IMMUNOPOTENTIATING EFFECTS OF ADJUVANTS ON BOVINE HUMORAL AND CELLULAR IMMUNE RESPONSES TO *BRUCELLA ABORTUS* VACCINES

By

GLADSTONE KWABLA DZATA

Doctor of Veterinary Medicine
University of Zagreb
Zagreb, Yugoslavia
1982

Master of Science
Sam Houston State University
Huntsville, Texas
1984

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of the Oklahoma State University
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Thesis Approved:

Thesis Adviser

Dean of Graduate College

PREFACE

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CHAPTER I

AN INTRODUCTION WITH A REVIEW OF THE LITERATURE

Bovine Brucellosis

Introduction

Brucellosis is an acute and chronic infectious disease of domestic animals transmissible to man causing undulant fever (McCullough, 1970). Bovine brucellosis caused by Brucella abortus is characterized principally by abortion or delivery of a non-viable calf, reduction in fertility, and decreased milk production. Except countries where it has been eradicated, bovine brucellosis is prevalent on every continent and is of economic importance because it results in reduced beef production due to the loss of calf crop from abortion, non-viable calves and decreased fertility (Alton et al., 1975; Matyas and Fujikura, 1984).

The magnitude of the global economic losses can be envisaged because the annual losses in Latin America alone has been estimated to be $700 million (Adbussalam and Fein, 1976). The seriousness of brucellosis in terms of human illness and economic loss has been a matter of major concern to national, public, and international organizations, particularly the World Health Organization and Food and Agriculture Organization of the United Nations (Matyas and Fujikura, 1984).

Etiology and Pathogenesis

Brucella abortus, the major etiological agent of bovine brucellosis, is a small, gram-negative, short rod or coccobacillus (0.5 - 0.7 um by 0.6 - 1.5 um). The organism is usually arranged singly and does not possess a capsule, flagellae, or endospores (Gillepsie and Timoney, 1981). *Brucella abortus* is divided into nine biotypes. The criteria used for biotyping are CO_2 requirement for growth, production of hydrogen sulfide, growth on dye media (basic fuchsin and thionin), and agglutination in monospecific antisera (A&M antigens) (Morgan, 1984). Although there does not appear to be differences in virulence among the biotypes, 85% of all documented infections are due to biotype 1 (Nicoletti, 1980).

Brucella abortus is primarily a facultative intracellular bacterium because it is capable of surviving and multiplying within host macrophages (Braude, 1951). The bacteria have a predilection for the reticuloendothelial system, particularly mononuclear phagocytes. However, intracellular growth occurs in other cell types such as fibroblasts, endothelial cells, and chorionic trophoblasts. Theobald Smith (1919) was the first to report the intracellular habitat of *B. abortus* by observing the localization of the bacterium within epithelial cells of the bovine fetal membrane.

The most important means for transmission of the disease is by direct contact of a susceptible animal with an infected animal, its tissues or secretions and excretions. Mucous membranes are the usual portal of entry. These include mucous membranes of the oropharynx, upper respiratory tract, and conjunctivae (McCullough, 1970). Most

cattle acquire natural infection by ingesting infected or contaminated material. Experimentally, the conjunctivae is used most commonly as a route of inoculation.

Besides the reticuloendothelial system, B. abortus has a marked predilection for the gravid uterus (Pearce et al., 1962), which it reaches hematogenously. Following initial invasion of the host, the organism localizes in the regional lymph nodes and subsequently spreads to other lymphoid tissues including the spleen. Corner et al. (1987), investigated the distribution of B. abortus in naturally and experimentally (conjunctival challenge) infected cattle by isolation through bacterial culture. They showed that, in pregnant cows, the mammary lymph node was the most frequently infected tissue, whereas in nonpregnant heifers, B. abortus was isolated most frequently from the mandibular lymph node. Due to the intracellular nature of the bacterium, bacteremia is rarely detected during the incubation period or in an animal with latent infection. However, following pregnancy, there is massive proliferation of the organism in the uterus. Several factors may account for this. Pearce et al. (1962), demonstrated that erythritol, a carbohydrate substance present in the placenta and fetal fluids, is a strong growth stimulant of B. abortus. B. abortus preferentially utilizes erythritol over glucose; therefore, the presence of erythritol in these tissues may account for the massive proliferation of B. abortus in the gravid uterus (Pearce et al., 1962). Also, ruminant placentae produce progesterone (Heap et al., 1983), which has been shown to suppress cell-mediated immunity during pregnancy (Szekeres et al., 1981). These factors could depress the host's immune system reducing the host's ability to control the

replication of B. abortus.

Following invasion of the pregnant uterus, B. abortus first localizes in the uterine wall and then spreads to the uterine lumen (intercotyledonary spaces) leading to a severe ulcerative endometritis with subsequent destruction of the villi (Payne, 1959). Trophoblasts are believed to be the primary cell type involved in the entry, localization, and replication of B. abortus in placentae (Anderson et al., 1986). Abortion occurs typically in the last three months of gestation with the incubation period dependent on the stage of gestation, dose of bacteria, and age and immunological status of the host (Nicoletti, 1980). The important fetal lesions in brucellosis are multifocal bronchitis and bronchopneumonia with a predominant mononuclear cell infiltration (Jubbs and Kennedy, 1985).

Vaccines

Strain 19 vaccine. The most economically feasible and effective way to control bovine brucellosis is through an effective vaccination and epidemiological survey/elimination program. B. abortus strain 19 (S19) vaccine is presently the most effective vaccine for the control of bovine brucellosis. This vaccine is used in the United States and in many countries where the disease is prevalent. S19 was originally isolated in 1923. Buck (1930) reported S19 to be of reduced virulence compared to field isolates, and it was efficacious when used as a vaccine for cattle (Alton et al., 1975). S19 vaccine was officially approved for use in the United States brucellosis program in 1939 (Nelson, 1977). Vaccination of heifers with S19 effectively increases the resistance to brucellosis and thereby helps control the disease.

However, despite the beneficial effects of S19, residual post-vaccinal titers, aberrant serologic reactions, and chronic infections with S19 may occur and interfere with diagnostic serologic tests (Corner and Alton, 1981; Sutherland et al., 1982). Furthermore, S19 is a live vaccine, capable of infecting humans and constitutes a public health hazard.

Other B. abortus vaccines. Another vaccine that is used for immunizing cattle against brucellosis is the killed B. abortus strain 45/20 (S45/20) vaccine. S45/20 is a rough strain of B. abortus and lacks the outer o-polysaccharide of the LPS (Moreno et al., 1984). Therefore, S45/20 vaccine induces low levels of agglutinating antibody and does not interfere with diagnostic agglutination tests. Furthermore, being a killed vaccine it is not infectious to humans. This vaccine was first described by McEven and Samuel (Cunningham, 1977) and is used predominantly in Ireland and Austalia. The official vaccine K45/20A contains a standard adjuvant. Two vaccinations are necessary to induce immunity, and it is ineffective when given before six months of age (Cunningham, 1977). The advantages and disadvantages of S19 and K45/20A vaccines have been compared and reviewed (Cunningham, 1977).

Several other vaccines have been examined as potential immunizing agents against brucellosis. These vaccines include the mucoid (M) vaccine of Huddleson (1947), the B. melitensis strain H38 of Renoux (Renoux et al., 1964), and several extractable soluble substances from B. abortus (Gillepsie and Timoney, 1981; Confer et al., 1987). These vaccines have not gained acceptance.

Role of Antibody in Resistance

The host responds to *Brucella* sp. infection by producing a variety of serum antibodies representing most classes of immunoglobins (Sutherland, 1980). However, serum antibody responses do not correlate with immunity since heifers vaccinated with S19 may be immune to challenge despite low antibody titers within a few months after vaccination (McDiarmid, 1957; Cunningham and O'Reilly, 1968). In fact, high serum antibody titers correlate with chronic infection rather than resistance (Kreutzer et al., 1979). However, in the murine model of brucellosis, humoral immune responses may contribute to protection (Sultzeanu, 1965; Plommet and Plommet, 1983; Winter et al., 1988).

Role of Cell-Mediated Immunity (CMI) in Resistance

B. abortus is a facultative, intracellular bacterium and development of immunity requires activation of host cell-mediated immune responses (Sutherland, 1980; Plommet et al., 1985; Person et al., 1987). Acquired resistance to brucellosis requires antigen-specific T-cells (Jones and Buening, 1983) and activated macrophages (Cheers and Pagram, 1979). Macrophages from immunized cattle have increased capacity to kill the organism (Holland and Picket, 1958). Fitzgeorge et al. (1967), demonstrated that *B. abortus* multiplied more slowly in the macrophages of vaccinated cattle than in control animals. Adoptive transfer of resistance in mice using immune T-cells was reported (Pavlov et al., 1982; Madraso and Cheers, 1984; Plommet

et al., 1985).

The lymphocyte blast transformation (LBT), the macrophage migration inhibition test (MMIT), leukocyte migration inhibition test (LMIT), and delayed type hypersensitivity (DTH) test are generally used to assess CMI function following exposure to antigen (Sutherland, 1980). The LBT is a test for the general assessment of T-cell function and measures the cell division of lymphocytes following stimulation by antigen or mitogen. In bovine brucellosis the LBT has been used to evaluate the CMI responses to antigens in infected and immunized animals (Kaneene et al., 1978; Klesius et al., 1978; Baldwin et al., 1984). The use of the LBT assay was proposed as a diagnostic test for bovine brucellosis (Kaneene et al., 1978); however, it has not been used widely.

Potential Mechanisms of *Brucella* Immunity

The Role of the Macrophage in Host Defense

The first defense of the immune system after pathogenic invasion involves the phagocytes and antigen nonspecific cytotoxic cells. Macrophages, in particular, function as the first line of defense against invading microorganisms via nonspecific or opsonic phagocytosis. The mechanisms of activation of phagocytes and their role in immunity against different pathogens have been described (McCullough, 1970; Adam et al., 1984; Lukacs et al., 1985).

B. abortus is primarily an intracellular parasite to which protective immunity may be dependent on antigen-specific effector T-cells and macrophage bactericidal activity (Cheers, 1984). Several

investigators described the mechanisms of anti-bacterial CMI (Mackaness, 1971; North, 1974; Collins, 1978; Kaufmann, 1987; Mielke et al., 1988).

Neutrophils are capable of phagocytizing Brucella sp. organisms, but they have limited bactericidal capacity. Virulent B. abortus inhibits the myeloperoxidase halide antibacterial reaction of neutrophils due to specific suppression of degranulation of peroxidase-positive granules (Caning et al., 1985; Bertran et al., 1986). However, opsonization of B. abortus with specific antibody stimulates an oxidative metabolic burst (Canning et al., 1988).

Using the murine system model, it was demonstrated that the macrophage is the most important host cell for killing Brucella organisms (McCullough, 1970). Mononuclear phagocytes, tissue macrophages, and fixed phagocytic cells of the reticuloendothelial system are all responsible for the elimination of Brucella sp. in both the normal and the immune animal (McCullough, 1970). Following introduction of B. abortus into host tissues (by intravenous, subcutaneous, per os, intradermal, intranasal, intraconjunctival, or aerogenic routes), the organism encounters different populations of resident phagocytic cells. The bacteria can then reach the blood stream via infected macrophages within lymphatics and colonize the liver and spleen (Collins et al., 1977). Although a small amount of the inoculum reaches the liver and spleen, this splenic inoculum appears to determine the degree of systemic immunity expressed by the immunized host (Collins et al., 1977; Collins and Campbell, 1982). In the absence of splenic involvement, there is a minimal systemic resistance mounted against the bacteria (Collins and Carter, 1974).

Resident macrophages play an important role during the induction of the immune response. By means of phagocytosis, they limit the growth of Brucella sp. organisms in the extracellular environment, serve as antigen presenting cells (APC) for helper T-cells, and prevent cellular damage by detoxifying enzymes and metabolic products released into the tissues by the rapidly multiplying bacteria (Collins and Campbell, 1982). However, unless activated, macrophages have little bactericidal capacity for Brucella sp. In the murine listeriosis model, Mackaness (1969) demonstrated that macrophage activation and granuloma formation depend on specific lymphocytes, especially T-cells. T-cells activate macrophages via the production of soluble products (lymphokines), in particular macrophage activating factor (MAF), now designated interferon-gamma (IFN-g) (Nathan et al., 1983). Lymphokines are active in both macrophage recruitment and macrophage activation. MAF increases Fc receptors and promote phagocytosis via the C3b receptor. The immunologically-activated macrophage is larger, more mobile, and metabolically more active with increased bactericidal activity (Mackaness, 1970). Macrophage activation is nonspecific, and activated macrophages can kill tumor cells and intracellular parasites more effectively. Macrophage killing of intracellular microbes requires the generation of oxygen intermediates, i.e., hydroxyl radical and singlet oxygen or hydrogen peroxide. Thus, with regards to infection with Brucella sp., the most important attribute of the activated macrophage is enhanced bactericidal capacity. In fact, macrophages from immunized cattle have increased capacity to kill Brucella sp. (Holland and Pickett, 1958; Fitzgeorge et al., 1967). Therefore, macrophages are the effector

cells, and T-cells are mediators in the induction of CMI in brucellosis (Cheers, 1984).

