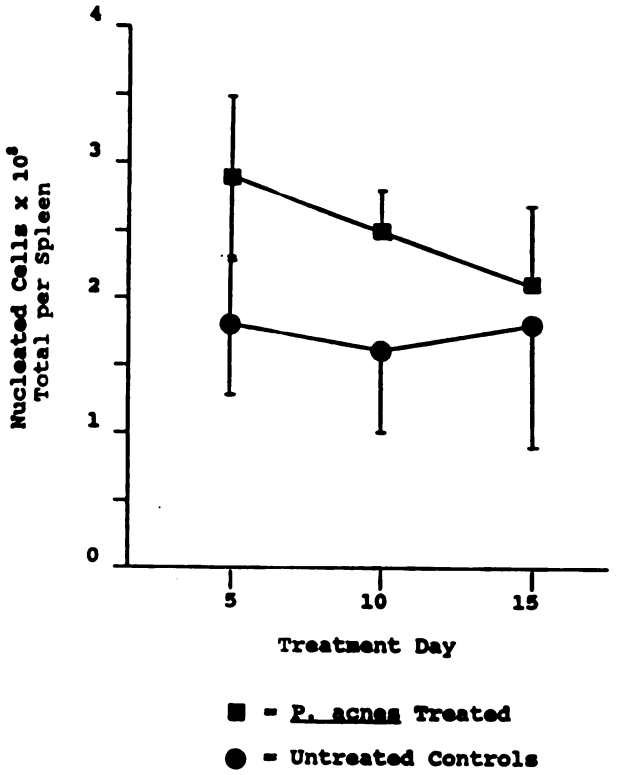


Figure Experiment 3.1

Increased spleen cell numbers were accompanied by significant ($p=0.01$) increases in both relative and absolute numbers of IgM ISC (Figures Exp 3.2 and 3.3). Significant ($p=0.01$) changes in the numbers of IgM ISC were noted with time along with a significant interaction between time and P. acnes treatment.

The total number of IgM ISC per spleen was maximal on day 5, averaging 4.3 times control values. Relative IgM ISC values for treated mice also peaked at this time with 2.7 times more IgM ISC per 10^6 spleen cells than controls. Relative expansion of the IgM ISC population was responsible for 81.6% of the increase in IgM ISC while enlargement of the overall spleen cell pool only contributed to 18.4% of the increased IgM ISC numbers. Therefore, augmentation of spleen cell numbers was not the

result of a uniform expansion of all spleen cell types. Expansion of select spleen cell subpopulations, such as IgM ISC, appeared to be favored. It cannot be determined from these data if expansion of the IgM ISC was the result of increased cellular division, increased cellular maturation or a combination of both.

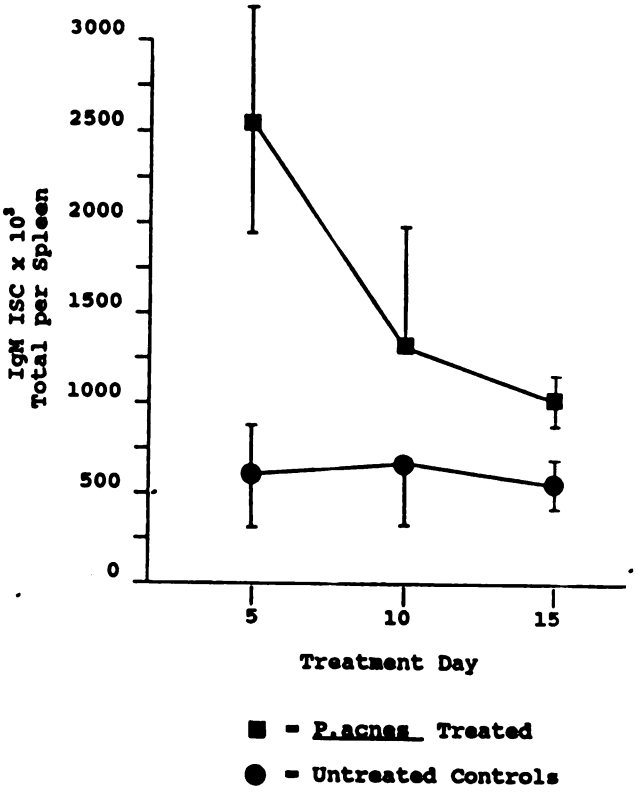


Figure Experiment 3.2

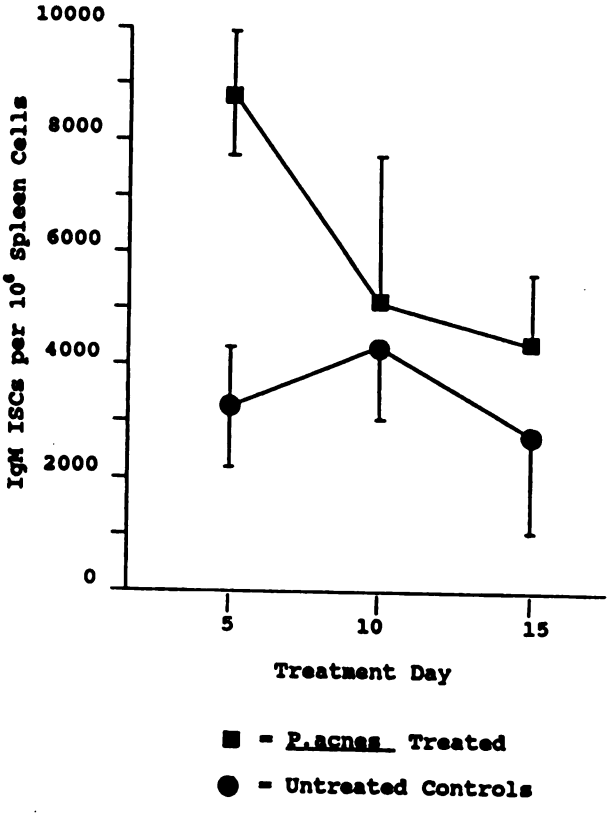


Figure Experiment 3.3

Expansion of splenic IgM ISC was accompanied by significant ($p=0.01$) increases in the spontaneous production of IgM anti-TNP and anti-FITC antibodies (Figure Experiment 3.4-3.7). Significant ($p=0.01$) increases in absolute and relative numbers were noted with maximal numbers coinciding on day 5 when total IgM production was at its greatest.