The Role of the T-cell in the Induction of Acquired Resistance

Infections by facultative intracellular bacteria, such as *Brucella* sp., are characterized by the formation of granulomas macrophage activation, and the development of DTH (Mackaness, 1962, 1971). It was recently shown that T-cells are mediators for the induction of acquired resistance (CMI) to brucellosis (Cheers, 1984; Plommet, 1985). However, expression of the CMI response requires interaction between macrophages and immunocompetent T-cells (Mackaness, 1971; North, 1974; Splitter and Everlith, 1986).

In contrast to antibodies which bind free antigen directly, T-cells recognize foreign antigen in association with products encoded by the major histocompatibility complex (MHC) on the surface of host cells. Macrophages phagocytize foreign antigen, process it biochemically, and present the processed antigen to helper T-cells in association with MHC class II molecules (Unanue and Allen, 1987). During this process, APC produce a lymphocyte activating factor, interleukin-1 (IL-1).

IL-1 activates the T-cell and induces the expression of receptors for the T-cell derived lymphokine interleukin-2 (T-cell growth factor) (Dinarello, 1984). Activated T-cells are capable of proliferating in response to IL-2. Antigen and IL-2 also cause pre-cytotoxic T-cells to proliferate and differentiate into effector cells. The expansion of antigen- specific (i.e., *Brucella* sp. specific) T-cells is induced and

sustained by IL-2. Activated T-cells release a number of lymphokines such as macrophage chemotactic factor (MCF), macrophage-migration-inhibition factor (MIF), and IFN-g, which induce macrophage accumulation and activation. Thus, the most important function of Brucella-sensitized helper T-cells is the attraction, focusing, and activation of macrophages at the infected site (i.e., to the site of a DTH response to an injection of Brucella antigen). Also, IFN-g induces expression of Ia (immune response associated) molecules on the macrophage (Steeg et al., 1982).

Immunocompetent T-cells have the ability to recognize foreign antigen entering the tissues. Sensitized T-cells are extremely mobile (Koster et al., 1971) and migrate to infective foci, thereby increasing their chances of association and recognition of sensitizing antigens. As T-cells migrate in increasing numbers into the inflammatory focus, they come into contact with processed antigen presented by the infected macrophages (Collins, 1979). These cells will then undergo blast transformation which causes a concomitant clonal expansion of immunocompetent T-cells with subsequent manifestation of the immune response. Stimulated T-cells release a number of previously described lymphokines into the tissues which, in turn, activate surrounding macrophages and can result in the eventual resolution of the infection (Mackaness, 1971a,b).

Acquired resistance to most facultative intracellular parasites is relatively short-lived. The immune response is mediated by a population of short-lived small lymphocytes, which following infection, increase sharply in numbers then rapidly decline (North, 1973). A decline in memory cells has been observed in response to

Brucella sp.- and Salmonella sp.-infected mice (Collins, 1971). Despite the rapid reduction in the numbers of detectable sensitized T-cells, the convalescent host can upon challenge mount an accelerated recall of the earlier immune response (North, 1975). This anamnestic response is mediated by a population of long-lived memory T-cells thought to persist within the spleen and lymph nodes, even in the absence of the sensitizing antigen (Collins and Campbell, 1982). When exposed to the triggering antigen or active infection, such memory cells will undergo rapid clonal expansion resulting in large numbers of specifically sensitized T-cells capable of mediating an anamnestic response (Cohen and Livnat, 1976).

Recently, Kaufmann (1988) showed that cytotoxic T-cells could lyse Listeria-infected Ia-positive bone marrow macrophages in vitro. This contradicts the widely accepted idea that activated macrophages are the main defense against facultative intracellular bacteria. However, it has been demonstrated that both helper and cytotoxic T-cell subsets are important in protection against Listeria monocytogenes, a facultative intracellular bacterium (Bishop et al., 1987; Czupryinski et al., 1987). This mechanism has been demonstrated for other intracellular microbes (Orme, 1987; Pedrazzini, 1987). Recently, cytotoxic T-cells were shown to potentially protect mice from malaria infection (Schofield et al., 1987; Mogil et al., 1987). Lysis of Brucella sp.- infected cells by cytotoxic T-cells has not been demonstrated. Kaufmann (1987) proposed that since virtually all nucleated host cells express MHC class I molecules, association of bacterial antigens with class I molecules permits identification of infected cells independent of their type and origin. Thus, class I-

restricted cytotoxic T-cells, with specificity for bacterial antigens, could identify and lyse most infected host cells.

Suppressor T-cells, Immunosuppression and Tolerance

The immune response of an individual following introduction of antigens via vaccination consists of a rapid recruitment of immunocompetent cells with release of soluble factors. The hallmarks of this response are specificity, an enormous repertoire for recognition, and an ability to recall previous immunogenic challenges (Bankert et al., 1981). However, as with any physiological system, it must be regulated to be efficient. Immunologic reactions are regulated and represent the net balance of stimulatory and suppressor functions. During an immune response, suppressor cells and anti-idiotypic antibodies are considered to have regulatory functions in the immune response. Depending upon the relative balance between inhibiting factors and reactive lymphocytes, a lymphoproliferative response may or may not be detected. Stimulation of helper T-cells ultimately results in induction of suppressor T-cells circuits (Flood et al., 1986). This is important to avoid overproduction of helper T-cells and their factors. When IFN-g, IL-1, or IL-2 reach a minimum level a suppressive activity is induced through the suppressor T-cell circuit (Hausman et al., 1986).

Gershon (1970) was the first to describe suppressor T-cells while investigating immune tolerance in mice. In a synthetic peptide terpolymer system, Pierce et al (1979) have described two suppressor factors, one specific and the other nonspecific.

Suppressor T-cells are important in the regulation of the immune response and in tolerance, as they are able to suppress either helper or effector T-cells (Miller, 1975). Following activation by antigen on APC, suppressor inducer T-cells produce an antigen-specific factor TsF1 (T-lymphocyte suppressor factor one). This factor activates the suppressor T-cell precursor, which may bind TsF1 through specific recognition of the TsF1 idiotype. The precursor differentiates into or activates pre-existing effector suppressor T-cells. These suppressor cells may down regulate the immune response by two postulated mechanisms: 1) suppressor cells may bind helper T-cells through recognition of the idiotype of the helper T-cell antigen receptor, blocking the binding of antigen to the helper T-cell; 2) suppressor T-cells may release soluble suppressor factors that bind antigen bound helper T-cells, leading to shutdown of helper T-cell activity (Johnston, 1985).

A variety of products of microbial origin have been shown to modulate the immune system (Schwab, 1977), and some bacterial agents can suppress various phases of specific immunity (Schwab, 1975, 1983). Mostly facultative or obligate intracellular bacteria are implicated in immunosuppression. These organisms can survive and multiple in macrophages. Several examples of natural and experimental chronic infections have been described in which progressive uncontrolled infection is associated with a depressed CMI. These include tuberculosis, leprosy, pseudomoniasis, and trypanosomiasis (Watson et al., 1981; Schwab, 1983; Liew et al., 1987; Nakamura et al., 1989).

A decreased lymphoproliferative response to phytohemaglutinin (PHA) was described in humans with acute and chronic brucellosis

(Renoux and Renoux, 1977). In experimental murine brucellosis, inhibition of lymphoblastogenesis to several mitogens was attributed to splenic macrophages (Riglars and Cheers, 1980). Macrophages infected with Brucella sp. inhibit or suppress in vitro proliferation of T-cells (Splitter and Everlith, 1989). Macrophage-mediated immunosuppression was reported in several diseases (Allison, 1978; Elmer, 1978; Snyder et al., 1982). The susceptibility of mice to chronic infection with B. abortus is believed to be mediated by a suppressor mechanism (Ho and Cheers, 1982).

Macrophages have a dual role in the regulation of the immune response because they supply both activating factors for lymphocytes as well as inhibitory factors (Chouaib and Fradelizi, 1982). Many studies have shown that arachidonic acid metabolites synthesized by macrophages have important immunoregulatory functions (Goodwin et al., 1980; Stenson et al., 1980; Petit et al., 1985). Activated macrophages could inhibit T-cell-macrophage interaction during antigen presentation by regulating Ia expression through prostaglandin synthesis (Petit et al., 1985). IL-1 produced by activated macrophages can induce the production of adrenocorticotrophic hormone, which increases plasma cortisol levels. High plasma cortisol levels have been shown to be associated with depressed immune responses in calves (Roth and Kaeberle, 1983; Blecha and Baker, 1986).

Suppression of CMI to L. monocytogenes, may be mediated by prostaglandins secreted by activated macrophages (Petit et al., 1985). Thus, the mechanism responsible for the control of facultative intracellular bacteria may result in the inhibition of specific T-cell functions.

Immune stimulation with high doses of antigen often induces tolerance. Under these conditions the regulatory system enhances the suppressor T-cell. A more normal immunogenic dose enhances helper and contrasuppressor T-cells (Johnston, 1985).

The Role of Cellular Mediators

Soluble factors produced by immunocompetent cells in response to antigenic stimulation represent one communication link by which the immune system regulates its activities (Webb et al., 1983). Soluble mediators secreted by one cell bind to and modify the biological activity of another cell (Johnston, 1985).

Soluble factors that serve in the regulation of cellular hypersensitivity and immunity were discovered with the demonstration of MIF associated with DTH (Bloom and Bennet, 1966). Soluble mediators secreted by lymphocytes or monocytes are called lymphokines or monokines, respectively. Lymphokines are released by activated T-cells when they react with the antigen to which they were specifically sensitized.

IL-1, a protein secreted by stimulated macrophages, induces the production of IL-2 by helper T cells and is, therefore, necessary for lymphocyte proliferation. Besides inducing proliferation, IL-1 stimulates the liver to secrete acute-phase proteins, which are important in acute inflammatory responses. Bovine IL-1 can be measured using a murine IL-1 dependent cell line (D10.G4:1) (Everlith and Splitter, 1989).

Tumor necrosis factor (cachectin) (TNF-a), a monokine secreted mainly by macrophages, exerts profound and important biological

effects on a variety of cells in the host (Nathan, 1987). TNF has also been shown to act synergistically with IFN-g to enhance the macrophage activation (Esparza et al., 1987). In fact, TNF is important in host defense against L. monocytogenes infection (Nakane et al., 1988).

T-cells secrete a variety of lymphokines which are important in regulating the activity of the entire immune system. CMI is mediated through the collective action of lymphokines and cytotoxic T-cells. IL-2 is a glycoprotein secreted by T-lymphocytes after antigen and IL-1 stimulation. It is required for proliferation and clonal expansion of antigen- or mitogen- activated T-cells by acting on IL-2 receptors that are induced on activated T-cells. It also induces the synthesis of other lymphokines (Bendtzen, 1985; Alm, 1987). Besides IL-2, activated T-cells secrete a variety of other soluble factors including IFN-g, MIF, MCF, and GM-CSF. IFN-g induces expression of Ia molecules on macrophages and other APC, is a potent activator of macrophages, enhances the production of IL-1, and enhances intracellular killing of bacteria by macrophages. MCF recruits macrophages to the site of infection, whereas MIF keeps the attracted cells localized the at the focal site of antigen release. In addition, T-cells secrete suppressor factors that inhibit helper T-cell activity and down regulate the immune response.

In summary, potential mechanisms of immunity to Brucella sp. could be postulated as follows: Brucella organisms are phagocytized by macrophages and proliferate within cells. Macrophages degrade the bacteria, process antigens for presentation, and subsequently activate specific T-cells. Recognition of Brucella sp. antigens leads to the induction of CMI and DTH. Sensitized T-cells proliferate or secrete

lymphokines which have three effects: enhanced macrophage bactericidal activity, recruitment of monocytes from the hemopoietic tissue to the site of infection, and local proliferation of macrophages. Activated macrophages form granulomas and eliminate intracellular bacteria. Suppressor T-cells may block these pathways by inhibiting either the interaction between T-cells and APC or by the release of lymphokines. The immune response is further mediated by lymphokines and monokines.

The identification of the cell type responsible for most effective protection against brucellosis and the characterization of the cellular events that leads to its induction are crucial in the development of efficacious vaccines against Brucella sp..

Adjuvants for Enhancing Immune Mechanisms

An adjuvant may be defined as any agent that acts nonspecifically to augment an immune response to a specific antigen (Allison, 1979). Vaccination has long been considered as the most effective way of protecting an organism against pathogenic microorganisms. The success of a vaccination program depends both on the immunogenicity of the vaccine and the capacity of the host to mount an adequate immune response to the vaccine (Bizzini, 1984).

One main application of adjuvants is the potentiation of vaccines either by increasing the immunogenic potency of the vaccine and/or enhancing immunological responsiveness to a vaccine. By augmenting the efficacy of vaccination, an appropriate adjuvant could induce long-lasting immunity using a low dose of antigen and thus minimizing side effects of vaccination. An adjuvant can enhance humoral or cell-

mediated immunity or both. Thus, the choice of an adjuvant for any vaccine should be determined by the relative role of humoral and CMI in protecting the host against the infectious agent.