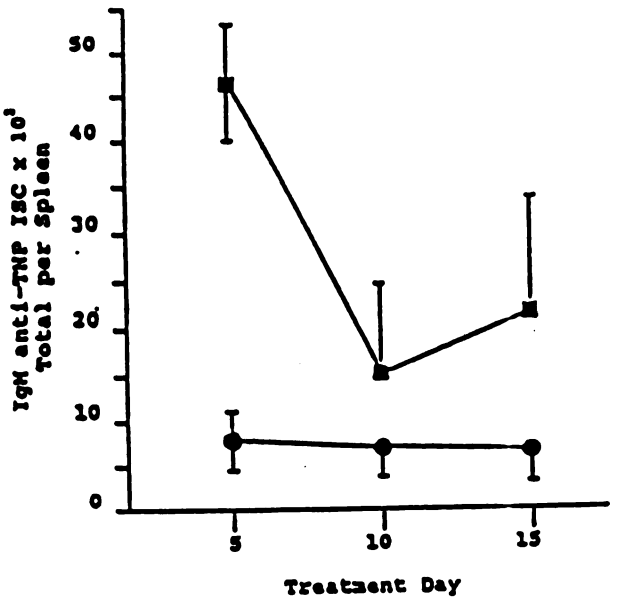


Figure Experiment 3.4

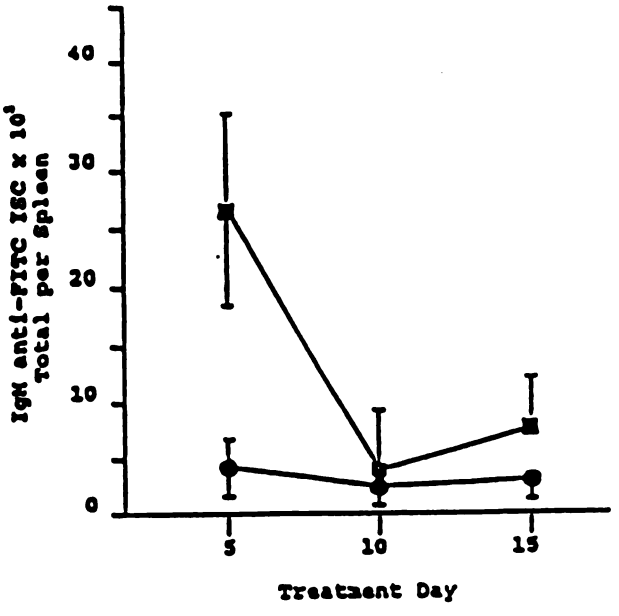


Figure Experiment 3.5

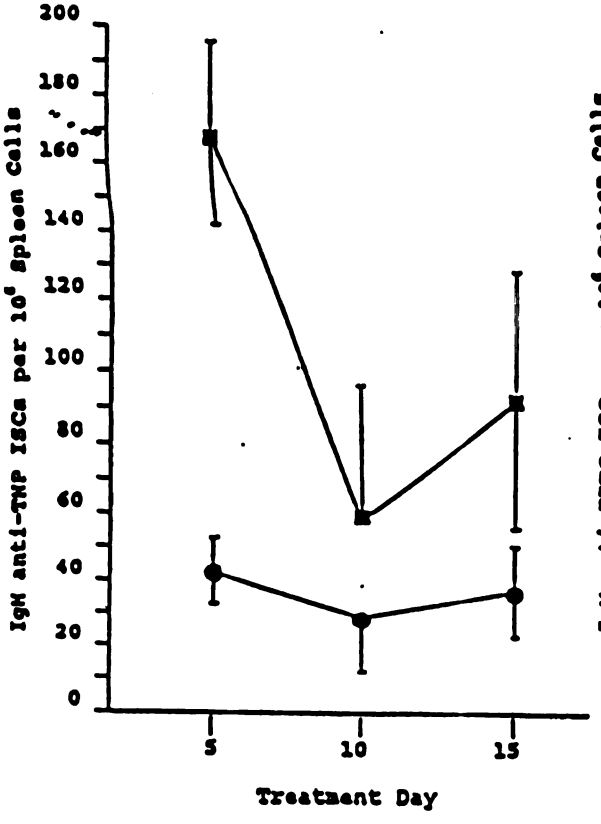


Figure Experiment 3.6

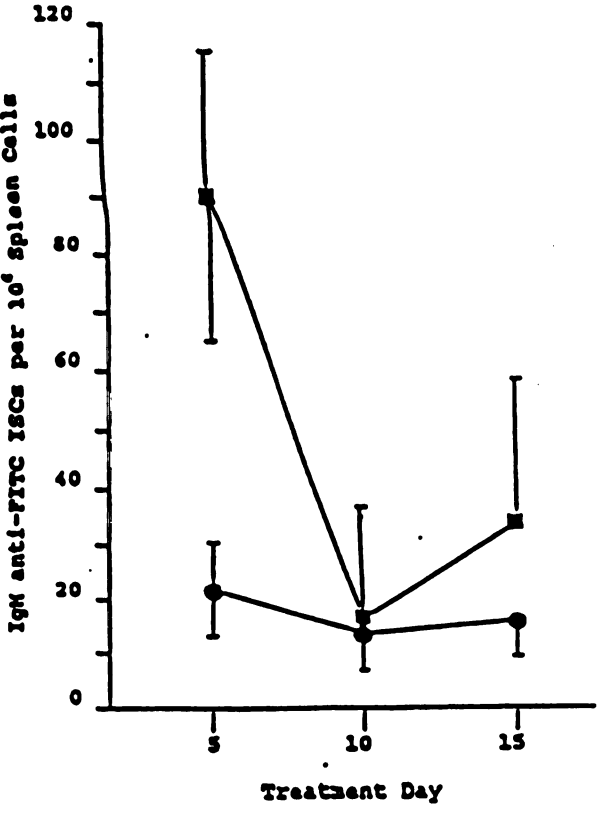


Figure Experiment 3.7

The increased ISC anti-hapten activity dropped rapidly toward control values by day 10. Approximately 13% and 12% of the increased anti-TNP and anti-FITC peak activity was respectively the result of overall spleen cell pool expansion and 87% and 88% was respectively due to elevations in relative anti-hapten ISC numbers. Although, 1.7 times more IgM anti-TNP ISC than IgM anti-FITC ISC were detected on experimental day 5, the magnitude of both anti-hapten responses was nearly equal being 5.7 and 6.3 times respective control values. This suggests that the two IgM anti-hapten subpopulations were being stimulated to similar relative functional levels.

Significant ($p=0.01$) increases in serum IgM anti-TNP antibody levels were noted throughout the experiments duration (Figure Experiment 3.8). Maximal values were present on day 5 corresponding with the peak in IgM anti-TNP ISC activity. The difference between the average control and treated percent positive ratio was also maximal at this time. Percent positive ratios changed significantly ($p=0.01$) over time with a significant ($p=0.01$) interaction between P. acnes treatment and experimental duration.

Serum IgM anti-FITC percent positive ratios were significantly elevated ($p=0.05$) on day 5 and 15 (Figure Experiment 3.9). The interaction between P. acnes treatment and time had the same level of significance ($p=0.05$). The highest average percent positive ratio for treated mice was found on experimental day 15. However, maximal differences in treated and control values were present on experimental day 5 when IgM anti-FITC ISC numbers were at their peak.

Other polyclonal B cell activators are capable of activating different anti-hapten clones in different lymphoid organs to different levels of antibody production. It is possible that peak splenic ISC

values may not have equated with peak serum antibody levels since the ELISPOT only evaluated splenic and not systemic lymph node anti-hapten ISC numbers while serum antibody quantification measured antibody production from both.

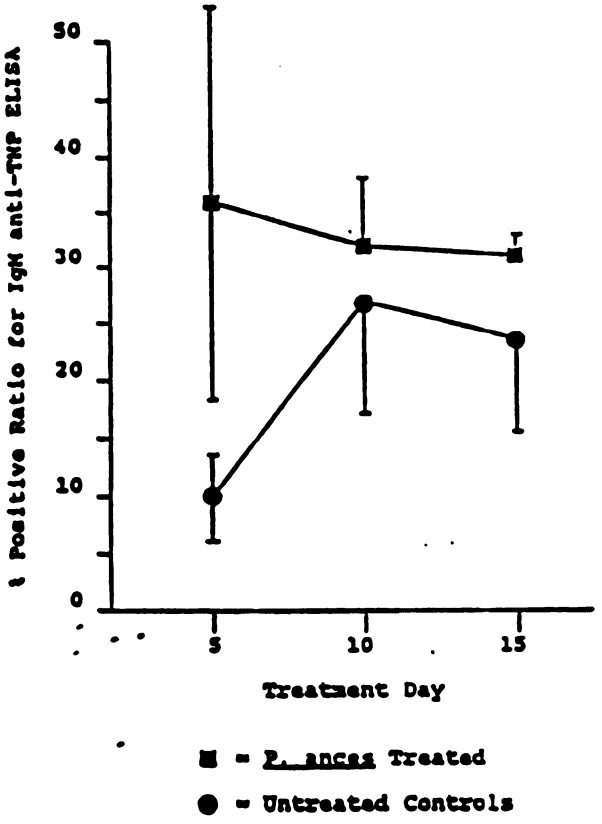


Figure Experiment 3.8

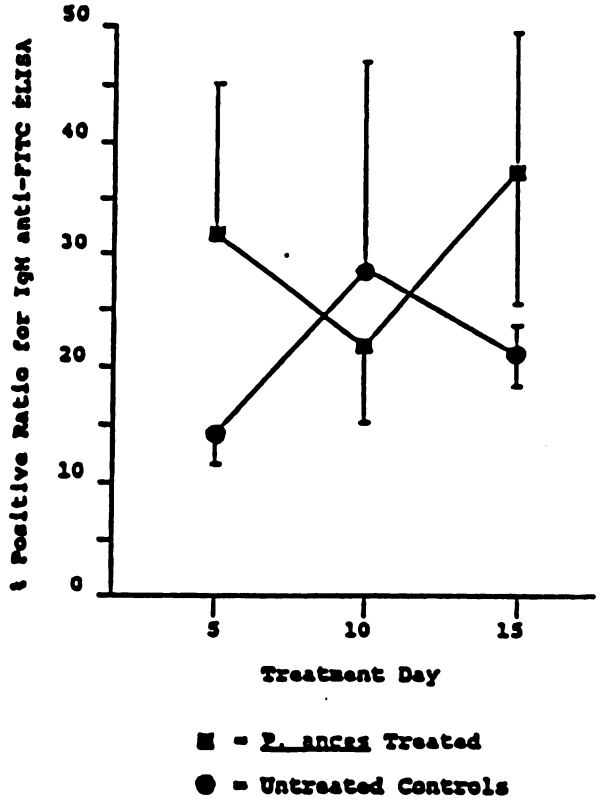


Figure Experiment 3.9

Concurrent significant increases in the absolute ($p=0.05$) and relative ($p=0.01$) numbers of IgG ISC were also noted (Figure Experiment 3.10 and 3.11). Maximal relative and absolute IgG ISC were noted on day 10. At this time, the absolute number of IgG ISC for P. acnes treated mice averaged 3.9 times greater than controls. The relative number of IgG ISC per 10^6 spleen cells was 2.0 times greater than control values. IgG ISC numbers for treated mice did not show as rapid a decline toward control values by day 15 as was noted with IgM ISC. In fact, no

significant change in absolute or relative IgG ISC numbers was noted with time and no significant interaction between time and treatment was detected. This suggests that IgG ISC numbers rapidly increase and remain elevated for a longer duration than IgM ISC activity.

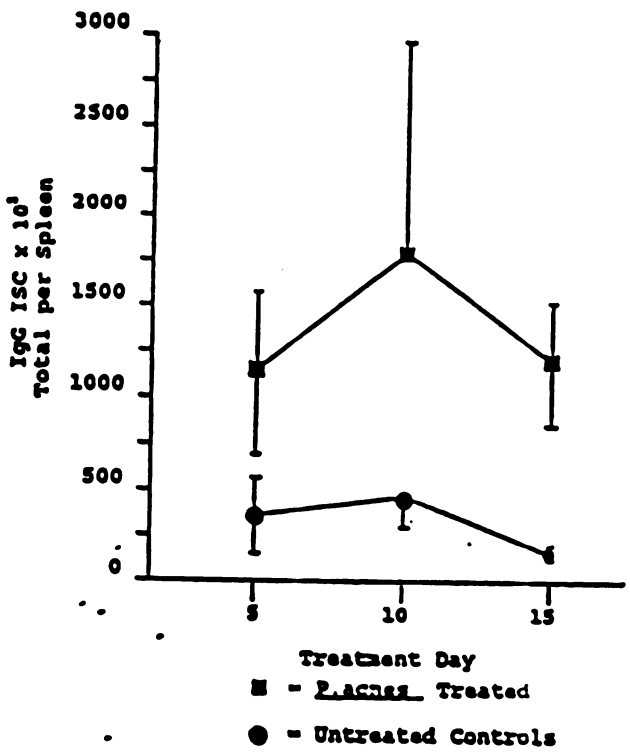


Figure Experiment 3.10

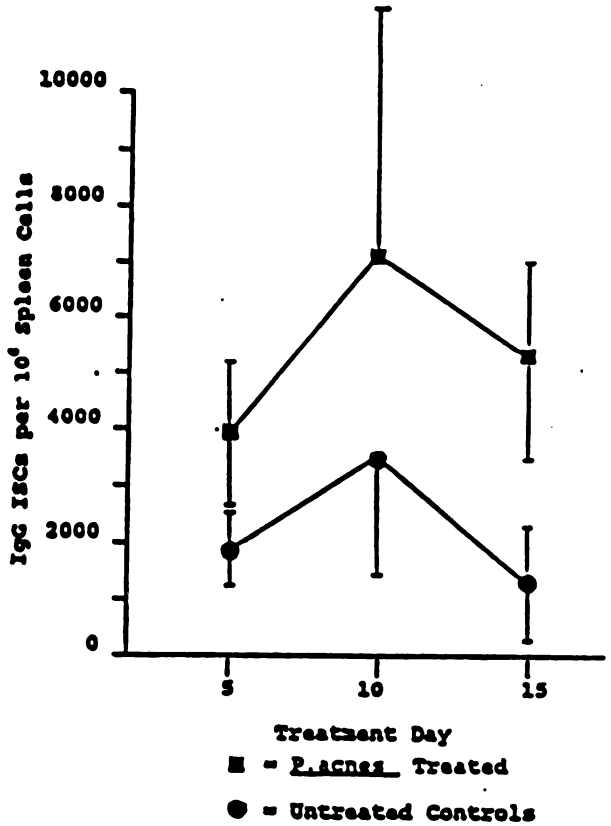


Figure Experiment 3.11

When peak IgG ISC values were present, 62% of the increase was due to overall expansion of the spleen cell pool. In addition, 38% of IgG ISC expansion was the result of relative increases in the IgG ISC population. Similar to IgM ISC, P. acnes produced a favorable expansion stimulus for IgG ISC in comparison to the overall spleen cell pool.

IgG anti-TNP absolute ISC numbers (Figure Experiment 3.12) paralleled the changes noted in overall IgG ISC activity. A significant ($p=0.05$) treatment effect was present. A significant effect of time alone

or a time and treatment interaction was not noted. P. acnes did not produce any significant effect on the relative numbers of IgG anti-TNP ISC in the spleen nor any significant elevation in serum anti-TNP antibody levels (Figure Experiment 3.14 and 3.16). Many of the IgG immunospots were small and of weak staining intensity which suggests that the antibody dected was being produced in low quantities or was of low affinity. Even though increased numbers of IgG anti-TNP ISC were noted, low levels of IgG production by indivdual cell may have adversely affected serum antibody levels.

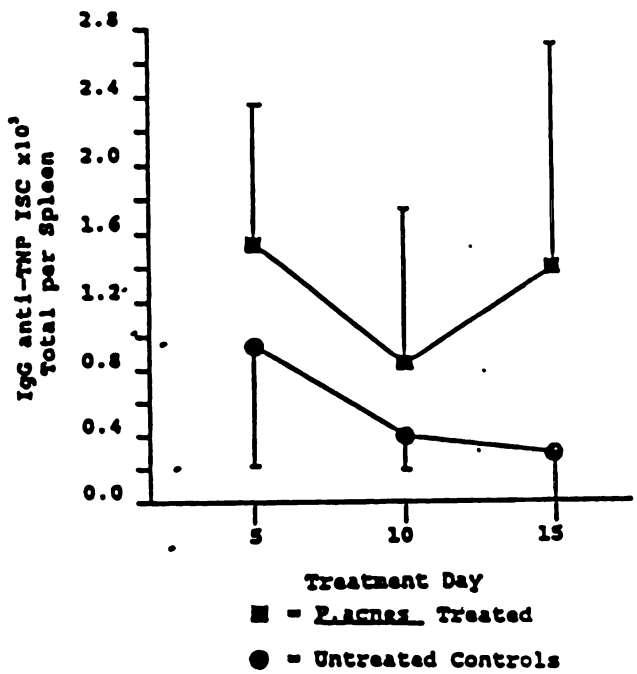


Figure Experiment 3.12

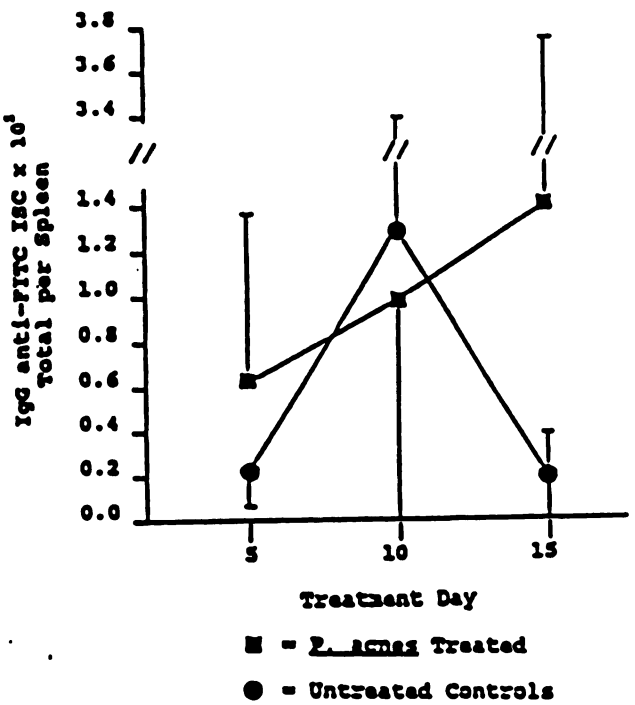


Figure Experiment 3.13

No significant change in the absolute or relative numbers of IgG anti-FITC ISC was noted with P. acnes therapy (Figure Experiment 3.13 and 3.15). However, treated mice had significantly ($p=0.05$) elevated serum IgG anti-FITC antibody levels compared to controls (Figure Experiment 3.17).

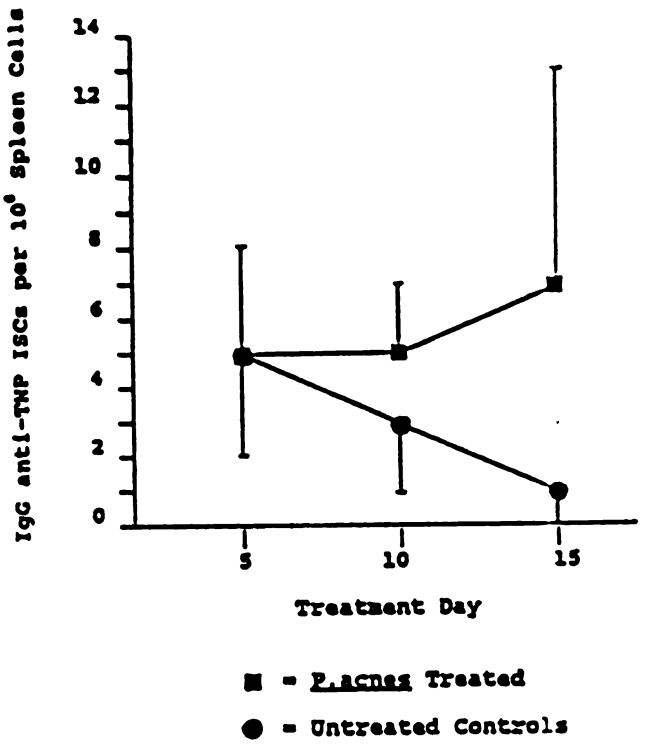


Figure Experiment 3.14

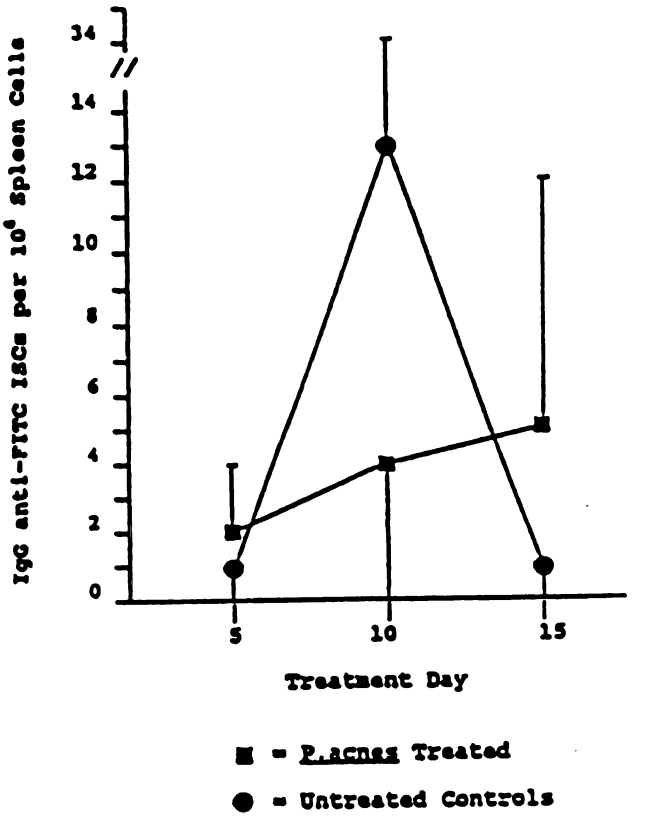


Figure Experiment 3.15

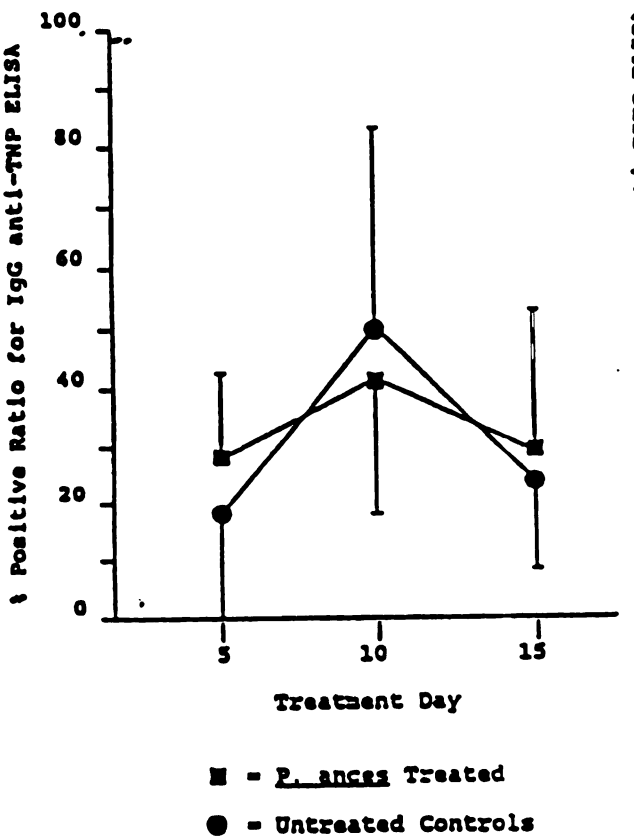


Figure Experiment 3.16

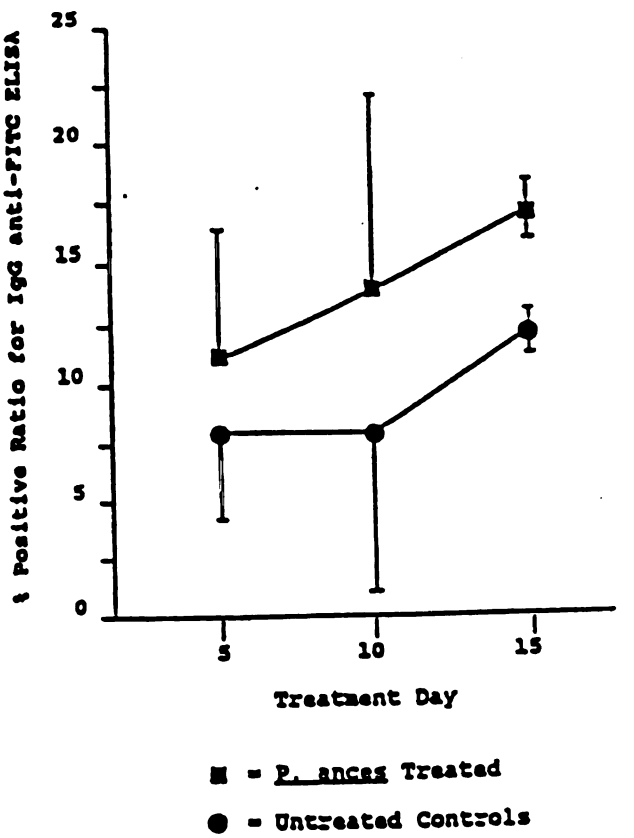


Figure Experiment 3.17

Apparent discrepancies between IgG anti-hapten ISC and serum antibody values may be related to differences in splenic and systemic antibody production or may reflect a type I statistical error. In addition, differences in experimental protocols between ELISA and ELISPOT may have produced variations in the ability to detect antibodies with low affinity and therefore resulted in differences in assay sensitivity. A recent report suggests that the standard ELISPOT protocol does not always detect low affinity antibodies or ISC with low levels of antibody production.[84]

Discussion

P. acnes is an in vivo polyclonal B cell activtor (PBA) producing, as documented in this experiment, spontaneous IgM anti-hapten antibody production. Detection of spontaneous anti-hapten antibody production is the standard means of documenting polyclonal B cell activity.[66] PBA trigger distinct subpopulations of B cells which may be at various stages of differentiation and in disparate lymphoid organs.[94,96,78] When activated by PBA, the immunocompetent B cell essentially performs the function of an antigen-activated cell and initiates production of the antibody for which it is genetically programmed and which it is capable of expressing at the current stage of differentiation.[47] Polyclonal activation is therefore capable of inducing B cells to produce antibodies against a variety of haptenic determinants even without appropriate antigen stimulation.