A myriad of immunopotentiating agents have been described in the literature, but only a few have been used in human and veterinary vaccination programs. This review of adjuvants is not intended to be comprehensive. It is a brief discussion of adjuvants used for humoral and cellular immunity, especially those capable of enhancing cellular response for Brucella sp. vaccines. The nature, chemistry, and action of immunological adjuvants of human and veterinary importance have been the subject of numerous reviews in the last 10 years (Aubry et al., 1979; Allison, 1979; Osebold, 1982; Cancelotti and Galassi, 1984; Adam, 1985; Lefrancier, 1985; Warren et al., 1986; Nervig et al., 1986; Dalsgaard, 1987).

Adjuvants for Humoral Immunity

Aluminum salts are widely used adjuvants for immunogens that require antibody responses to induce protection. Aluminum forms a precipitate with the antigen (alum-antigen precipitate). The alum-antigen precipitate localizes the antigen at the site of subcutaneous or intramuscular injection. The delayed absorption of the antigen makes it available to the immune system for a prolonged period, with enhancement of the immune response resulting from exaggerated, long term secondary stimulations (Kaeberle, 1986). The adjuvant effect of aluminum enhances antigen-specific IgG responses and is thought to be due to the delayed rate of absorption of the precipitated antigen (Bunn et al., 1986). It was also reported that aluminum can activate

complement (Ramanathan et al., 1979), which may in turn activate macrophages and increase their phagocytic activity. Because of their safety, aluminum salts have been used in human and veterinary biologics. In veterinary biologics, aluminum hydroxide gel is the most commonly used adjuvant.

Saponin is another adjuvant that has been used in a variety of veterinary vaccines, in particular foot and mouth disease virus (Dalsgaard, 1978). Saponin is a glycoside of plant origin and it is a surface-active adjuvant due to its amphipathic nature. It augments humoral responses and does not appear to stimulate CMI responses (Bomford, 1980). Saponin interacts with cell membranes and activates phospholipase A, with resultant formation of surface-active lysophosphatidylcholine, which inhibits prostaglandin synthesis. Inhibition of prostaglandin synthesis reduces the negative effect of prostaglandin on lymphoid cells, with a resultant augmentation of immune responses (Kaeberle, 1986).

The oil adjuvants (water-in-oil) consist of a highly purified mineral oil combined with an emulsifying agent such as Arlacel-A. The oil adjuvants retain a portion of the antigen at the site of injection, leading to a gradual and continuous release for stimulating antibody production. They alter lymphocyte recirculation by localizing lymphocytes in draining lymph nodes and they enhance the accumulation of lymphocytes and macrophages in lymph nodes draining sites of inflammation, infection or antigenic challenge. This facilitates interaction between cells that participate in immune responses (McKercher, 1986). Oil adjuvants increase the immune

response to most antigens and generally prolong the duration of immunity. They have been used to increase antibody responses for vaccines against such diseases as rabies, canine distemper, infectious canine hepatitis, hog cholera and foot and mouth disease (McKercher, 1986). However, the greatest use of oil adjuvants appear to have been with foot and mouth disease vaccines, particularly in those for use in swine.

Nonionic block copolymers, and Freund's incomplete adjuvant (FIA) potentiate humoral immune responses (Hunter and Bennett, 1986; Opdebeeck and Norcross, 1984). The nonionic block copolymers are surface-active agents consisting of a central polymer of polyoxypropylene (POP) which is hydrophobic, flanked by polymers of polyoxyethylene (POE) which is hydrophilic. The copolymers are adhesive molecules which can promote adhesion of proteins to one another or to cells. They promote adhesion of antigens to APC leading to effective presentation of antigen to T-cells. They also activate complement by the alternate pathway and C3b on the surface may facilitate localization of antigens on the surface of APC (Hunter and Bennett, 1986). FIA is an oil adjuvant without killed tubercle bacilli. The mode of action of FIA is similar to that of the oil adjuvant described above. FIA causes production of soft local granulomas that are rich in macrophages, lymphocytes, and plasma cells. FIA induces secondary antigen depots in lymph nodes, spleen, and lungs. Localization of antigen at injection site provides a persistent stimulus for antibody production (Osebold, 1982).

Adjuvants for Cell-Mediated Immunity

Freund's complete adjuvant (FCA), an emulsion of mineral oil and killed Mycobacteria sp., is one of the most potent adjuvants known for stimulating both humoral and CMI. Sulfatides from Mycobacteria sp. inhibit phagosome-lysosome fusion in macrophages (Goren et al., 1976). The resultant incomplete digestion of the phagocytosed bacteria apparently contributes to the induction of a CMI response (Kaeberle, 1986). CMI responses to both tuberculoproteins and test antigens are induced by FCA. FCA has limited clinical applications in domestic animals because it produces granulomas at the site of injection, fever, and toxic effects as well as tuberculin hypersensitivity. Bordetella pertussis (Tamura et al., 1985), Propionibacterium acnes (formerly called Corynebacterium parvum; Bomford, 1980), muramyl dipeptide (MDP, Audibert et al., 1985), dimethyl dioctadecyl ammonium bromide (DDA, Gordon et al., 1980), and trehalose dimycolate (TDM) (Lederer et al., 1987) are adjuvants capable of potentiating CMI. Other adjuvants capable of stimulating CMI are levamisole (Brunner and Muscoplat, 1980), sodium diethyl dithiocarbamate (DTC) (imuthiol) (Adam, 1985), polyribonucleotides (Adam, 1985), and azimexon (Chirigos, 1984).

The principal components of Bordetella pertussis are lipopolysaccharide (LPS) and pertussis toxin (PT). PT is a lymphocytosis-promoting factor that potentiates the CMI response by its ability to alter recirculation of T-lymphocytes by localizing lymphocytes in draining lymph nodes (Warren et al., 1986). PT also increases DTH responses. B. pertussis stimulates macrophages to

release molecules that stimulates helper T-lymphocyte functions. The macrophage appears to be the mediator for many of the immunostimulatory activities induced by P. acnes as well and it is believed that macrophage activation is primarily responsible for augmentation of CMI responses (Adlam and Scott, 1973; Filice et al., 1980). P. acnes-activated macrophages exhibit enhanced antigen processing (Wiener and Bandieri, 1975), phagocytic and bacteriostatic activity (Fauve and Herin, 1971).

DDA, a synthetic lipophilic quartenary amine, and a cationic surface active lipid, enhances expression for Fc receptors, increased interleukin-1 production, and increased lysosomal enzyme activity in macrophages (Gordon et al., 1980; Kraaijeveld et al., 1982; Prager, 1985). Inhibited phagosome-lysosome fusion in macrophages and enhanced DTH reactions result from use of DDA as an adjuvant (Snippe et al., 1982).

MDP is the minimal structure capable of replacing Mycobacteria sp. in FCA and TDM is a glycolipid (cord factor) associated with cell wall of Mycobacteria sp. The macrophage is the main target cell for MDP and TDM. These compounds activate macrophage metabolism with a resultant increase in bactericidal and cytostatic activities and increased IL-1 production (Guenounou et al., 1985; Garrec et al., 1986; Lederer et al., 1987).

Levamisole and DTC are sulfur-containing compounds; however, DTC lacks the imidazole ring. They both enhance macrophage and T-lymphocyte function and reduce suppressor T-cell function (Renoux and Renoux, 1979; Brunner and Muscoplat, 1980; Pompidou et al., 1985). The polyribonucleotides enhance helper T-cell activity through

increased synthesis of lymphokines and modulation of membrane receptors. Polyribonucleotides also activate macrophages and NK cell activity (Adam, 1985). Azimexon increases DTH responsiveness, stimulate T-lymphocyte functions and macrophage phagocytosis and natural killer cell activity (Chirigos, 1984). LPS, although long considered to be a potent polyclonal activator of B-cells can stimulate CMI responses as measured by DTH (Ohta et al., 1982). A number of these adjuvants (MDP, TDM, DDA, P. acnes, and FCA) have been employed in experimental Brucella sp. vaccines in cattle and laboratory animals (Woodard et al., 1980; Woodard and Jasman, 1981; Winter et al., 1983; Montaras and Winter, 1986; Panangala et al., 1986; Confer et al., 1987; Winter et al., 1988).

Biological Response Modifiers

Biologicals are products of the mammalian genome, and biological response modifiers (BRM) are agents when administered to an animal are capable of modifying the normal host defense mechanisms or immune responses. BRM are generally divided into two groups: 1) Those that are produced by the host and are a product of the host genome e.g. IL-1, IL-2, and interferons; and those that are not products of the mammalian genome but which can potentially alter the individual's biologic response, e.g. MDP, TDM, and DDA. A brief discussion of the first group will be considered here.

IL-1, a lymphocyte activating factor, is a protein secreted by activated macrophages. It stimulates the production of IL-2 by helper T-cells and is, therefore, necessary for lymphocyte proliferation. Thus, IL-1 serves as an endogenous adjuvant, wherein it is a co-factor

during lymphocyte activation primarily inducing the synthesis of IL-2 and the activation of the resting T-cell (Staruch and Wood, 1983).

IL-2, a T-cell growth factor, is produced by activated T-lymphocytes and is required for clonal expansion of activated T-cells. IL-2 also activates natural killer cells, enhances thymocyte proliferation, and induces cytotoxic T-cell activity. Bovine IL-2 has been produced *in vitro*, purified, characterized, and cloned (Cerretti et al., 1986; Magnuson et al., 1987). DNA recombinant techniques have produced purified products of IL-1 and IL-2, making it feasible to investigate their role as adjuvants. The in vivo effects of exogenously administered IL-1 and IL-2 on the immune system have been documented (Staruch and Wood, 1983; Rouse et al., 1985; Sharma et al., 1985).

Bovine IFN-a and IFN-g have been produced through recombinant DNA technology and evaluated *in vivo* in cattle and *in vitro* on bovine cells (Bielefeldt-Ohmann and Babuik, 1984; Steinbeck et al., 1986).

Conclusion and Statement of the Thesis Problem

Its beneficial effects not withstanding, the current S19 vaccine induces post-vaccinal antibody titers, aberrant serologic reactions, and chronic infections which may interfere with routine diagnostic serologic tests. The problems associated with S19 have spurred interest in the development of a *B. abortus* subunit vaccine which combines a few essential macromolecules of the *Brucella* surface. Numerous investigators have studied the immunogenicity of various subcellular fractions of *Brucella* (Montaraz and Winter, 1986; Confer et al., 1987; Winter and Rowe, 1988). However, subunit vaccines have

essentially low immunogenicity and have to be administered with an adjuvant to augment the immune response.

The studies in this dissertation were conducted to evaluate, in cattle, the immunopotentiating effects of various adjuvants for use with non-living B. abortus antigens by comparing serum antibody and CMI responses induced by these experimental vaccines. Additionally, the CMI responses were assessed with regard to functional lymphocyte and mononuclear phagocyte activities from the peripheral blood of calves vaccinated with S19 or non-living vaccines.

Three experiments were conducted. In experiments I and II, cattle were immunized with B. abortus soluble antigen (BASA) plus various adjuvant preparations. In experiment III, the immunizing antigen was a soluble extract of gamma-irradiated B. abortus (GBASA) which had reduced LPS content as compared to BASA. The CMI responses were evaluated using the lymphocyte-blast transformation assay and cutaneous DTH responses. The humoral immune responses were measured using standard serologic tests and immunoassays.

These studies were conducted using the following working hypothesis: (1) protective immunity could be achieved in bovine brucellosis using a non-living vaccine with an appropriate adjuvant; and (2) the mechanistic basis of adjuvant actions in bovine brucellosis could be delineated by evaluating the CMI responses with regard to helper and suppressor cell activity and IL-1 and IL-2 production of lymphocytes from calves vaccinated with strain 19 or non-living vaccines.

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CHAPTER II

COMPARATIVE EFFECTS OF DIFFERENT ADJUVANTS ON THE IMMUNE RESPONSES IN CATTLE VACCINATED WITH *BRUCELLA ABORTUS* SOLUBLE ANTIGEN

Introduction

Bovine brucellosis, caused by *Brucella abortus*, is a complex disease associated with abortion and infertility in cows. Despite an eradication program with a live *B. abortus* strain 19 (S19) vaccine, brucellosis remains a problem to the cattle industry in several regions of the country (Woodard, 1981). Vaccination of heifers with S19 induces resistance against brucellosis and thereby provides a measure of disease control. However, despite the beneficial effects of S19, interference with routine diagnostic serologic tests from residual post-vaccinal antibody titers, aberrant serologic reactions, and chronic infections with S19 may occur (Corner and Alton, 1981; Sutherland et al., 1982). Furthermore, S19, although attenuated for cattle is virulent for humans and thus, constitutes a human health hazard. Some of these problems associated with S19 have been decreased but not eliminated by using a reduced vaccinal dose (Barton et al., 1980). There is a need for an effective *Brucella* vaccine that would induce effective immunity without causing the problems currently associated with S19 (Alton, 1977).

It has been postulated that protective immunity can be induced for bovine brucellosis with a subunit vaccine with an appropriate adjuvant (Winter et al., 1983). An advantage of a subunit vaccine for brucellosis compared to a whole cell bacterin is the elimination of bacterial components that do not contribute to immune-mediated protection. Therefore, the presence of only one or two essential antigens in a subunit vaccine should eliminate the immune response against many irrelevant antigenic determinants of the organism (Bachrach, 1982). However, inactivated and purified soluble antigens often are weak immunogens and require a safe and effective adjuvant to effectively induce a strong immune response.