Increased serum IgM anti-hapten antibody levels post P. acnes treatment were accompanyied by increased relative and absolute numbers of splenic IgM anti-TNP and anti-FITC ISC. Although P. acnes is a B-cell mitogen for mouse spleen cells,[347] it is not apparent if the increased antibody secreting activity noted in this experiment resulted from an

increase in cell division, an increase in antibody secreting activity due to increased functional maturation or a combination of both.

Considerable variability in the response to polyclonal B cell activation has been documented between specific PBA. In mice, it has been shown that dextran sulphate, a PBA, stimulates relatively immature B cells and gives rise mainly to an increased DNA synthesis. In contrast, PPD produces polyclonal B cell activation by acting on relatively mature cells and by producing a marked increase in antibody formation with only a modest increase in DNA synthesis. LPS and type III pneumococcal polysaccharide induce polyclonal B cell activation by stimulating cells between these two extremes.[95]

Among bacteria, polyclonal B cell activation is not unique to P. acnes. Many bacteria, such as E. coli, B pertussis, and M. bovis to name a few, are capable of eliciting a polyclonal B cell response in murine models. In fact, many commonly evaluated PBA are bacteria or bacterial products. One extensively studied PBA is LPS. LPS is derived from the cell walls of gram negative bacteria. The active biologic constituent of P. acnes has also been isolated to the cell wall although no chemical similarities with LPS have been found.

Although their active components vary, the P. acnes induced spontaneous anti-hapten antibody production detected in this experiment resembles, in many respects, that induced by LPS. The increased IgM activity produced by P. acnes has a similar temporal relationship.[245] Maximal IgM antibody production was present in P. acnes treated mice on experimental day 5. LPS results in maximal IgM antibody production 4 days post treatment of spleen cell cultures and between 5 and 7 days post treatment of peripheral blood lymphocyte cultures.[150,284]

Relative numbers of IgM anti-TNP and anti-FITC ISC post P. acnes treatment were comparable to those obtained with LPS using the same C57Bl mouse strain. On experimental day 5 when IgM antibody activity was maximal, P. acnes induced 159 ± 27 IgM anti-TNP ISC per 10^6 spleen cells and 91 ± 25 IgM anti-FITC ISC per 10^6 spleen cells. For comparison, LPS administration to C57Bl/6 mice resulted in 55.9 ± 18.5 IgM anti-TNP and 95.2 ± 8.4 IgM anti-FITC ISCs per 10^6 spleen cells.[282] In other mouse strains LPS induced IgM anti-TNP ISC activity ranging from 18 to 184 ISC per 10^6 spleen cells.[19]

The spontaneous anti-TNP and anti-FITC antibody response to P. acnes is also similar to that observed in the NZB and MRL/l systemic lupus erythematosus mouse models. Both of these autoimmune mouse strains produce, between 1 and 5 months of age, increased levels of IgM anti-hapten antibodies without any detectable IgG anti-hapten activity. IgM anti-TNP ISC cells averaged 30.5 ± 9.8 and 26.4 ± 9.7 per 10^6 spleen cells for NZB and MRL/l mice respectively. Spontaneous IgM anti-FITC ISC activity averaged 76.1 ± 10.0 and 32.3 ± 4.6 per 10^6 splenn cells for NZB and MRL/l mice respectively. In addition to the comparable levels of magnitude in polyclonal B cell activation, P. acnes treated mice and young autoimmune mice both lacked significant IgG anti-hapten activity. Other PBA are capable of inducing autoantibodies [83] and it would be of interest to examine the potential autoimmune repertoire that P. acnes might induce.

Evidence for spontaneous IgG anti-hapten antibody production after P. acnes treatment is equivocal. Only absolute numbers of IgG anti-TNP ISC and serum IgG anti-FITC antibody levels were significantly (0.05) elevated. IgG anti-TNP or anti-FITC absolute and relative numbers as well as serum antibody levels were not as a group consistently elevated above

control values. Discrepencies in the significance of IgG anti-hapten assays used to evaluated spontaneaous anti-TNP or anti-FITC antibody production, may be the result of a type I statistical error, differences in assay sensitivities to low affinity antibodies, variations in the quantities of antibody produced by individual ISC, or diffences in the ability to P. acnes to stimulate clones of ISC in the spleen versus other lymphoid organs.

Although IgG ISC numbers were significantly elevated in P. acnes treated mice, it is possible that the increase in IgG ISC resulted as part of the antibody response directed against P. acnes or augmentation of established antibody responses. P. acnes does induce the production of anti-P. acnes antibodies and has known adjuvant activities for antigen induced B-cell immune responses. Additional work is required before the effect of P. acnes on spontaneous IgG antibody production can be delineated.

Numbers of anti-hapten ISCs for control animals tended to fluctuate between experimental days. This is most apparent for the anti-IgG FITC ISC results. Marked elevations in control anti-IgG FITC ISCs were mainly the result of 2 mice with unusually high ISC values (see Appendix Figure 3.11, 3.14, 3.15). No cause for these fluctuations was apparent. However, IgM and IgG ISCs from the same individual has been reported to differ when tested at different times.[245] Unintentional variability in testing protocol could account for such differences. Also, subclinical infections may influence polyclonal B cell activation.[357] "Healthy" human patients who suffered from upper respiratory tract infections have reported concurrent elevated ISC numbers. The possibility of exposure to infectious agents during this experiment is possible since mice were housed in an open colony.

SUMMARY AND CONCLUSIONS

P. acnes, when administered intraperitoneally to mice, induces an IgM polyclonal B cell response. This response is characterized by spontaneous anti-hapten antibody production against FITC and TNP. Relative and absolute numbers of IgM anti-FITC and anti-TNP secreting spleen cells maximally increase 5 days post P. acnes treatment. Anti-hapten activity rapidly declines toward control values by 15 days. Serum IgM anti-TNP and anti-FITC concentrations elevate and decline in parallel with splenic antibody secreting activity. Significant changes in IgG anti-TNP and anti-FITC activity are not detectable.

The magnitude, timing and nature of the polyclonal antibody response induced by P. acnes is similar to that produced by bacterial LPS. LPS is not a component of P. acnes, therefore, P. acnes is inducing polyclonal B cell activation by another, as yet unknown, mechanism. Additional investigations will be required to determine whether P. acnes produces polyclonal activation via its potent effect on the monocyte-macrophage system or by a direct stimulation of B cell clones.

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APPENDIX

	% Concentration of FCS Used to Sensitize IgM anti-FCS ELISA Solid Phase				
	0	0.008	0.040	0.200	1.000
Control A	0.005	0.004	0.001	0.001	0.002
Control B	0.006	0.010	0.002	0.001	0.008
FITC Immunized A	0.006	0.008	0.003	0.000	0.003
FITC Immunized B	0.005	0.005	0.002	0.000	0.003
TNP Immunized A	0.006	0.006	0.002	0.004	0.007
TNP Immunized B	0.005	0.008	0.000	0.002	0.004
Mean +/ ISO	0.006 +/0.001	0.007 +/0.002	0.000 +/0.002	0.001 +/0.002	0.005 +/0.002

	% Concentration of FCS Used to Sensitize IgG anti-FCS ELISA Solid Phase				
	0	0.008	0.040	0.200	1.000
Control A	0.001	0.003	0.002	0.003	0.002
Control B	0.002	0.004	0.001	0.003	0.004
FITC Immunized A	0.008	0.003	0.006	0.003	0.007
FITC Immunized B	0.007	0.006	0.001	0.004	0.002
TNP Immunized A	0.003	0.005	0.000	0.004	0.001
TNP Immunized B	0.004	0.001	0.002	0.005	0.003
Mean +/ ISO	0.004 +/0.003	0.004 +/0.002	0.001 +/0.003	0.004 +/0.001	0.003 +/0.002

Data Experiment Figure 1.1
Absorbance Values for IgM and IgG anti-FCS ELISA

	IgM anti-TNP		IgG anti-TNP		IgM anti-FITC		IgG anti-FITC	
	0.0	0.2	0.0	0.0	0.0	0.2	0.0	0.2
Control A	0.006	0.006	0.008	0.005	0.109	0.004	0.097	0.010
Control B	0.001	0.003	0.015	0.005	0.100	0.003	0.074	0.008
TNP Immunized A	0.090	0.049	0.126	0.093	0.115	0.000	0.040	0.002
TNP Immunized B	0.093	0.061	0.126	0.099	0.140	0.010	0.062	0.002
FITC Immunized A	0.072	0.012	0.014	0.004	0.174	0.094	0.154	0.092
FITC Immunized B	0.066	0.034	0.023	0.006	0.190	0.031	0.164	0.118

Absorbance

	IgM anti-TNP		IgG anti-TNP		IgM anti-FITC		IgG anti-FITC	
	0.0	0.2	0.0	0.0	0.0	0.2	0.0	0.2
Control A	6.7	6.7	6.3	4.0	62.6	0.0	63.0	6.4
Control B	0.0	3.3	11.9	4.0	57.5	0.0	48.1	5.2
TNP Immunized A	100.0	54.4	100.0	73.8	66.1	0.0	26.0	1.3
TNP Immunized B	103.3	67.8	100.0	78.6	80.5	5.7	40.3	1.3
FITC Immunized A	80.0	13.3	11.1	0.0	100.0	54.0	100.0	60.0
FITC Immunized B	73.3	37.8	18.3	4.8	109.2	17.8	106.5	76.6

Percent Positive Control

	IgM anti-TNP		IgG anti-TNP		IgM anti-FITC		IgG anti-FITC	
	0.0	0.2	0.0	0.0	0.0	0.2	0.0	0.2
Control	3.4 +/4.7	5.0 +/2.4	9.1 +/4.0	4.0 +/0.0	60.0 +/3.6	0.0 +/0.0	55.6 +/10.5	5.8 +/0.8
TNP Immunized	101.7 +/2.3	61.1 +/9.3	100.0 +/0.0	76.2 +/3.4	73.3 +/10.2	2.9 +/4.0	33.2 +/10.1	1.3 +/0.8
FITC Immunized	76.7 +/4.7	25.6 +/17.3	14.7 +/5.1	2.4 +/3.4	104.6 +/6.5	35.9 +/25.6	103.3 +/4.6	68.3 +/-11.7

Sample Mean +/ SD

Figure Experiments 1.2, 1.3, 1.4, 1.5
Percent FCS in Solid Phase Sensitizing Buffer

	Absorbance				% Positive Ratio			
	ug. of TNP-FCS Protein/ml of Buffer				ug. of TNP-FCS Protein/ml of Buffer			
	3	14	72	360	3	14	72	360
Control	-0.002	-0.005	0.008	0.027	0.0	0.0	8.8	29.7
FITC Immunized	0.001	0.001	0.012	0.092	1.1	1.1	13.2	101.1
TNP Immunized	0.014	0.029	0.063	0.091	15.4	31.9	69.2	100.0