Recently, Montaraz and Winter (1986) demonstrated protection of BALB/C mice against experimental B. abortus infection at 1 week, but not at 4 weeks, following vaccination using B. abortus cell envelopes, killed whole cells, or outer membrane proteins; therefore, the use of nonliving vaccines for control of bovine brucellosis may be feasible. However, Woodard and Jasman (1983) failed to protect cattle against brucellosis using a trehalose dimycolate-B. abortus strain 45/20 bacterin. Confer et al (1987) failed to protect cattle from experimental brucellosis using a salt-extractable B. abortus antigen in Freund's complete adjuvant. Most experiments regarding the immunopotentiating capacities of various adjuvants in brucellosis have been studied in laboratory animals (Bosseray et al., 1984; Montaraz and Winter, 1986; Woodard et al., 1980), and these findings may not correlate well to such studies in cattle under field conditions.

The purpose of these studies was to evaluate the immunopotentiating effects of various adjuvants in conjunction with a nonliving

soluble B. abortus immunogen preparation through comparison of serum antibody and cell-mediated immune responses.

Materials and Methods

Cattle. Sixty-four weanling crossbred beef steers, approximately 250 to 300 kg each, were used. These steers were obtained from herds that had no clinical signs of brucellosis and were serologically negative for B. abortus. They were transported to the Livestock Health Research Center in Hugo, Oklahoma, maintained on a bermuda grass pasture, and fed a protein supplement.

Lymphoproliferation assay. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-hypaque density centrifugation as described (Confer et al., 1985). Two antigen preparations were used for the in vitro stimulations; heat-killed B. abortus S1119 (HKA) prepared as previously described (Dorsey and Deyoe, 1982) or gamma-irradiated B. abortus S19 (GBA, irradiated with 1.4 Mrad from a ^{60}Co source, obtained from Dr. B.L. Deyoe, Ames, IA). The lymphoproliferation (LP) assays were conducted in triplicate in 96-well microtiter plates (COSTAR, Cambridge, MA) as previously described (Confer et al., 1985). PBMC were used at a concentration of 5×10^5 cells per well. Antigen-driven LP responses were determined to HKA (2 ug per well) (Experiment 1), and to HKA and GBA at concentrations of 1 ug, 2 ug, and 4 ug per well (Experiment 2). Pokeweed mitogen (PWM) was used as a positive stimulation control. The LP responses were determined by ^3H-thymidine uptake (0.8 uCi per well, specific activity 30 mCi) over the final 18 hours of culture. A stimulation index (SI) was calculated for each sample: SI = mean

counts per minute (CPM) for HKA-, GBA-, or PWM-stimulated cultures/mean CPM for unstimulated cultures.

Serologic tests. The standard serologic tests used were the brucellosis card test (CARD), rivanol precipitation-plate agglutination test (RIV), and a microtiter complement fixation test (CF) (Alton et al., 1975).

A quantitative fluorometric immunoassay [Fluorescent Immunoassay System (FIAX), (Whittaker M.A. Biologics) was performed according to the method of Hall et al (1984) using dialyzed B. abortus soluble antigen (BASA-d, Tabatabai and Deyoe, 1984). Sera were diluted 1/51 (IDT Automatic Pipettor) in PBS containing 0.15% Tween 20. Fluorescein isothiocyanate-conjugated rabbit anti-bovine IgG (light and heavy chain specific) (Cappel Labs, Cockranville, PA) was diluted to 1/800. A fluorometer was used to determine fluorescence for duplicate serum samples. The average ng of IgG binding was evaluated from a standard curve.

The enzyme-linked immunosorbent assay (ELISA) was performed as previously described (Confer et al., 1985). Briefly, 100 ul of BASA-d in carbonate buffer (pH 9.6) were used to coat the wells of polystyrene microtiter plates. Sera were used at a dilution of 1/250. Brucella-specific antibodies were identified with affinity purified rabbit antibovine IgG (light and heavy chain specific) conjugated with horseradish peroxidase (Pel Freeze, Rogers, AR). The color reaction was developed using o-phenylenediamine and H_2O_2. Mean OD_{490} was determined on duplicate samples and the ng of IgG per well determined from a standard curve.

Delayed type hypersensitivity. As an in vivo correlate of cell-

mediated immunity, delayed type hypersensitivity (DTH) to BASA was determined 35 days after vaccination (Experiment 2). Each steer was tested by intradermal injection of 50 ug of BASA antigen in a volume of 0.1 ml using tuberculin syringes with 25-gauge needles. A second skin site was injected with 0.1 ml of phosphate-buffered saline as a negative control. The double skin fold thickness was measured with calipers before injection of antigen or PBS and 30 hours later. An increase in double skin fold thickness of 2 mm or greater than the initial measurement prior to injection of antigen was considered as a positive reaction (Nicoletti, 1983).

Adjuvants. The adjuvants used were of three general types: 1) an antigen depot adjuvant - Freund's incomplete adjuvant (FIA); 2) bacterial adjuvants - Bordetella pertussis, Propionibacterium acnes (formerly designated Corynebacterium parvum), and the synthetic adjuvant-muramyl dipeptide (MDP), which is considered to be the minimal structure capable of replacing mycobacteria in complete Freund's adjuvant; and 3) a surface-acting adjuvant, dimethyldioctadecyl-ammonium bromide (DDA). The immunopotentiating activities of these adjuvants have been documented in cattle and other species (Bomford, 1980; Bomford, 1980; Snippe et al., 1982; Winter et al., 1983).

Antigen. All steers receiving the soluble B. abortus preparation were immunized with Brucella abortus soluble antigen (BASA) (obtained from the National Veterinary Services Laboratories, Ames, IA) (Berman et al., 1980). In both experiments, 2 mg of BASA (1 ml containing 2 mg protein) was mixed with either an equal volume of the relevant adjuvant or sterile saline plus the adjuvant to make a final volume of

2 ml. With each antigen-adjuvant preparation, a total volume of 2 ml was injected subcutaneously in the cervical region anterior to the scapula.

Experiment 1. Thirty crossbred steers were equally allotted into 6 groups. Group 1 (control) was not immunized. Group 2 was immunized with the standard reduced dose of S19. Group 3 was immunized with BASA only. Group 4 received a combination of BASA + MDP (2 mg/dose) (Sigma Chemical Co., St. Louis, MO) emulsified in an equal volume of FIA (Difco Laboratories, Detroit, MI). Group 5 received a water-in-oil emulsion made up of equal volumes of BASA and FIA. Group 6 received a combination of equal volumes of BASA and DDA (Prolong^{TM}, Diamond Scientific, Des Moines, IA). Jugular blood samples were collected from all steers at the time of vaccination (day 0) and at intervals thereafter for serological assays (days 9, 14, 21, 28, 42, 56) and for the LP assay (days 3, 9, 14, 21, 28, 42, 56).

Experiment 2. Thirty-four crossbred steers were allocated into nine groups. Group 1 (control, n=4) was not immunized. Group 2 (n=4) was vaccinated with the standard reduced dose of S19. Groups 3 (n=5) received BASA only. Group 4 (n=5) received BASA + heat-killed P. acnes (obtained from ImmunoVet, Tampa, FL, (5 mg P. acnes/dose). Group 5 (n=5) received a solution of BASA + heat-killed B. pertussis (Lederle Laboratories, Pearl River, New York, strain 130, (5 mg B. pertussis/dose). Group 6 (n=5) received BASA + DDA as described in experiment 1. Group 7 (n=2) received P. acnes alone, at the same dose as in group 4. Group 8 (n=2) received B. pertussis only, at the same dosage as group 5. Group 9 (n=2) received 1 ml of DDA alone. Jugular blood samples were collected from all steers at the time of

vaccination (day 0) and at intervals thereafter for serological assays (days 6, 14, 21, 28, 42, 56) and for LP assay (days 4, 6, 11, 14, 21, 28, 35, 42, 49, 56). On day 35, the cattle were skin tested as described above.

Statistical analysis. Results were analyzed by the least significant difference (LSD) method (SAS, 1982).

Results

Serology. In the first experiment, the maximum number of steers positive by the CARD test was on days 14 and 21 (Table 1). By ELISA, an antibody response was detected in all groups by 9 days after vaccination (Fig. 1). In general, antibody responses for vaccinates peaked between days 28 and 56 as measured by ELISA and FIAX (Figs. 1 and 2) and on day 14 as measured by the CF and RIV tests, with the exception that steers vaccinated with BASA + FIA reached a maximum antibody response on days 28, 42, and 56 by the RIV and CF tests respectively (Figs. 3 and 4). The mean serum antibody responses, as determined by the FIAX test and ELISA, were significantly higher ($P < 0.05$) for vaccinates than for control steers at days 21, 42, and 56 with FIAX and day 56 with ELISA. There were no significant differences ($P > 0.05$) between antibody responses for steers vaccinated with S19 or with BASA + MDP. Mean antibody titers for BASA + MDP vaccinates were significantly higher ($P < 0.05$) than for BASA + DDA vaccinates on day 56 as measured by the FIAX and RIV tests. In many instances, antibody titers for S19 and BASA + adjuvant vaccinates were significantly ($P <0.05$) elevated over those for the steers that received BASA alone. By all serological tests, the highest mean titers

occurred in those groups receiving BASA + MDP and BASA + FIA.

In the second experiment, the number of seropositive steers as determined by the CARD test reached a maximum by day 14 (Table 1). Control steers and those receiving P. acnes, B. pertussis, or DDA alone were consistently seronegative by all four serological tests. Overall, the maximum antibody responses for most groups occurred on day 56 (Figs. 5-8). At that time, antibody responses for steers vaccinated with S19, BASA + B. pertussis, or BASA + DDA were significantly higher ($P < 0.05$) than for steers vaccinated with BASA alone or BASA + P. acnes. As measured by the ELISA and the RIV test, S19 vaccinates had significantly higher antibody levels ($P < 0.05$) than did all other treatment groups on days 28 and 42. By all four serological tests, S19-vaccinated steers consistently had significantly higher titers than did control steers ($P < 0.05$) between days 14 and 56. There were no significant differences ($P < 0.05$) in antibody responses between steers receiving BASA alone and BASA + P. acnes. Often mean antibody titers for the BASA + B. pertussis and BASA + DDA groups were significantly higher ($P < 0.05$) than for those receiving BASA alone or BASA + P. acnes.

LP assay. For each sampling time, LP responses for both experiments were compared relative to responses in the control steers. Steers immunized with BASA alone had the lowest overall responses, in that the mean LP responses for those groups were substantially below day 0 values.

In experiment 1, the LP responses of steers to HKA showed considerable individual variation with no consistent pattern in any of the groups (Fig. 9). The most distinct responses occurred from days 21

and 42, reaching a peak on day 42. The lowest LP responses in all groups occurred on day 14. Between days 21 and 42, LP responses for the BASA + DDA groups were insignificantly elevated over the responses of BASA + MDP and BASA + FIA groups. On day 21, the LP responses for S19, BASA + MDP and BASA + DDA groups were significantly different ($P < 0.05$) from those for steers receiving BASA alone. On day 42, the highest LP responses occurred in S19 vaccinates. Except for day 21, significant differences in LP responses were not seen among groups because of the small sample size per group ($n = 5$) and within group variations.

In experiment 2, LP responses to HKA (20 ug/ml) for all groups were markedly elevated over control responses on days 4 and 21 (Fig. 10). This was followed by a decline with only insignificant ($P > 0.05$) sporadic increases in group means. In general, LP responses in the S19 group were higher than for other treatment groups. LP response in the BASA + DDA group reflected a similar pattern of response as in the S19 group. Overall, S19 vaccinates had consistently higher LP responses than did control steers. DDA alone elicited markedly elevated LP responses which, in most instances, equaled or exceeded responses in S19 vaccinates (Fig. 11). However, as in experiment 1, significant differences were not observed among groups due to the small sample size and variation in LP responses within groups.

Delayed type hypersensitivity. In experiment 2, steers in all groups were skin tested and two out of four S19-immunized, two out of five BASA + DDA-immunized, and one out of five BASA + *P. acnes* immunized steer had highly positive responses to BASA. None of the steers in group 1 (control) or the other groups responded positively

to BASA. Overall, steers receiving BASA + DDA had the highest mean DTH response (Fig. 12). The least response was observed in steers receiving BASA alone.

Discussion

The capacity of various adjuvant preparations to augment both the cellular and humoral immune responses to experimental vaccine preparations have been documented (Bomford, 1980; Bomford, 1980; Edelman, 1980; Wells et al., 1982). A principal purpose for employing immunologic adjuvants is to elicit a more durable antibody and/or cell-mediated immune response by employing a smaller antigenic mass in a fewer number of doses. Adjuvants are also used to augment the induction of immunity against poorly or nonimmunogenic tumor cells or cells infected with intracellular microbes (e.g., Brucella) that are not adequately destroyed by naturally induced immune responses (Ribi, 1986). In this study, we attempted to potentiate the immune response to a soluble antigen (BASA) by employing different adjuvants. A single administration of antigen was used to study the primary response in comparison to the single inoculation with S19 as a standard. There was marked variability in the ability of different adjuvants to augment both humoral and cell-mediated immune responses when administered with BASA.