Figure Experiment 1.6
TNP-FCS Titration for IgM anti-TNP ELISA

	Absorbance				% Positive Ratio			
	ug. of TNP-FCS Protein/ml of Buffer				ug. of TNP-FCS Protein/ml of Buffer			
	3	14	72	360	3	14	72	360
Control	0.000	0.000	0.005	0.019	0.0	0.0	4.0	15.2
FITC Immunized	-0.001	0.003	0.006	0.030	0.0	2.4	4.8	24.0
TNP Immunized	0.016	0.061	0.099	0.125	12.8	48.8	79.2	100.0

Figure Experiment 1.7
TNP-FCS Titration for IgG anti-TNP ELISA

	Absorbance				% Positive Ratio			
	ug. of TNP-FCS Protein/ml of Buffer				ug. of TNP-FCS Protein/ml of Buffer			
	3	14	72	360	3	14	72	360
Control	-0.011	0.001	0.000	0.033	0.0	0.9	0.0	28.2
FITC Immunized	0.051	0.104	0.106	0.117	43.6	88.9	90.6	100.0
TNP Immunized	0.003	0.010	0.037	0.072	0.0	8.5	31.6	61.5

Figure Experiment 1.8
FITC-FCS Titration for IgM anti-FITC ELISA

	Absorbance				% Positive Ratio			
	ug. of TNP-FCS Protein/ml of Buffer				ug. of TNP-FCS Protein/ml of Buffer			
	3	14	72	360	3	14	72	360
Control	0.020	0.048	0.058	0.700	13.8	33.1	40.0	48.3
FITC Immunized	0.103	0.139	0.138	0.145	71.0	95.9	95.2	100.0
TNP Immunized	0.005	0.005	0.025	0.067	0.0	3.4	17.2	46.2

Figure Experiment 1.9
FITC-FCS Titration for IgG anti-FITC ELISA

	Absorbance			% Positive Ratio		
	Serum Dilution			Serum Dilution		
	1:100	1:200	1:400	1:100	1:200	1:400
Control	0.005	0.006	0.001	6.2	7.4	1.2
FITC Immunized	0.013	0.011	0.007	16.0	13.6	8.6
TNP Immunized	0.081	0.036	0.021	100.0	44.4	25.9

Figure Experiment 1.10
Mouse Sera Titration for IgM anti-TNP ELISA

	Absorbance			% Positive Ratio		
	Serum Dilution			Serum Dilution		
	1:100	1:200	1:400	1:100	1:200	1:400
Control	0.059	0.002	0.004	37.8	1.3	2.6
FITC Immunized	0.061	0.013	0.005	39.1	8.3	3.2
TNP Immunized	0.156	0.097	0.068	100.0	62.2	43.6

Figure Experiment 1.11
Mouse Sera Titration for IgG anti-TNP ELISA

	Absorbance			% Positive Ratio		
	Serum Dilution			Serum Dilution		
	1:100	1:200	1:400	1:100	1:200	1:400
Control	-0.002	0.003	0.004	0.0	2.4	3.1
FITC Immunized	0.127	0.110	0.042	100.0	86.6	33.1
TNP Immunized	0.007	-0.006	0.002	5.5	0.0	1.6

Figure Experiment 1.12
Mouse Sera Titration for IgM anti-FITC ELISA

	Absorbance			% Positive Ratio		
	Serum Dilution			Serum Dilution		
	1:100	1:200	1:400	1:100	1:200	1:400
Control	0.030	-0.004	0.001	18.3	0.0	0.6
FITC Immunized	0.164	0.152	0.083	100.0	92.7	50.6
TNP Immunized	0.022	0.003	0.002	13.4	1.8	1.2

Figure Experiment 1.13
Mouse Sera Titration for IgG anti-FITC ELISA

	Absorbance			% Positive Ratio		
	Peroxidase Labelled anti-IgM Dilution			Peroxidase Labelled anti-IgM Dilution		
	1:100	1:200	1:400	1:100	1:200	1:400
Control	0.001	0.000	-0.001	1.9	0.0	0.0
FITC Immunized	0.017	0.011	0.004	31.5	20.4	7.4
TNP Immunized	0.045	0.052	0.054	83.3	6.3	100.0

Figure Experiment 1.14
Peroxidase Labelled anti-IgM Titration
for IgM anti-TNP ELISA

	Absorbance			% Positive Ratio		
	Peroxidase Labelled anti-IgG Dilution			Peroxidase Labelled anti-IgG Dilution		
	1:250	1:500	1:10000	1:250	1:500	1:1000
Control	0.031	0.016	0.018	27.4	14.2	15.9
FITC Immunized	0.037	0.013	0.010	37.2	19.5	18.8
TNP Immunized	0.113	0.092	0.089	100.0	81.4	78.8

Figure Experiment 1.15
Peroxidase Labelled anti-IgG Titration
for IgG anti-TNP ELISA

	Absorbance			% Positive Ratio		
	Peroxidase Labelled anti-IgM Dilution			Peroxidase Labelled anti-IgM Dilution		
	1:100	1:200	1:400	1:100	1:200	1:400
Control	0.021	0.015	0.009	25.3	18.1	10.8
FITC Immunized	0.083	0.080	0.066	100.0	96.4	79.5
TNP Immunized	0.014	0.009	0.003	16.9	10.8	3.6

Figure Experiment 1.16
Peroxidase Labelled anti-IgM Titration
for IgG anti-FITC ELISA

	Absorbance			% Positive Ratio		
	Peroxidase Labelled anti-IgG Dilution			Peroxidase Labelled anti-IgG Dilution		
	1:250	1:500	1:10000	1:250	1:500	1:1000
Control	0.037	0.025	0.030	27.6	18.7	22.4
FITC Immunized	0.134	0.109	0.120	100.0	81.3	89.6
TNP Immunized	0.008	0.000	0.010	6.0	0.0	7.5

Figure Experiment 1.17
Peroxidase Labelled anti-IgG Titration
for IgG anti-FITC ELISA

		% Positive Ratio					Within Run		
	Sample	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Mean	SD	CV
RUN 1	Control A	0.0	10.2	0.0	0.0	20.4	6.1	9.1	
	Control B	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
	FITC Immunized A	42.9	59.2	57.2	38.9	63.3	52.3	10.8	
	FITC Immunized B	36.7	57.1	36.7	46.9	53.1	46.1	9.3	
	TNP Immunized A	100.0	85.7	100.0	102.0	114.3	100.4	10.2	10.2
	TNP Immunized B	112.2	134.7	112.2	106.1	91.8	111.4	13.8	12.4
RUN 2	Control A	3.4	20.7	13.8	32.8	27.6	19.7	11.6	
	Control B	0.0	0.0	0.0	1.7	0.0	0.3	0.8	
	FITC Immunized A	67.2	56.9	55.2	44.8	65.5	57.9	9.0	
	FITC Immunized B	56.9	53.4	58.6	67.2	53.4	57.9	5.7	
	TNP Immunized A	100.0	94.8	108.6	110.3	89.7	100.7	8.8	8.7
	TNP Immunized B	148.3	127.6	136.2	139.7	122.4	134.8	10.2	7.6
RUN 3	Control A	2.3	0.0	0.0	0.0	9.3	2.3	4.0	
	Control B	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
	FITC Immunized A	55.8	58.1	69.8	90.7	90.7	73.0	17.0	
	FITC Immunized B	62.8	55.8	60.5	86.0	104.7	74.0	20.8	
	TNP Immunized A	100.0	102.3	97.7	111.6	132.6	108.8	14.3	13.1
	TNP Immunized B	183.7	172.1	146.5	141.9	165.1	161.9	17.5	10.8

Data Figure Experiment 1.18
IgM anti-TNP ELISA Within Run Precision

		Absorbance					Within Run		
	Sample	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Mean	SD	CV
RUN 1	Control A	-0.003	0.005	-0.002	-0.001	0.010	0.002	0.006	
	Control B	-0.010	-0.005	-0.006	-0.006	-0.007	-0.004	0.006	
	FITC Immunized A	0.021	0.029	0.028	0.019	0.031	0.026	0.005	
	FITC Immunized B	0.018	0.028	0.018	0.023	0.026	0.023	0.005	
	TNP Immunized A	0.049	0.042	0.049	0.050	0.056	0.049	0.005	10.2
	TNP Immunized B	0.055	0.066	0.055	0.052	0.045	0.055	0.008	14.5
RUN 2	Control A	0.002	0.012	0.008	0.019	0.016	0.011	0.007	
	Control B	-0.001	0.000	-0.005	0.001	-0.004	-0.002	0.002	
	FITC Immunized A	0.039	0.033	0.032	0.026	0.038	0.034	0.005	
	FITC Immunized B	0.033	0.031	0.034	0.039	0.031	0.034	0.003	
	TNP Immunized A	0.058	0.055	0.063	0.064	0.052	0.058	0.005	8.6
	TNP Immunized B	0.086	0.074	0.079	0.081	0.071	0.078	0.006	7.7
RUN 3	Control A	0.001	-0.003	-0.005	-0.001	0.004	-0.001	0.003	
	Control B	0.001	-0.007	-0.005	-0.002	-0.008	-0.004	0.004	
	FITC Immunized A	0.024	0.025	0.030	0.039	0.039	0.031	0.007	
	FITC Immunized B	0.027	0.024	0.026	0.037	0.045	0.032	0.009	
	TNP Immunized A	0.043	0.044	0.042	0.048	0.057	0.047	0.006	12.8
	TNP Immunized B	0.079	0.074	0.063	0.061	0.071	0.070	0.008	11.4

Data Figure Experiment 1.18 (continued)
IgM anti-TPN ELISA Precision

		% Positive Ratio					Within Run		
	Sample	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Mean	SD	CV
RUN 1	Control A	18.4	47.4	43.0	45.6	45.6	40.0	12.2	
	Control B	18.4	24.6	25.4	26.3	24.6	23.9	3.1	
	FITC Immunized A	13.2	11.4	21.2	20.2	26.3	18.4	6.1	
	FITC Immunized B	10.5	19.3	15.8	25.4	31.6	20.5	8.3	
	TNP Immunized A	100.0	98.2	108.8	116.7	119.3	108.6	9.5	8.7
	TNP Immunized B	112.3	126.3	124.6	126.3	121.9	122.3	5.9	4.8
RUN 2	Control A	38.1	43.1	26.2	38.1	35.7	36.4	6.4	
	Control B	21.4	21.4	23.8	25.4	20.6	22.5	2.0	
	FITC Immunized A	12.7	11.9	15.9	19.0	11.9	14.3	3.1	
	FITC Immunized B	12.7	11.9	15.9	19.0	14.3	16.7	2.0	
	TNP Immunized A	100.0	104.8	103.2	104.8	100.8	102.7	2.2	2.2
	TNP Immunized B	114.3	111.1	106.3	108.7	105.6	109.2	3.6	3.3
RUN 3	Control A	25.7	42.2	42.2	44.0	37.6	38.3	7.5	
	Control B	9.2	11.9	8.3	19.3	12.8	12.3	4.3	
	FITC Immunized A	0.0	5.5	6.4	7.3	6.4	5.1	2.9	
	FITC Immunized B	1.8	5.5	7.3	9.2	4.6	5.7	2.8	
	TNP Immunized A	100.0	103.7	105.5	100.9	105.5	103.1	2.6	2.5
	TNP Immunized B	104.6	124.8	110.1	116.5	103.7	111.9	8.8	7.9

Data Figure Experiment 1.18 (continued)
IgG anti-TNP ELISA Within Run Precision

		Absorbance					Within Run		
	Sample	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Mean	SD	CV
RUN 1	Control A	0.021	0.054	0.049	0.052	0.052	0.046	0.014	
	Control B	0.021	0.028	0.029	0.030	0.028	0.027	0.004	
	FITC Immunized A	0.015	0.013	0.024	0.023	0.030	0.021	0.007	
	FITC Immunized B	0.012	0.022	0.018	0.029	0.036	0.023	0.009	
	TNP Immunized A	0.114	0.112	0.124	0.133	0.136	0.124	0.011	8.7
	TNP Immunized B	0.128	0.144	0.142	0.144	0.139	0.139	0.007	4.8
RUN 2	Control A	0.048	0.055	0.033	0.048	0.045	0.046	0.008	
	Control B	0.027	0.027	0.030	0.032	0.026	0.028	0.003	
	FITC Immunized A	0.016	0.015	0.020	0.024	0.015	0.018	0.004	
	FITC Immunized B	0.019	0.021	0.023	0.024	0.018	0.021	0.003	
	TNP Immunized A	0.126	0.132	0.130	0.132	0.127	0.129	0.003	2.3
	TNP Immunized B	0.144	0.146	0.134	0.137	0.133	0.139	0.006	4.2
RUN 3	Control A	0.028	0.046	0.046	0.048	0.041	0.042	0.008	
	Control B	0.010	0.013	0.009	0.021	0.014	0.013	0.005	
	FITC Immunized A	0.001	0.006	0.007	0.008	0.007	0.005	0.004	
	FITC Immunized B	0.002	0.006	0.008	0.010	0.005	0.006	0.003	
	TNP Immunized A	0.109	0.113	0.115	0.110	0.115	0.112	0.003	2.5
	TNP Immunized B	0.114	0.136	0.120	0.127	0.113	0.122	0.010	7.9

Data Figure Experiment 1.18 (continued)
IgG anti-TNP ELISA Precision

		% Positive Ratio					Within Run		
	Sample	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Mean	SD	CV
RUN 1	Control A	5.6	0.0	0.0	0.0	8.3	2.7	3.9	
	Control B	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
	FITC Immunized A	100.0	100.0	81.8	83.3	91.7	91.4	8.7	9.5
	FITC Immunized B	150.0	150.0	134.7	127.8	147.2	141.9	10.1	7.1
	TNP Immunized A	6.9	12.5	0.0	0.0	12.5	6.4	6.3	
	TNP Immunized B	6.9	2.8	0.0	4.2	9.7	4.7	3.7	
RUN 2	Control A	2.2	7.6	15.2	6.3	19.0	14.9	6.8	
	Control B	1.3	0.0	0.0	1.3	0.0	0.5	0.7	
	FITC Immunized A	100.0	93.7	96.2	94.9	103.8	97.7	4.1	4.2
	FITC Immunized B	154.4	148.1	165.8	143.0	146.8	151.6	8.9	5.9
	TNP Immunized A	5.1	0.0	6.3	1.3	0.0	2.5	3.0	
	TNP Immunized B	19.0	8.9	15.2	7.6	10.1	12.2	4.8	
RUN 3	Control A	0.0	1.1	0.0	0.0	0.0	0.2	0.5	
	Control B	0.0	1.2	0.0	0.0	1.2	0.5	0.7	
	FITC Immunized A	100.0	101.1	86.2	94.3	88.5	94.0	6.7	7.1
	FITC Immunized B	146.0	129.9	129.9	133.3	128.7	133.6	7.2	5.4
	TNP Immunized A	1.1	9.2	0.0	0.0	1.1	2.3	3.9	
	TNP Immunized B	9.2	13.8	0.0	0.0	4.6	5.5	6.0	

Data Figure Experiment 1.18 (continued)
IgM anti-FITC ELISA Within Run Precision

		Absorbance					Within Run		
	Sample	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Mean	SD	CV
RUN 1	Control A	0.004	0.002	0.005	0.004	0.006	0.000	0.005	
	Control B	0.002	0.003	0.001	0.013	0.003	0.005	0.007	
	FITC Immunized A	0.072	0.072	0.059	0.060	0.066	0.066	0.006	9.1
	FITC Immunized B	0.108	0.108	0.097	0.092	0.106	0.102	0.007	6.9
	TNP Immunized A	0.005	0.009	0.001	0.001	0.009	0.005	0.005	
	TNP Immunized B	0.005	0.002	0.004	0.003	0.007	0.003	0.004	
RUN 2	Control A	0.017	0.006	0.012	0.005	0.015	0.011	0.005	
	Control B	0.001	0.002	0.000	0.001	0.001	0.000	0.001	
	FITC Immunized A	0.079	0.074	0.076	0.075	0.082	0.077	0.003	3.9
	FITC Immunized B	0.122	0.117	0.131	0.113	0.116	0.120	0.007	5.8
	TNP Immunized A	0.004	0.000	0.005	0.001	0.001	0.002	0.003	
	TNP Immunized B	0.015	0.007	0.012	0.006	0.008	0.010	0.004	
RUN 3	Control A	0.000	0.001	0.005	0.004	0.006	0.003	0.003	
	Control B	0.005	0.001	0.006	0.008	0.001	0.003	0.004	
	FITC Immunized A	0.087	0.088	0.075	0.082	0.077	0.082	0.006	7.3
	FITC Immunized B	0.127	0.113	0.113	0.116	0.112	0.116	0.006	5.2
	TNP Immunized A	0.001	0.008	0.006	0.005	0.001	0.000	0.006	
	TNP Immunized B	0.008	0.012	0.000	0.000	0.004	0.005	0.005	

Data Figure Experiment 1.18 (continued)
IgM anti-FTC ELISA Precision

		% Positive Ratio					Within Run		
	Sample	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Mean	SD	CV
RUN 1	Control A	41.3	41.3	44.4	36.5	35.7	39.8	3.7	
	Control B	31.7	31.0	33.3	38.1	35.7	34.0	2.9	
	FITC Immunized A	100.0	103.2	99.2	102.4	104.0	101.8	2.1	2.1
	FITC Immunized B	116.7	121.4	116.7	115.9	123.0	118.7	3.2	2.7
	TNP Immunized A	0.0	11.1	14.3	11.1	5.6	8.4	5.7	
	TNP Immunized B	8.7	7.1	7.1	7.1	9.5	7,9	1.1	
RUN 2	Control A	55.0	58.3	46.4	48.3	43.7	50.3	6.1	
	Control B	40.4	39.1	35.1	30.5	33.8	35.8	4.0	
	FITC Immunized A	100.0	100.0	97.4	96.7	93.4	97.5	2.7	2.8
	FITC Immunized B	106.6	115.9	111.9	106.6	108.6	109.9	4.0	3.6
	TNP Immunized A	21.9	13.9	15.9	11.3	11.3	14.9	4.4	
	TNP Immunized B	11.3	6.6	4.0	5.3	8.6	7.2	2.9	
RUN 3	Control A	23.1	28.2	29.2	44.4	47.9	34.6	10.9	
	Control B	23.1	21.4	24.8	23.1	28.2	24.1	2.6	
	FITC Immunized A	100.0	105.1	110.3	107.7	117.9	108.2	6.6	6.1
	FITC Immunized B	123.1	124.8	126.5	129.9	130.8	127.0	3.3	2.6
	TNP Immunized A	0.0	3.4	5.1	0.9	4.3	2.7	2.2	
	TNP Immunized B	0.0	0.0	0.0	0.0	1.7	0.3	0.8	

Data Figure Experiment 1.18 (continued)
IgG anti-FITC ELISA Within Run Precision

		Absorbance					Within Run		
	Sample	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Mean	SD	CV
RUN 1	Control A	0.052	0.052	0.056	0.046	0.045	0.050	0.005	
	Control B	0.040	0.039	0.042	0.048	0.045	0.043	0.004	
	FITC Immunized A	0.126	0.130	0.125	0.129	0.131	0.128	0.003	2.3
	FITC Immunized B	0.146	0.153	0.142	0.146	0.155	0.148	0.005	3.4
	TNP Immunized A	-0.004	0.014	0.018	0.014	0.007	0.010	0.009	
	TNP Immunized B	0.011	0.009	0.009	0.009	0.012	0.010	0.001	
RUN 2	Control A	0.083	0.088	0.070	0.073	0.066	0.076	0.009	
	Control B	0.061	0.059	0.053	0.046	0.051	0.054	0.006	
	FITC Immunized A	0.151	0.151	0.147	0.146	0.140	0.147	0.005	3.4
	FITC Immunized B	0.167	0.175	0.169	0.167	0.164	0.168	0.004	2.4
	TNP Immunized A	0.033	0.021	0.024	0.017	0.017	0.022	0.007	
	TNP Immunized B	0.017	0.010	0.006	0.008	0.013	0.011	0.004	
RUN 3	Control A	0.027	0.003	0.035	0.052	0.056	0.035	0.021	
	Control B	0.027	0.025	0.029	0.027	0.033	0.028	0.003	
	FITC Immunized A	0.117	0.123	0.129	0.126	0.138	0.127	0.008	6.1
	FITC Immunized B	0.144	0.146	0.148	0.152	0.153	0.149	0.004	2.6
	TNP Immunized A	-0.002	0.004	0.006	0.001	0.005	0.003	0.003	
	TNP Immunized B	-0.005	-0.005	-0.007	-0.004	0.002	-0.004	0.003	

Data Figure Experiment 1.18 (continued)
IgG anti-FTC ELLISA Precision

		% Positive Ratio			Between Run		
	Sample	Mean Run 1	Mean Run 2	Mean Run 3	Mean	SD	CV
IgM Anti-TNP	Control A	6.1	19.7	2.3	9.4	9.1	
	Control B	0.0	0.3	0.0	0.1	0.2	
	FITC Immunized A	52.3	57.9	73.0	61.1	10.7	
	FITC Immunized B	46.1	57.9	74.0	59.3	14.0	
	TNP Immunized A	100.4	100.7	108.8	103.3	4.8	4.6
	TNP Immunized B	111.4	134.8	161.9	136.0	25.3	18.6
TgG Anti-TNP	Control A	40.0	36.4	48.4	48.2	1.8	
	Control B	23.9	22.5	12.3	19.6	6.3	
	FITC Immunized A	18.4	14.3	5.1	12.6	6.8	
	FITC Immunized B	20.5	16.7	5.7	14.3	7.7	
	TNP Immunized A	108.6	102.7	103.1	104.8	3.3	3.1
	TNP Immunized B	122.3	109.2	111.9	114.5	6.9	6.0
IgM Anti-FITC	Control A	2.7	13.9	0.2	5.6	7.3	
	Control B	0.0	0.5	0.5	0.3	0.3	
	FITC Immunized A	91.4	97.7	94.4	94.4	3.2	3.4
	FITC Immunized B	141.9	151.6	133.6	142.4	9.0	6.3
	TNP Immunized A	6.4	2.5	2.3	3.7	2.3	
	TNP Immunized B	4.7	12.2	5.5	7.5	4.1	
IgG Anti-FITC	Control A	39.8	50.3	34.6	41.6	8.0	
	Control B	34.0	35.8	24.1	31.3	6.3	
	FITC Immunized A	101.8	97.5	108.2	102.5	5.4	5.3
	FITC Immunized B	118.7	109.9	127.0	118.5	8.6	7.3
	TNP Immunized A	8.4	14.9	2.7	8.7	6.1	
	TNP Immunized B	7.9	7.2	0.3	5.1	4.2	