In experiment 1, the greatest serum antibody responses to B. abortus were induced by BASA in conjunction with the adjuvants FIA and MDP. Steers receiving BASA + DDA had antibody responses of a magnitude intermediate between S19-vaccinated and control steers. FIA appeared to provide the greatest stimulus for antibody production.

Cell-mediated immune responses as determined by LP assay was most evident in S19 and BASA + DDA vaccinates, with the highest responses occurring in S19 vaccinates. Although MDP and FIA induced high antibody titers, they failed to stimulate LP responses at levels equivalent to S19-vaccinated cattle. DDA appeared to be the most efficacious adjuvant with respect to induction of LP responses.

In experiment 2, the S19 group had the highest immunologic responses, i.e., antibody titers, LP response, and DTH responses. Among the adjuvant groups, BASA + DDA elicited the greatest CMI response as measured by LP and DTH tests. However, steers receiving DDA alone had marked LP responses on sporadic days, especially on day 4 indicating nonspecific stimulation of LP responses may have occurred.

In most instances, antibody responses for steers receiving BASA + B. pertussis were higher than those receiving BASA + DDA. Generally, antibody responses of the BASA + P. acnes group were similar to those immunized with BASA alone. Increases in mean antibody titers persisted up to 56 days.

The LP responses in the BASA + P. acnes and BASA + B. pertussis groups were often comparable to those of the BASA + DDA groups. However, the former groups failed to respond in the DTH test as did the latter group. In both experiments, humoral and CMI responses were lowest when no adjuvant was included with the antigen. On the basis of serological, LP and DTH results, DDA seemed to be the most effective adjuvant.

Our results indicate that DDA promotes a marked CMI response with a low antibody response. DDA is a synthetic lipophilic quartenary

amine and a cationic surface-active lipid. The adjuvanticity and biological properties of DDA have been extensively reviewed (Baechtel and Prager, 1982; Chiba and Egashira, 1978; Gordon et al., 1980; Snippe et al., 1977; Snippe et al., 1982). One advantage of DDA over other adjuvants, especially those of microbial origin, is that it has no immunogenic components that might cross-react with B. abortus (Corbel, 1985). DDA has been shown, in mice, to be effective in the immunopotentiation of Listeria sp. vaccines and also superior to Freund's complete adjuvant in stimulating a DTH reaction in mice (Snippe et al., 1977; Van der Meer et al., 1979). Woodard et al (1980) failed to potentiate acquired resistance to Brucella in guinea pigs using a DDA-B. abortus strain 45/20 vaccine. However, the dose of DDA used in that study may have caused deterioration of cellular integrity and lysis leading to lack of antigen processing (Prager, 1985).

Development of an improved and effective nonliving B. abortus vaccine would constitute major progress toward the goal of eradicating bovine brucellosis in the United States. These studies demonstrated that, in cattle, DDA stimulates both humoral and CMI responses when administered with a nonliving B. abortus antigen preparation. Evaluation of DDA as an adjuvant for a Brucella vaccine using purified B. abortus antigens requires further study.

TABLE 1. Numbers of serologically positive cattle as detected by the *Brucella abortus* CARD test.

Group	Vaccine	No. of Cattle	Days after vaccination (No. seropositive) 0	6	9	14	21	28	42	56
Experiment #1										
1	None (control)	5	0	ND[a]	0	0	0	0	0	0
2	S19	5	0	ND	4	5	5	5	5	5
3	BASA	5	0	ND	2	2	1	1	1	1
4	BASA + MDP	5	0	ND	5	5	5	5	5	5
5	BASA + FIA	5	0	ND	4	5	5	5	4	5
6	BASA + DDA	5	0	ND	4	3	4	3	2	1
Experiment #2[b]										
1	None (control)	4	0	0	ND	0	0	0	0	1
2	S19	4	0	4	ND	4	4	4	4	4
3	BASA	5	0	2	ND	4	3	1	3	3
4	BASA + *P. acnes*	5	0	3	ND	4	2	1	4	3
5	BASA + *B. pertussis*	5	0	5	ND	5	5	5	5	5
6	BASA + DDA	5	0	2	ND	2	5	5	5	5
7	*P. acnes* only	2	0	0	ND	0	0	0	0	0
8	*B. pertussis* only	2	0	0	ND	0	0	0	0	0
9	DDA only	2	0	0	ND	0	0	0	0	0

[a]not determined.
[b]cattle in experiment #2 were skin tested on day 35.

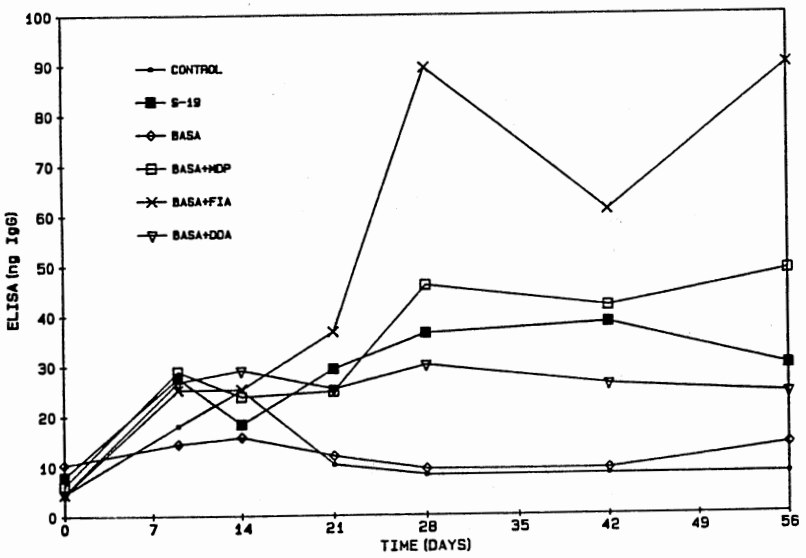

Figure 1. Mean antibody responses to B. abortus in Experiment 1 as measured by an enzyme-linked immunosorbent assay (ELISA).

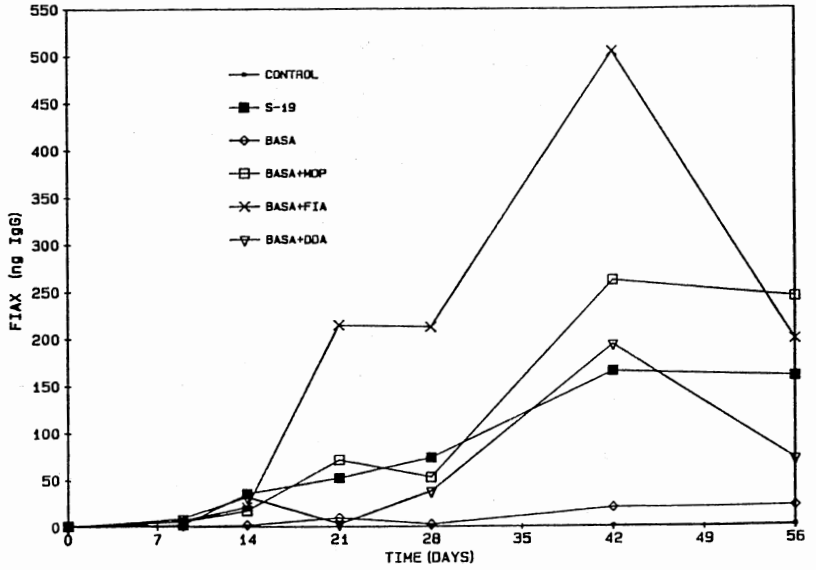

Figure 2. Mean antibody responses to B. abortus in Experiment 1 as measured by a fluorometric immunoassay (FIAX).

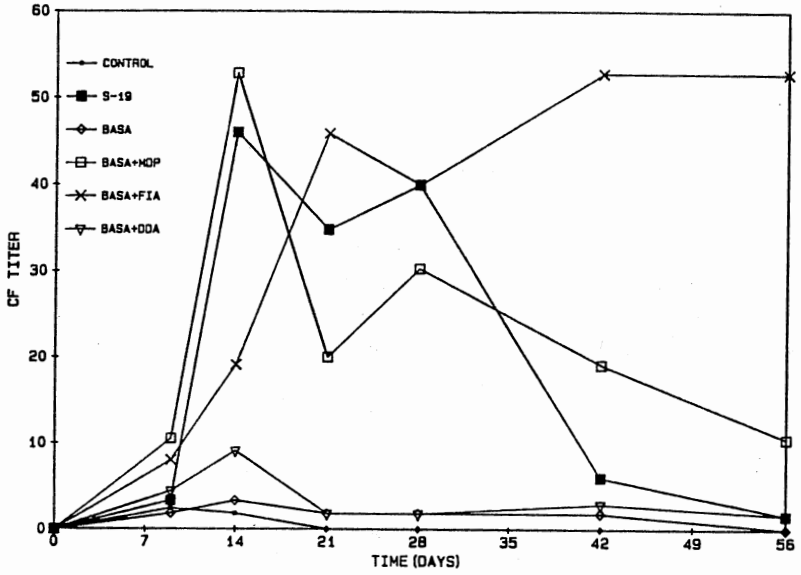

Figure 3. Mean antibody responses to B. abortus in Experiment 1 as detected by complement fixation (CF).

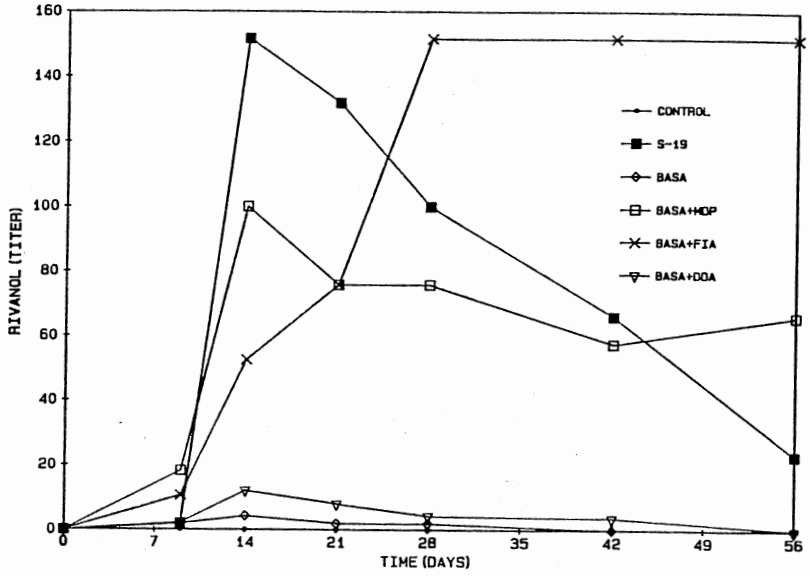

Figure 4. Mean antibody responses to B. abortus in Experiment 1 as measured by Rivanol agglutination (RIV).

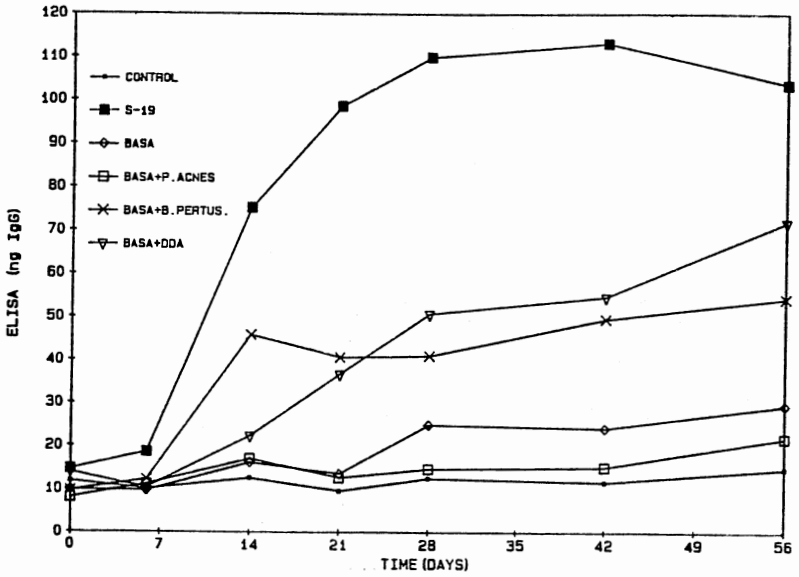

Figure 5. Mean antibody responses to B. abortus in Experiment 2 as measured by an enzyme-linked immunosorbent assay (ELISA). Antibody responses for steers receiving P. acnes, B. pertussis and DDA alone were similar to responses for steers in the control group.