Data Figure Experiment 1.18 (Continued)
Anti-Hapton ELISA Between Run Precision

		Absorbance			Between Run		
	Sample	Mean Run 1	Mean Run 2	Mean Run 3	Mean	SD	CV
IgM Anti-TNP	Control A	0.002	0.001	0.001	0.004	0.006	
	Control B	0.004	0.002	0.004	0.003	0.001	
	FITC Immunized A	0.026	0.034	0.031	0.030	0.004	
	FITC Immunized B	0.023	0.034	0.031	0.029	0.006	
	TNP Immunized A	0.049	0.058	0.047	0.051	0.006	11.8
	TNP Immunized B	0.055	0.078	0.070	0.068	0.012	17.6
TgG Anti-TNP	Control A	0.046	0.046	0.042	0.045	0.002	
	Control B	0.027	0.028	0.013	0.023	0.008	
	FITC Immunized A	0.021	0.018	0.005	0.015	0.009	
	FITC Immunized B	0.023	0.021	0.006	0.017	0.009	
	TNP Immunized A	0.124	0.129	0.112	0.122	0.009	7.4
	TNP Immunized B	0.139	0.139	0.112	0.130	0.016	12.3
IgM Anti-FITC	Control A	0.000	0.011	0.003	0.003	0.007	
	Control B	0.005	0.000	0.003	0.003	0.003	
	FITC Immunized A	0.066	0.077	0.082	0.075	0.008	10.7
	FITC Immunized B	0.102	0.120	0.116	0.113	0.009	8.0
	TNP Immunized A	0.005	0.002	0.000	0.002	0.003	
	TNP Immunized B	0.003	0.010	0.005	0.006	0.004	
IgG Anti-FITC	Control A	0.050	0.076	0.035	0.054	0.021	
	Control B	0.043	0.054	0.028	0.042	0.013	
	FITC Immunized A	0.128	0.147	0.127	0.134	0.011	8.2
	FITC Immunized B	0.148	0.168	0.149	0.155	0.011	7.1
	TNP Immunized A	0.010	0.022	0.003	0.012	0.010	
	TNP Immunized B	0.010	0.011	0.004	0.005	0.008	

Data Figure Experiment 1.18 (Continued)
Anti-Hapton ELiSA Between Run Precision

	Pre Immunization			Post Immunization		
	No Inhibition	FCS-TNP Inhibited	FCS-FITC Inhibited	No Inhibition	FCS-TNP Inhibited	FCS-FITC Inhibited
IgM Anti-TNP	0.0	0.0	0.0	100.0	0.0	85.5
IgG Anti TNP	0.0	0.0	0.0	100.0	0.0	100.0
IgM Anti-FITC	2.3	3.1	0.0	100.0	71.8	0.0
IgG Anti-FITC	9.1	3.5	0.0	100.0	82.5	0.0

Percent Positive Ratio

	Pre Immunization			Post Immunization		
	No Inhibition	FCS-TNP Inhibited	FCS-FITC Inhibited	No Inhibition	FCS-TNP Inhibited	FCS-FITC Inhibited
IgM Anti-TNP	0.001	-0.001	0.005	0.056	-0.011	0.065
IgG Anti TNP	0.000	-0.001	-0.002	0.021	-0.004	0.021
IgM Anti-FITC	0.003	0.004	-0.006	0.131	0.094	-0.001
IgG Anti-FITC	0.013	0.005	-0.001	0.143	0.118	-0.001

Absorbance

Figure Experiment 1.19
Positive Control Sera

	Control Mouse		Treated Mouse	
5 Day	5C1	1.4	5T1	3.6
	5C2	1.9	5T2	3.1
	5C3	1.5	5T3	2.0
	5C4	1.6	5T4	3.2
	5C5	2.6	5T5	2.7
	X +/ SD	1.8+/0.5	X +/ SD	2.9+/0.6
10 Day	10C1	1.0	10T1	2.8
	10C2	1.5	10T2	2.1
	10C3	2.4	10T3	2.6
	10C4	0.9	10T4	2.4
	10C5	2.2	10T5	2.5
	X +/ SD	1.6+/0.6	X +/SD	2.5+/0.3
15 Day	15C1	2.0	15T1	3.1
	15C2	1.3	15T2	1.7
	15C3	0.4	15T3	2.3
	15C4	2.5	15T4	1.9
	15C5	2.6	15T5	3.0
	X +/ SD	1.8+/0.9	X +/ SD	2.4+/0.6

Figure Experiment 3.1
Total Nucleated Cells x 10^8 per Spleen

	Control				Treated			
	Mouse	Anti TNP	Anti FITC	Total IgM ISC Activity	Mouse	Anti TNP	Anti FITC	Total IgM ISC Activity
5 Day	5C1	4.34	2.10	386.96	5T1	42.12	27.00	2,669.04
	5C2	10.83	6.84	964.63	5T2	48.98	40.61	3,045.44
	5C3	5.55	2.55	472.05	5T3	35.00	20.00	1,613.40
	5C4	6.88	2.40	340.32	5T4	49.60	24.00	3,275.84
	5C5	12.48	7.02	833.82	5T5	51.03	19.98	2,327.40
	X+/-SD	8.02 +/3.49	4.18 +/2.51	599.56 +/281.43	X+/SD	45.35 +/-6.73	26.32 +/8.52	2,586.22 +/ 653.20
10 Day	10C1	2.00	1.40	421.40	10T1	24.64	3.92	1,333.92
	10C2	4.65	2.25	535.05	10T2	0.42	0.63	145.74
	10C3	0.72	1.92	645.60	10T3	9.62	0.13	1,653.86
	10C4	4.05	0.72	509.13	10T4	18.48	0.96	2.005.68
	10C5	9.46	5.50	1,250.48	10T5	23.00	13.50	1,242.50
	X+/-SD	4.18 +/3.35	2.36 +/1.85	672.33 +/332.95	X+/SD	15.23 +/-10.13	3.83 +/5.61	1.276.34 +/ 699.50
15 Day	15C1	8.80	5.40	624.00	15T1	44.33	6.82	1,026.10
	15C2	7.54	2.34	727.09	15T2	15.64	3.91	1,085.28
	15C3	1.04	0.36	530.80	15T3	15.64	6.21	1,126.31
	15C4	5.50	3.25	571.75	15T4	21.47	15.20	817.76
	15C5	10.40	3.64	379.60	15T5	14.40	5.40	906.90
	X+/-SD	6.66 +/3.61	3.00 +/1.87	566.65 +/127.80	X+/SD	22.30 +/-12.62	7.51 +/4.44	992.47 +/ 127.97

Figure Experiment 3.2, 3.4, 3.5
Total IgM ISC x 10^3 per Spleen

		Anti-FCS	Anti-TNP*			Anti-FITC*			Total IgM ISC Activity		
		Plate 1	Plate 1	Plate 2	*Avg	Plate 1	Plate 2	*Avg	Plate 1	Plate 2	*Avg
5 Day	5C1	0	29	33	31	10	19	15	2,327	3,200	2,764
	5C2	0	45	69	57	35	37	36	4,720	5,433	5,077
	5C3	0	38	36	37	15	18	17	3,727	2,567	3,147
	5C4	1	62	23	43	15	15	15	2,787	1.467	2,127
	5C5	0	63	33	48	28	25	27	3,980	2,433	3,207
	X+/-SD		43 +/- 10			22 +/- 9			3,264 +/- 1.101		
10 Day	10C1	0	33	26	20	1	17	14	4,427	4,000	4,214
	10C2	0	25	37	31	9	12	15	4,133	3,000	3,567
	10C3	1	5	1	3	11	5	8	3,180	2,200	2,690
	10C4	1	57	32	45	4	12	8	5,480	5,833	5,657
	10C5	2	37	48	43	28	22	25	5,300	6,067	5,684
	X+/-SD		28 +/- 17			14 +/- 7			4,362 +/- 1,311		
15 Day	15C1	0	44	43	44	29	24	27	2,973	3,267	3,120
	15C2	0	66	49	58	18	18	18	5,353	5,833	5,593
	15C3	0	23	28	26	11	6	9	1,320	1,333	1,327
	15C4	1	22	21	22	19	7	13	3,007	1,567	2,287
	15C5	0	40	+	40	16	12	14	1,153	1,767	1,460
	X+/-SD		38 +/- 14			16 +/- 7			2,757 +/- 1,741		

* Corrected for Anti-FCS background + Unable to quantitate due to excessive precipitate

Figure Experiment 3.3, 3.6, 3.7
IgM ISC per 10^6 Spleen Cells

		Anti-FCS	Anti-TNP*			Anti-FITC*			Total IgM ISC Activity		
		Plate 1	Plate 1	Plate 2	*Avg	Plate 1	Plate 2	*Avg	Plate 1	Plate 2	*Avg
5 Day	5T1	2	93	141	117	86	64	75	7,027	7,800	7,414
	5T2	0	169	146	158	127	134	131	10,080	9,567	9,824
	5T3	0	212	137	175	95	105	100	8,800	7,333	8,067
	5T4	0	163	146	155	70	79	75	9,073	11,400	10,237
	5T5	0	180	197	189	75	72	74	7,967	9,273	8,620
	X+/-SD		159 +/- 27			91 +/- 25			8,833 +/- 1,183		
10 Day	10T1	1	80	95	88	13	14	14	4,893	4,633	4,764
	10T2	1	1	2	2	1	4	3	587	800	694
	10T3	0	29	45	37	7	2	5	5,921	6,800	6,361
	10T4	1	71	82	77	5	3	4	8,213	8,500	8,357
	10T5	2	104	80	92	53	54	54	4,773	5,167	4,970
	X+/-SD		59 +/- 39			16 +/- 22			5,029 +/- 2,816		
15 Day	15T1	0	149	136	143	21	23	22	2,920	3,700	3,310
	15T2	0	105	78	92	29	17	23	6,200	6,567	6,384
	15T3	0	67	69	68	21	33	27	4,693	5,100	4,897
	15T4	6	149	76	113	85	75	80	4,140	4,467	4,304
	15T5	0	69	27	48	13	23	18	2,613	3,433	3.023
	X+/-SD		93 +/- 37			34 +/- 26			4,383 +/- 1,349		