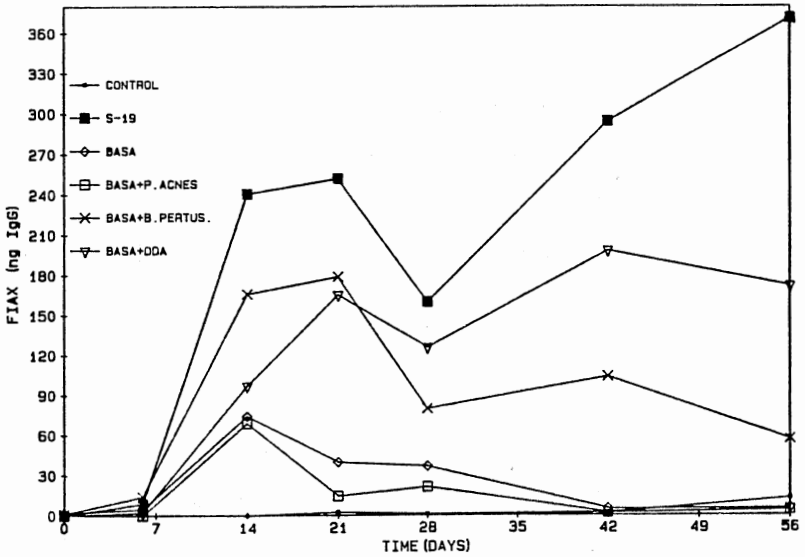

Figure 6. Mean antibody responses to B. abortus in Experiment 2 as measured by a fluorometric immunoassay (FIAX). Antibody responses for steers receiving P. acnes, B. pertussis and DDA alone were similar to responses for steers in the control group.

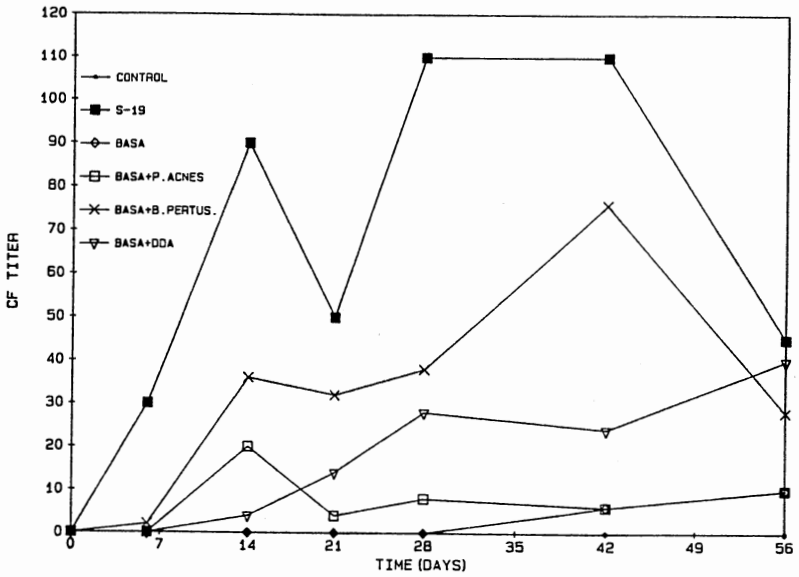

Figure 7. Mean antibody responses to B. abortus in Experiment 2 as measured by complement fixation (CF). Antibody responses for steers receiving P. acnes, B. pertussis and DDA alone were similar to responses for steers in the control group.

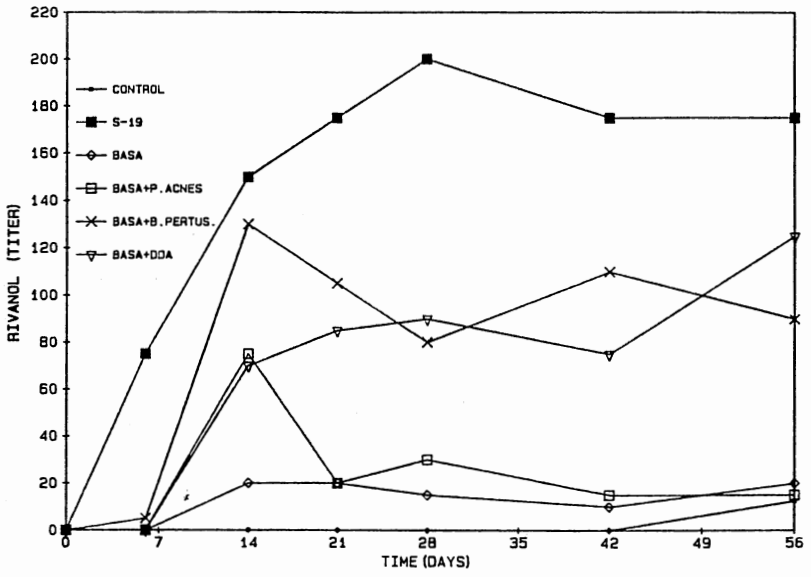

Figure 8. Mean antibody responses to B. abortus in Experiment 2 as measured by Rivanol agglutination (RIV). Antibody responses for steers receiving P. acnes, B. pertussis and DDA alone were similar to responses for steers in the control group.

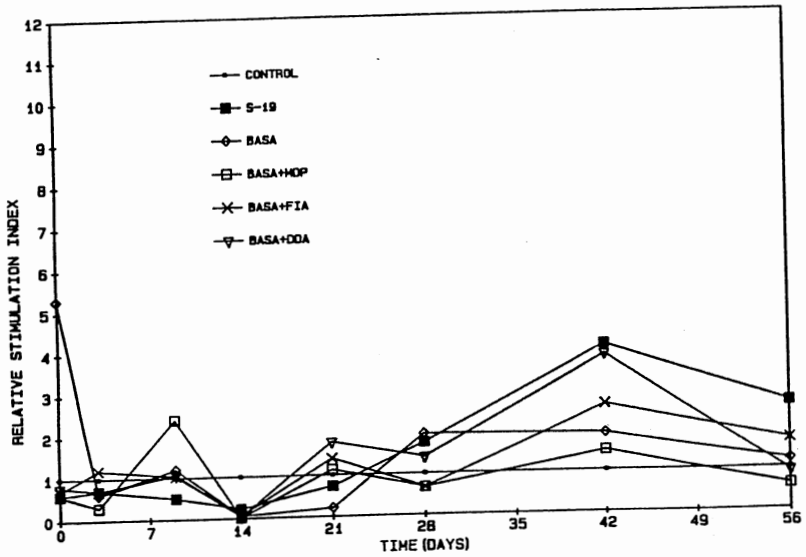

Figure 9. Mean stimulation indices to heat-killed B. abortus in Experiment 1 as determined by LP assay.

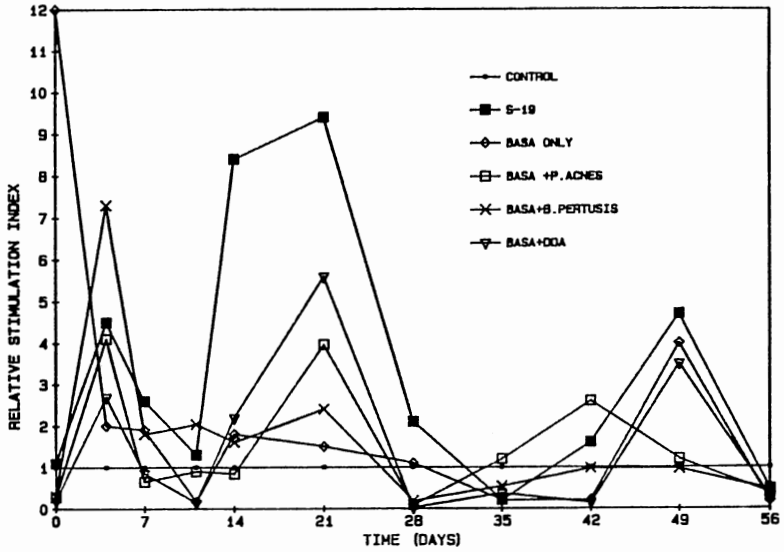

Figure 10. Mean stimulation indices to heat-killed B. abortus in Experiment 2 as determined by LP assay.

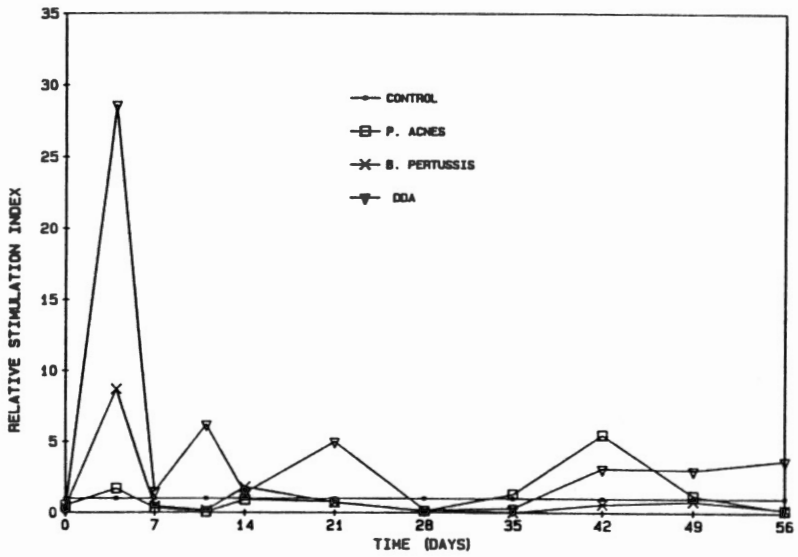

Figure 11. Mean stimulation indices to heat-killed B. abortus in Experiment 2 as determined by LP assay (adjuvant controls only).

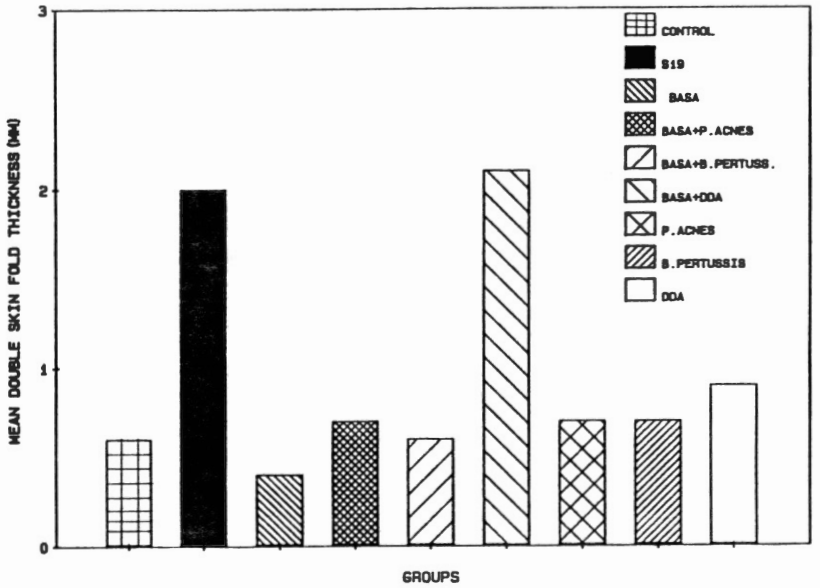

Figure 12. Mean delayed-type hypersensitivity (DTH) responses to BASA (Experiment 2).

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CHAPTER III

INFLUENCE OF IMMUNOMODULATORY AGENTS ON CELLULAR IMMUNE RESPONSES IN CATTLE VACCINATED WITH *BRUCELLA ABORTUS* SOLUBLE ANTIGEN

Introduction

Brucellosis continues to be a major worldwide problem from both economic and public health aspects (Matyas and Fujikura, 1984). Vaccination of heifers with strain 19 (S19) confers immunity against brucellosis and thereby helps control the disease. Despite the beneficial effects of S19, however, residual post-vaccinal antibody titers, aberrant serologic reactions, and chronic infections with S19 may occur and interfere with routine diagnostic serologic tests (Corner et al., 1981; Sutherland et al., 1982). These problems have stimulated interest in development of an effective *Brucella abortus* vaccine that would induce protective immunity but would not have the problems associated with S19 (Alton, 1977).

It was postulated that protective immunity can be induced against bovine brucellosis using a non-living (subunit) vaccine with an appropriate adjuvant (Winter et al., 1983). Numerous investigators have tested various non-living (subunit) vaccines in experimental bovine brucellosis (Woodard and Jasman, 1981; Confer et al., 1987;

Winter et al., 1988). Variable degrees of success were seen. The objectives of the present study were to: (1) evaluate in cattle the immunostimulatory effects of various adjuvants for use with non-living B. abortus antigens by comparing an in vitro correlate of cell-mediated immunity (CMI); (2) evaluate functional subset activities involved in CMI responses with regard to helper T-cell activity, macrophage activation, and suppressor cell activity of lymphocytes from calves immunized with S19 vaccine or a soluble B. abortus extract.

Materials and Methods

Cattle. Thirty-four weanling crossbred beef steers, approximately 250 to 300 kg each, were used. These steers were obtained from herds that had no clinical signs of brucellosis and were serologically negative for B. abortus. They were transported to the Livestock Health Research Center in Hugo, Oklahoma, maintained on a bermuda grass pasture, and fed a protein supplement. Thirty-four crossbred steers were placed into nine groups (see Table 1). The dose and source of adjuvant are the same as previously described in Chapter II. Jugular blood samples were collected from all steers at the time of vaccination (day 0) and at intervals thereafter for Interleukin 1 and 2 assays (days 6, 14, 21, 28, 35, 42, 42, 56) and for the LP and suppressor factor assays (days 4, 6, 11, 14, 21, 28, 35, 49, 56).

Adjuvants. Three adjuvants were used: Bordetella pertussis, Proprionobacterium acnes and dimethyl dioctadecyl-ammonium bromide (DDA). The capacity of these adjuvants as immune enhancers have been documented in cattle and other species (Bomford, 1980a,b; Snippe et

al., 1982; Wells et al., 1982).

Immunogens. Brucella abortus soluble antigen (BASA) was used as the vaccine antigen as previously described (Chapter II).

Lymphoproliferation assay (LP). The LP was conducted as previously described (Chapter II).

Delayed type hypersensitivity (DTH). DTH was conducted as previously described (Chapter II).

Preparation of culture supernates for IL-1 and IL-2 assay. Bovine peripheral blood mononuclear cells (PBMC, 5 x 10^6 cells/ml) were cultured in 96-well microtiter plates (Costar, Cambridge, MA) as previously described (Chapter II). Cultures were treated in triplicate with concanavalin A (Con A, 10 ug/ml), heat-killed B. abortus antigen (HKA, 20 ug/ml), gamma-irradiated B. abortus antigen (GBA, 20 ug/ml), or B. abortus soluble antigen (BASA, 40 ug/ml). After 48 hr. incubation in a humidified atmosphere containing 5% CO_2, the plates were centrifuged at 200 g for ten minutes. Culture supernatants were collected (50 ul and 75 ul for IL-1 and IL-2 assays respectively) into microtiter plates and frozen at -135^{o} C.

Interleukin-1 Assay

IL-1 was assayed using an IL-1-dependent murine helper T cell line (D10.G4.1) (obtained from American Type Culture Collection, ATCC, Rockville, Maryland). These cells were maintained by allostimulation on mitomycin C-treated H-2^b C57BL/6 splenocytes as previously described (Kaye et al., 1984). Culture supernatant fluids were replenished with 50 ul RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Briefly, 2 x 10^4 D10.G4.1 cells/per well were

cultured with culture supernatant fluid in a microtiter plate format for 48 hrs. and then pulsed with ^{3}H-TdR (1 uCi/well, specific activity = 30 mCi) for an additional 4 hrs. at which time the cells were harvested onto filter papers using a cell harvester (PhD, Cambridge Technology, Cambridge, MA) and radioactivity determined in a liquid scintillation counter (Packard, Downers Grove, IL.). The results were compared to serial titrations of a recombinant human IL-1-standard (obtained from Dr. Peter T. Lomedico, Hoffman-LaRoche, Inc., Nutley, NJ).

Interleukin-2 assay. IL-2 in culture supernatant fluid was assayed using bovine IL-2- dependent BT-2 cells (obtained from Dr. P.E. Baker, Immunex Corporation, Seattle, WA). These cells were originally isolated from a primary mixed lymphocyte reaction culture and has been shown to be dependent on IL-2 for continued growth in culture (Baker and Knoblock, 1982). The cells were maintained in culture in 6 well microtiter plates at 3.3 x 10^{4}/well in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% FBS with 1 ug/ml Con A and 10 U/ml recombinant bovine (rb) IL-2, (obtained from Dr. P.E. Baker, Immunex Corporation, Seattle, WA). The cells were passaged 2-3 times per week. The assay system used was an adaptation of the quantitative microtiter assay of Gillis, et al. (1978). BT-2 cells were washed 3x in DMEM to remove residual rb IL-2. Briefly, 2 x 10^{3} BT2 (in 25 ul of DMEM and 75 ml of supernate sample) cells per well were cultured with supernatant fluid from PBMC which has been exposed to different stimuli in a microtiter plate format for 48 hours and then radiolabelled with ^{3}H-TdR for 4 hours. Proliferative responses were determined by ^{3}H-TdR uptake as described above and

compared to parallel controls in which rb IL-2 has been titrated.

Suppressor Factor Assay

Suppressor factor assays were performed in conjunction with the LP assay. Culture supernatant fluid (50 ul/well) was collected from lymphocyte cultures after 6 days of incubation. The original wells were replenished with 50 ul of fresh media, RPMI 1640 medium supplemented with 50 ug/ml gentamicin. The harvested fluids were stored at -135^{o} C until assay. Suppressor factors were assayed by inhibition of blastogenesis of peripheral blood mononuclear cells (PBMC) induced with suboptimal levels of concanavalin A, by a modification of the assay previously described by Nelson et al. (1987). The Con A dose of 0.5 ug/ml was determined by titrating PBMC from a Hereford steer with different doses of Con A and a suboptimal concentration was chosen for the suppressor assay according to a dose-response curve. PBMC from this steer was used throughout the experiment to determine suppression. To assay the culture supernates, 50 ul of RPMI 1640 medium supplemented with 10% FBS plus 50 ug/ml gentamicin was added to 50 ul of the sample and 100 ul of media containing 1 x 10^{5} PBMC plus 0.5 ug of Con A was added to each well. After a 72 hr incubation each well was pulsed for 18 hr. with 0.8 uCi of ^{3}H-TdR. The cells were harvested onto filter papers and thymidine incorporation determined as previously described. Results are expressed as stimulation index.

Statistical analysis. Results were analyzed by the least significant difference (LSD) method (SAS, 1982). Statistical significance was considered at the $P < 0.05$ level.

Results

Data are shown as relative stimulation indices (RSI) and are expressed relative to the responses in the control steers. See Chapter II for the results and discussion of the LP and DTH responses.

IL-1 Production

With the exception of minor variations, IL-1 activity detected in supernatants from mitogen-/antigen-stimulated cultures reflected the same pattern of responses (Figures 1-4). The most distinct proliferative responses occurred in day 14 samples. This was followed by a decline with only insignificant ($P > 0.05$) sporadic increases in group means. On day 14, statistical analysis showed that IL-1 levels for groups 6, 8, and 9 were significantly ($P < 0.05$) greater than the levels for the control and other treatment groups. Often IL-1 levels for group 7 steers were insignificantly elevated ($P > 0.05$) over the control levels. The greatest and least stimuli for IL-1 production were respectively induced by BASA + DDA and S19. IL-1 dependent cells proliferated minimally when stimulated directly with mitogen (Con A) or antigen (HKA, GBA, BASA) alone. This suggests that the cells were not responding to residual antigen or mitogen in the culture supernatant fluid but to a factor elaborated by the stimulated PBMC used to generate the supernates.

IL-2 Production

IL-2 activity in Con A-stimulated culture supernatant fluid demonstrated considerable variation with no consistent pattern in any

group (Figure 5). The S19 group had a progressive rise in IL-2 production from day 0 to day 28 followed by a plateau and decline. On day 35, IL-2 levels in all treatment groups were elevated over the control levels. At that time, steers receiving P. acnes, B. pertussis, and DDA alone had significantly higher ($P < 0.05$) IL-2 levels than control steers. Except for minor variations, IL-2 activity in supernatant fluids from antigen-stimulated PBMC was maintained at similar levels (Figures 6-8). S19 vaccinates had the greatest stimulus for IL-2 production which peaked on day 21. At that time, the S19 group had significantly ($P < 0.05$) greater IL-2 levels than levels for the other treatment groups. Steers receiving DDA alone had transient insignificant ($P < 0.05$) rises in IL-2 levels. IL-2 production in the S19 group corresponded with the results of the lymphoproliferative assay. BT-2 cells failed to proliferate when cultured with Con A or antigen alone indicating no effect of any possible antigen or mitogen carried over in the supernates from the generating cultures of PBMC.

Suppressor Factor Assay

Data for soluble suppressor factor release in response to stimulation with mitogen and B. abortus antigens are illustrated in Figures 9-11. The group mean RSI to PWM and B. abortus antigens demonstrated a wide range of mean values. A non-significant ($P > 0.05$) depression in responsiveness to PWM occurred at day 7 in all treatment groups (Figure 9). The largest suppressive responses to PWM were observed in BASA + B. pertussis and BASA + DDA vaccinates in that the mean RSI for these groups were lower than the controls on six of

eleven bleeding days. HKA induced suppressive supernates on days 14 and 28 for all treatment groups (Figure 10). This was followed by insignificant ($P > 0.05$) sporadic depressions in group means. Responses to HKA induced supernates in BASA + B. pertussis vaccinates rebounded to above the control group at day 42 with a gradual increase in responsiveness to HKA. The largest HKA-induced suppressive supernates were observed in S19, BASA and P. acnes vaccinated steers. GBA induced suppressive supernates in all treatment groups on day 7 and in S19, BASA + DDA, P. acnes, B. pertussis and DDA vaccinated steers on days 14 and 28. The largest GBA-induced suppressive supernates were observed in S19, BASA + B. pertussis and P. acnes vaccinated steers.

Discussion

Adjuvant-induced modulation of immune responses to several B. abortus immunogens has been documented (Woodard et al., 1980; Winter et al., 1983; Montaraz and Winter, 1986; Confer et al., 1987; Winter et al., 1988). The mechanisms of actions of these adjuvants have not been clearly defined. Therefore in this study, we attempted to elucidate the basis for the mechanism of actions of adjuvants when administered with BASA by assaying for IL-1, IL-2, and soluble suppressor factor release in supernatant fluids from mitogens or B. abortus antigen-stimulated PBMC cultures.

Cell-mediated immune responses require interactions between immunocompetent cells and soluble factors. One of these factors is IL-2 (T-cell growth factor). IL-2, a glycoprotein produced by lymphocytes in response to antigenic or mitogenic stimulation sustains

the clonal expansion of activated T-cells, activates natural killer cells, enhances thymocyte mitogenesis, and induces cytotoxic T-cell activity (Magnuson, et al, 1987). Reduced IL-2 production is an important mechanism in pathogenesis of infectious disease (Wainberg et al., 1983; Orz et al., 1985; Modlin et al., 1984). The ability of an individual's lymphocytes to produce and respond to IL-2 may provide a quantitative measure of CMI responses to specific antigens (Miller-Edge and Splitter, 1986). In the present study, S19 provided the greatest stimulus for IL-2 production. For the BASA plus adjuvant groups, none of the lymphocyte cultures produced IL-2 in response to B. abortus antigens at levels equivalent to those for S19-vaccinated cattle. However, Con A-stimulated cells from each group produced equivalent IL-2 levels.

IL-2 production in S19-vaccinated cattle corresponded with the LP responses. Although the LP responses in BASA + DDA vaccinated cattle reflected a similar pattern as in S19 vaccinates, the former did not produce equivalent levels of IL-2. The reason for this disparity is not known. To exert its biological effects, IL-2 must interact with high affinity receptors on the lymphocyte membranes (Fletcher and Goldstein, 1987). Therefore, IL-2 levels measured by this assay system, reflect only the amount of free IL-2 in the supernatant fluid. Perhaps, the presence of an inhibitory factor in culture supernatants could have blocked IL-2 activity in the culture fluids (Lafferty et al., 1980). Proliferation of bovine PBMC is dependent on the presence of IL-2. The fact that LP responses in the BASA + DDA group paralleled responses in the S19 group suggests that low IL-2 levels in culture supernatants from BASA + DDA vaccinated cattle may be due to

the amount of IL-2 consumed by activated T-cells, rather than the presence of inhibitory factors in the medium.

Macrophages are important in the induction and expression of the immune response through direct cellular interactions and by production of monokines such as IL-1 (lymphocyte activating factor). IL-1 modulates many of the responses involved in the process of host defense against infection. IL-1 acts as an endogenous adjuvant, serving as a co-factor during lymphocyte activation primarily by inducing the synthesis of IL-2 and the activation of the resting T cell (Staruch and Wood, 1983). In the present study, DDA provided the strongest stimulus for IL-1 production. The least stimulus for IL-1 production was seen in the S19 group. Marginal levels of IL-1 were observed in groups 4 and 5.

The low level of IL-1 observed in the S19 group was unexpected because LP responses and IL-2 production were greatest in that group. Several factors may account for this. As with other polypeptide hormones, IL-1 exerts its biological effect on target cells by first binding to specific cell surface receptors. Several cell types express receptors for and are IL-1 responsive (Dower and Urdal, 1987). Lymphocytes from S19 vaccinated cattle may have had a greater responsiveness for IL-1 leading to increased consumption of IL-1 in the culture medium. IL-1 is expressed in both secreted and membrane-associated form in macrophage, monocytes, and B cell cultures (Oppenheim, 1986; Kurt-Jones et al., 1985). Recently, Splitter and Everlith (1989) demonstrated that B. abortus induces production of both membrane and secreted forms of IL-1 from macrophages. As a membrane-associated molecule, membrane IL-1 may serve as a persistent,

localized stimulant to activated T cells when present on the surface of antigen presenting cells at a site of infection (Oppenheim, 1986). It is, therefore, possible that PBMC from the S19 group preferentially express membrane IL-1 rather than secreted IL-1.