Figure Experiment 3.3, 3.6, 3.7 (continued)
IgM ISC per 10^6 Spleen Cells

	Mouse	IgM antiTNP	IgG antiTNP	IgM antiFITC	IgG antiFITC
Day 5	5C1	0.020	0.002	0.018	0.008
	5C2	0.005	0.033	0.019	0.019
	5C3	0.015	0.022	0.015	0.015
	5C4	0.013	0.024	0.029	0.010
	5C5	0.018	0.024	0.021	0.026
	Mean	0.014+/0.006	0.021+/0.012	0.020+/0.005	0.016+/0.007
Day 10	10C1	0.028	0.040	0.069	0.022
	10C2	0.062	0.046	0.043	0.020
	10C3	0.029	0.039	0.020	0.000
	10C4	0.033	0.030	0.034	0.027
	10C5	0.045	0.004	0.113	0.028
	Mean	0.039+/0.014	0.030+/0.020	0.056+/0.037	0.019+/0.011
Day 15	15C1	0.015	0.007	0.049	0.025
	15C2	0.041	0.017	0.042	0.023
	15C3	0.037	0.022	0.037	0.026
	15C4	0.030	0.041	0.038	0.024
	15C5	0.046	0.042	0.047	0.022
	Mean	0.034+/0.012	0.026+/0.015	0.043+/0.005	0.024+/0.002

Figure Experiment 3.8, 3.9, 3.16, 3.17
ELISA O.D. for Control Mice

	Mouse	IgM antiTNP	IgG antiTNP	IgM antiFITC	IgG antiFITC
Day 5	5T1	0.022	0.000	0.059	0.008
	5T2	0.036	-0.005	0.043	0.019
	5T3	0.059	-0.007	0.088	0.015
	5T4	0.085	0.048	0.079	0.010
	5T5	0.047	0.022	0.044	0.026
	Mean	0.059+/0.024	0.012+/0.023	0.063+/0.020	0.016+/0.007
Day 10	10T1	0.043	0.040	0.029	0.022
	10T2	0.032	0.024	0.033	0.020
	10T3	0.046	0.082	0.043	0.000
	10T4	0.054	0.023	0.050	0.027
	10T5	0.054	0.021	0.063	0.028
	Mean	0.046+/0.009	0.030+/0.026	0.044+/0.014	0.019+/0.011
Day 15	15T1	0.048	9.3	0.067	0.025
	15T2	0.043	22.7	0.065	0.023
	15T3	0.042	-0.008	0.116	0.026
	15T4	0.046	0.031	0.068	0.024
	15T5	0.042	0.025	0.054	0.022
	Mean	0.044+/0.003	-.016+/0.015	0.074+/0.024	0.024+/0.002

Figure Experiment 3.8, 3.9 (continued)
ELLISA O.D. for P. acnes Treated Mice

	Mouse	IgM antiTNP	IgG antiTNP	IgM antiFITC	IgG antiFITC
Day 5	5C1	14.1	2.7	9.2	4.1
	5C2	3.5	44.0	9.7	9.7
	5C3	10.6	29.3	7.7	7.7
	5C4	9.2	32.0	14.8	5.1
	5C5	12.7	32.0	10.7	13.3
	Mean	10.0+/4.1	28.0+/15.2	10.4+/2.7	8.0+/3.7
Day 10	10C1	19.7	53.3	35.2	11.2
	10C2	43.7	61.3	21.9	10.2
	10C3	20.4	52.0	10.2	0.0
	10C4	23.2	40.0	17.3	13.8
	10C5	31.7	0.0	57.7	14.3
	Mean	27.2+/10.1	41.3+/24.3	28.5+/18.7	7.9+/6.9
Day 15	15C1	10.6	9.3	25.0	12.8
	15C2	28.9	22.7	21.4	11.7
	15C3	26.1	2.7	18.9	13.3
	15C4	21.1	54.7	19.4	12.2
	15C5	32.4	56.0	24.0	11.2
	Mean	23.8+/8.5	29.1+/25.0	21.7+/2.7	12.2+/0.8

Figure Experiment 3.8, 3.9 (continued)
ELISA Percent Positive Ratio for Control Mice

	Mouse	IgM antiTNP	IgG antiTNP	IgM antiFITC	IgG antiFITC
Day 5	5T1	15.5	0.0	30.1	5.7
	5T2	25.4	0.0	21.9	13.6
	5T3	41.5	0.0	44.9	10.7
	5T4	59.9	64.0	40.3	7.1
	5T5	33.1	29.3	22.4	18.6
	Mean	35.1+/16.9	18.7+/28.3	31.9+/10.4	11.1+/5.2
Day 10	10T1	30.3	53.3	14.8	15.7
	10T2	22.5	32.0	16.8	14.3
	10T3	32.4	109.3	21.9	0.0
	10T4	38.0	30.7	25.5	19.3
	10T5	38.0	28.0	32.1	20.0
	Mean	32.3+/6.4	50.7+/34.3	22.2+/6.9	13.9+/8.1
Day 15	15T1	33.8	14.7	34.2	17.9
	15T2	30.3	29.3	33.2	16.4
	15T3	29.6	0.0	59.2	18.6
	15T4	32.4	41.3	34.7	17.1
	15T5	29.6	33.3	27.6	15.7
	Mean	31.1+/1.9	23.7+/16.4	37.8+/12.3	17.1+/1.2

Figure Experiment 3.8, 3.9 (continued)
ELISA Percent Positive Ratio for P. acnes Treated Mice

		Control			Treated			
	Mouse	Anti-TNP	Anti-FITC	Total IgG ISC Activity	Mouse	Anti-TNP	Anti-FITC	Total IgG ISC Activity
5 Day	5C1	0.56	0.14	244.16	5T1	2.16	0.72	1,046.52
	5C2	0.38	0.19	207.10	5T2	2.17	0.31	1,807.30
	5C3	0.30	0.15	368.55	5T3	0.60	0.00	636.00
	5C4	1.60	0.16	241.12	5T4	0.64	1.92	990.08
	5C5	1.82	0.52	716.82	5T5	2.16	0.27	1,308.69
	X+/-SD	0.93 +/0.72	0.23 +/0.16	355.55 +/211.08	X+/SD	1.55 +/-0.85	0.64 +/0.76	1,157.72 +/ 435.15
10 Day	10C1	0.40	4.90	625.30	10T1	1.96	0.84	1,494.36
	10C2	0.60	0.15	358.05	10T2	0.21	0.00	271.71
	10C3	0.24	0.00	639.12	10T3	0.13	3.38	3,356.60
	10C4	0.54	1.44	469.80	10T4	0.12	0.72	2,554.56
	10C5	0.22	0.00	250.14	10T5	1.75	0.25	1,371.00
	X+/-SD	0.40 +/0.17	1.30 +/2.10	468.48 +/168.51	X+/SD	0.83 +/-0.94	1.04 +/1.35	1,809.65 +/1,183.74
15 Day	15C1	0.00	0.20	190.80	15T1	0.31	5.58	901.17
	15C2	0.13	0.13	146.12	15T2	2.04	0.34	1,085.79
	15C3	0.04	0.08	124.00	15T3	3.45	0.69	1,769.62
	15C4	1.00	0.00	163.50	15T4	0.95	0.19	1,045.00
	15C5	0.26	0.52	140.40	15T5	0.30	0.30	1,220.10
	X+/-SD	0.29 +/0.41	0.19 +/0.20	152.96 +/ 25.43	X+/SD	1.41 +/-1.34	1.42 +/2.33	1,204.34 +/ 335.84

Figure Experiment 3.10, 3.12, 3.13
Total IgG ISC x 10^3 per Spleen

		Anti-FCS	Anti-TNP*			Anti-FITC*			Total IgG ISC Activity		
		Plate 1	Plate 1	Plate 2	*Avg	Plate 1	Plate 2	*Avg	Plate 1	Plate 2	*Avg
5 Day	5T1	0	6	6	6	1	2	2	2,513	3,300	2,907
	5T2	0	5	8	7	1	1	1	5,693	5,976	5,830
	5T3	0	1	4	3	0	0	0	3,560	2,800	3,180
	5T4	0	4	0	2	10	1	6	3,020	3,167	3,094
	5T5	0	5	10	8	1	0	1	4,893	4,800	4,847
	X+/-SD				5+/-3			2+/-2		3,972+/-1,299	
10 Day	10T1	0	5	9	7	6	0	3	5,240	5,433	5,337
	10T2	0	1	0	1	0	0	0	987	1,600	1,294
	10T3	0	3	6	5	7	19	13	10,720	15,100	12,910
	10T4	0	5	5	5	1	5	3	9,587	11,700	10,644
	10T5	0	8	5	7	1	0	1	5,000	5,967	5,484
	X+/-SD				5+/-2			4+/-5		7,134+/-4,630	
15 Day	15T1	10	2	0	1	2	33	18	3,447	2,367	2,907
	15T2	0	10	14	12	2	2	2	5,673	7,100	6,387
	15T3	6	22	7	15	3	3	3	5,987	9,400	7,694
	15T4	0	2	7	5	1	1	1	4,200	6,800	5,500
	15T5	0	1	0	1	1	0	1	+	4,067	4,067
	X+/-SD				7+/-6			5+/-7		5,311+/-1,884	

* Corrected for antiFCS ISC
\+ Backgruond too dense to quantitate

Figure Experiment 3.11, 3.14, 3.15
IgG ISC per 10^6 Spleen Cells

		Anti-FCS	Anti-TNP*			Anti-FITC*			Total IgG ISC Activity		
		Plate 1	Plate 1	Plate 2	*Avg	Plate 1	Plate 2	*Avg	Plate 1	Plate 2	*Avg
5 Day	5C1	0	3	5	4	0	1	1	1,287	2,200	1,744
	5C2	0	1	3	2	0	1	1	980	1,200	1.090
	5C3	0	2	1	2	1	0	1	2,047	2,867	2,457
	5C4	0	9	10	10	1	0	1	1,880	1,133	1,507
	5C5	0	5	8	7	0	4	2	3,280	2,233	2,757
	X+/-SD				5+/-3			1+/-1			1,911 +/- 685
10 Day	10C1	0	1	7	4	50	47	49	6,173	6,333	6,253
	10C2	0	2	5	4	1	1	1	2,273	2,500	2,387
	10C3	0	2	1	1	0	1	1	2,893	2,433	2,663
	10C4	0	5	7	6	19	13	16	5,107	5,333	5,220
	10C5	0	1	0	1	0	0	0	1,107	1,167	1,137
	X+/-SD				3+/-2			13+/-21			3,532 +/- 2,125
15 Day	15C1	0	0	0	0	0	1	1	940	967	954
	15C2	0	1	1	1	1	0	1	1,247	1,000	1,124
	15C3	0	2	0	1	4	0	2	3,300	2,900	3,100
	15C4	0	1	7	4	0	0	0	607	700	654
	15C5	0	0	2	1	0	4	2	580	500	540
	X+/-SD				1+/-2			1+/-1			1,274 +/- 1,047

Figure Experiment 3.11, 3.14, 3.15 (continued)
IgG ISC per 10^6 Spleen Cells