A variety of products of microbial origin can modulate the immune system (Schwab, 1977) and some bacterial agents can suppress various phases of specific immunity (Schwab, 1983). A decreased LP response to phytohemagglutinin was reported in humans with acute and chronic brucellosis (Renoux and Renoux, 1977). Inhibition of the LP response to several mitogens was described in experimental murine brucellosis (Riglars and Cheers, 1980). Furthermore, immunomodulatory agents may produce variable effects on the immune system from immunostimulation, immunosuppression, or no effect (Bizzini, 1984). In the present study, soluble suppressor factors were demonstrated in culture supernatant fluids from PBMC stimulated with PWM and B. abortus antigens. The least and greatest depressions in LP responses were seen in culture supernatants from PWM and antigen-stimulated lymphocytes respectively. The greatest suppressive responses were observed in groups 2, 5, and 7. The suppressive effects may be due to the effect of the adjuvant, because steers receiving adjuvants had depressed LP responses. However, we could not demonstrate a direct correlation between LP responses and the suppressive effect of culture supernatants.

The choice of an adjuvant for any vaccine should be determined by the relative role of humoral and cell-mediated immunity required for protecting the host against the infectious agent. In this study, DDA proved to be the most efficacious adjuvant with respect to CMI

responses i.e. increased LP and DTH responses (Chapter II), increased IL-1 production and decreased soluble suppressor factor production. DDA enhances macrophage and T-lymphocyte functions and reduces suppressor T-cell function (Gordon et al., 1980; Kraaijeveld et al., 1982). However, we were unable to demonstrate that DDA enhanced IL-2 production. The stimulatory effect of DDA on macrophages may be particularly important because B. abortus is an intracellular pathogen, and macrophages may be important in host defense in bovine brucellosis (Braude, 1951). The results obtained for P. acnes and B. pertussis were unexpected. These adjuvants failed to induce CMI responses comparable to DDA. These adjuvants are both potent stimulators of CMI responses (Bomford, 1980; Warren, 1986). Recently, it was demonstrated that P. acnes administered with S19 vaccine potentiates CMI responses in guinea pigs (Panangala et al., 1986). The effective dose of those adjuvants for use in cattle may need to be investigated.

The search for immune modulators is an important area of investigation because of the increased emphasis on the use of subunit vaccines. Numerous microbial-derived and synthetic compounds have been described to enhance humoral or cellular immune responses in animals (Edelman, 1980). Successful application of adjuvants to subunit vaccines will depend on the understanding of the mechanisms of action of the adjuvant and the appropriate factors and segments of the immune system that must be stimulated.

Table I

VACCINE AND ADJUVANT PREPARATIONS ADMINISTERD TO EACH GROUP OF EXPERIMENTAL ANIMALS

Group	(n)	Vaccine
1	4	None (control)
2	4	S19[a]
3	5	BASA[b]
4	5	BASA + *P. acnes*[c]
5	5	BASA + *B. pertussis*[d]
6	5	BASA + DDA[e]
7	2	*P. acnes* only
8	2	*B. pertussis* only
9	2	DDA only

[a] Standard reduced dose (1×10^9 CFU)
[b] 2 mg protein
[c] 5 mg *P. acnes*
[d] 5 mg *B. pertussis*
[e] 1 ml DDA

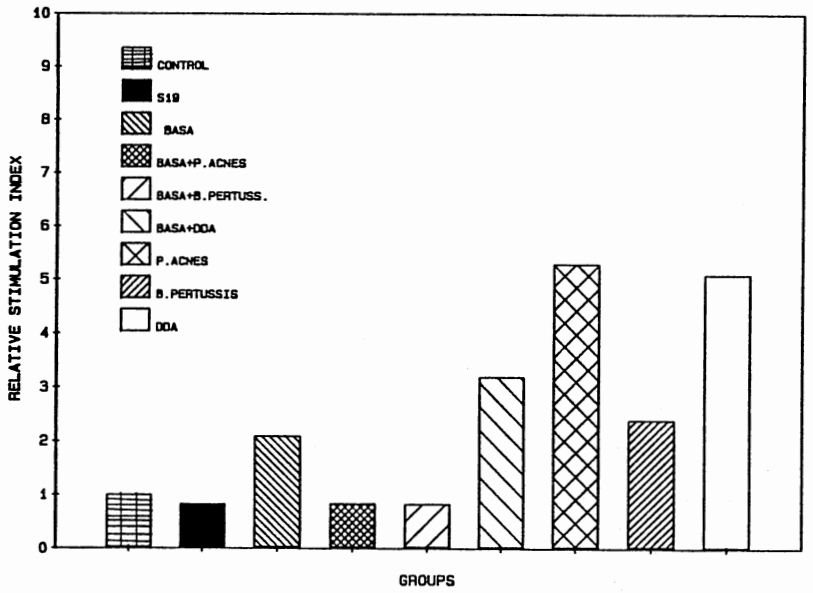


Figure 1. Mean relative stimulation indices to Con A-induced IL-1 production in culture supernatant fluid on day 14. The vaccine and adjuvant preparations administered to each group of animals are shown in ble 1.

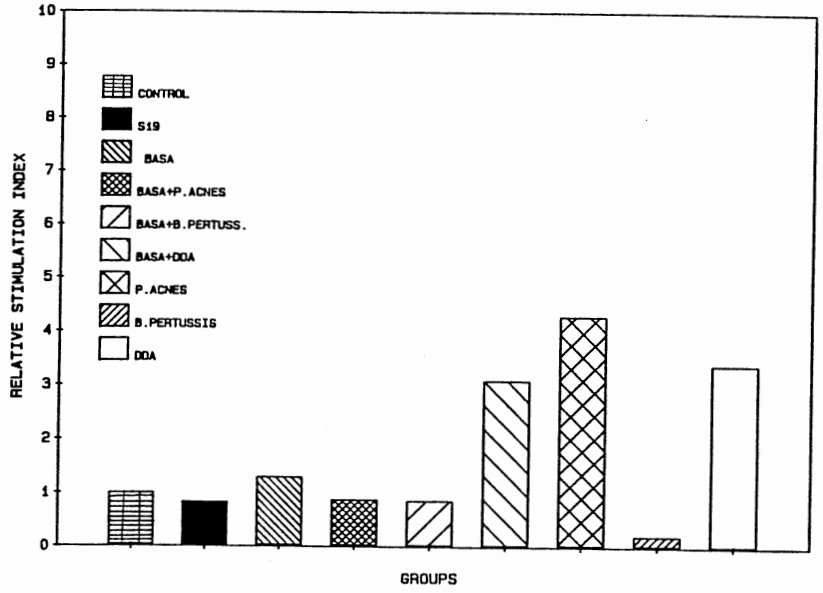


Figure 2. Mean relative stimulation indices to heat-killed B. abortus-induced IL-1 production in culture supernatant fluid on day 14. The vaccine and adjuvant preparations administered to each group of animals are shown in table 1.

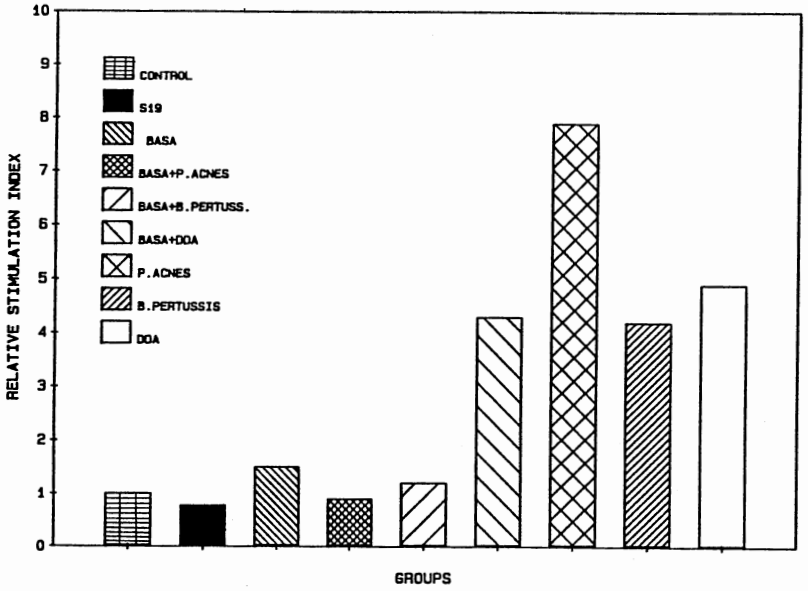


Figure 3. Mean relative stimulation indices to gamma-irradiated B. abortus-induced IL-1 production in culture supernatant fluid on day 14. The vaccine and adjuvant preparations administered to each group of animals are shown in table 1.

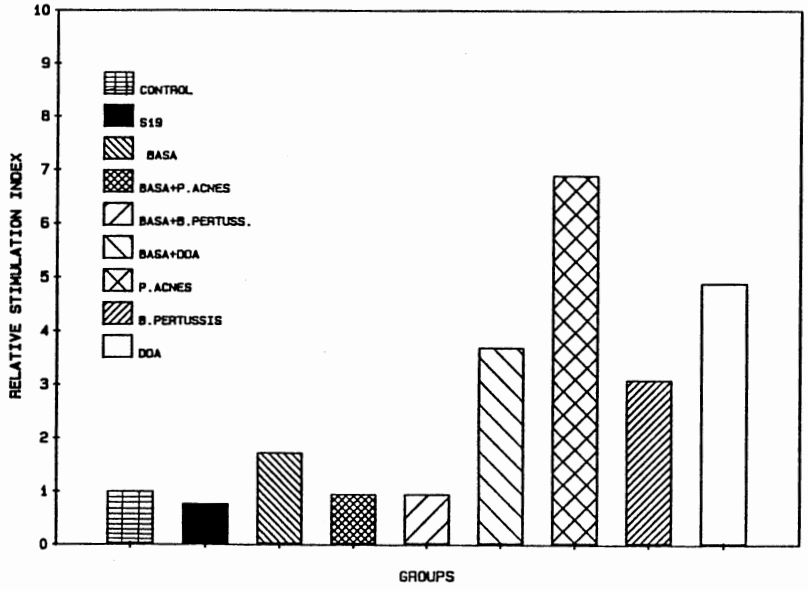
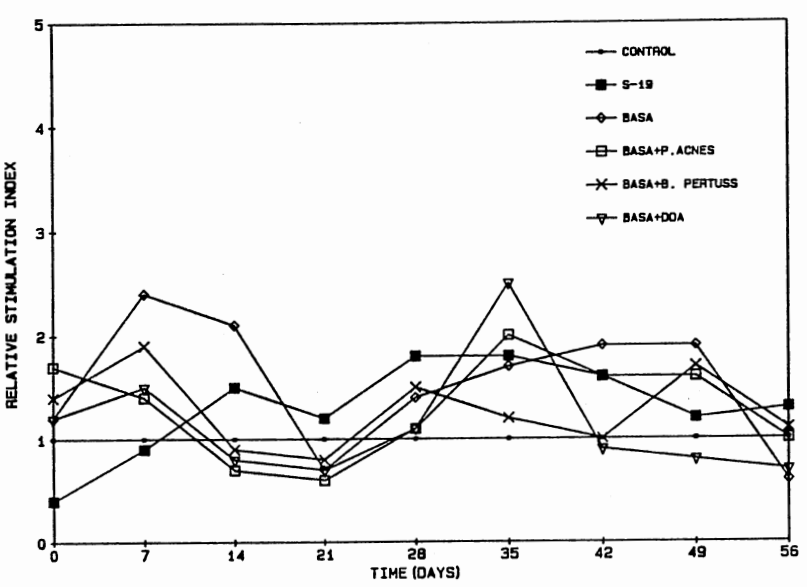


Figure 4. Mean relative stimulation indices to BASA-induced IL-1 production in culture supernatant fluid on day 14. The vaccine and adjuvant preparations administered to each group of animals are shown in table 1.



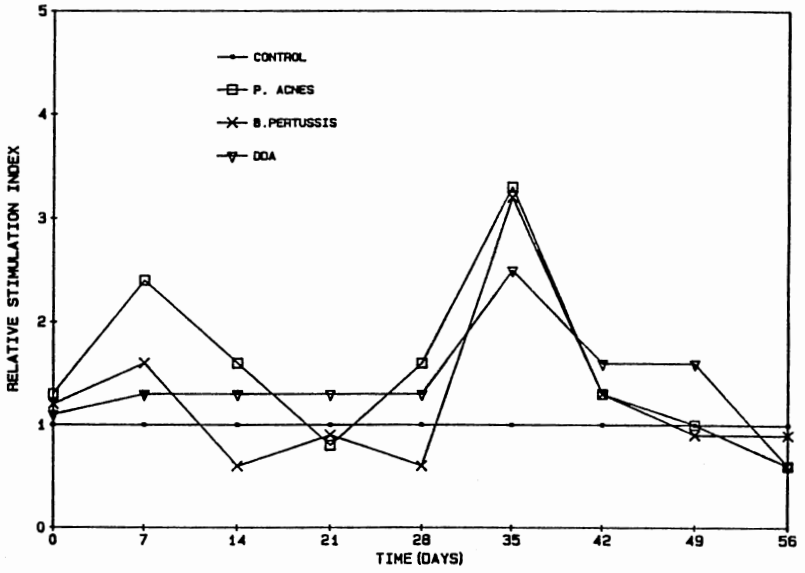


Figure 5A, B. Mean relative stimulation indices to Con A-induced IL-2 production in culture supernatant fluid. The vaccine and adjuvant preparations administered to each group of animals are shown in table 1.

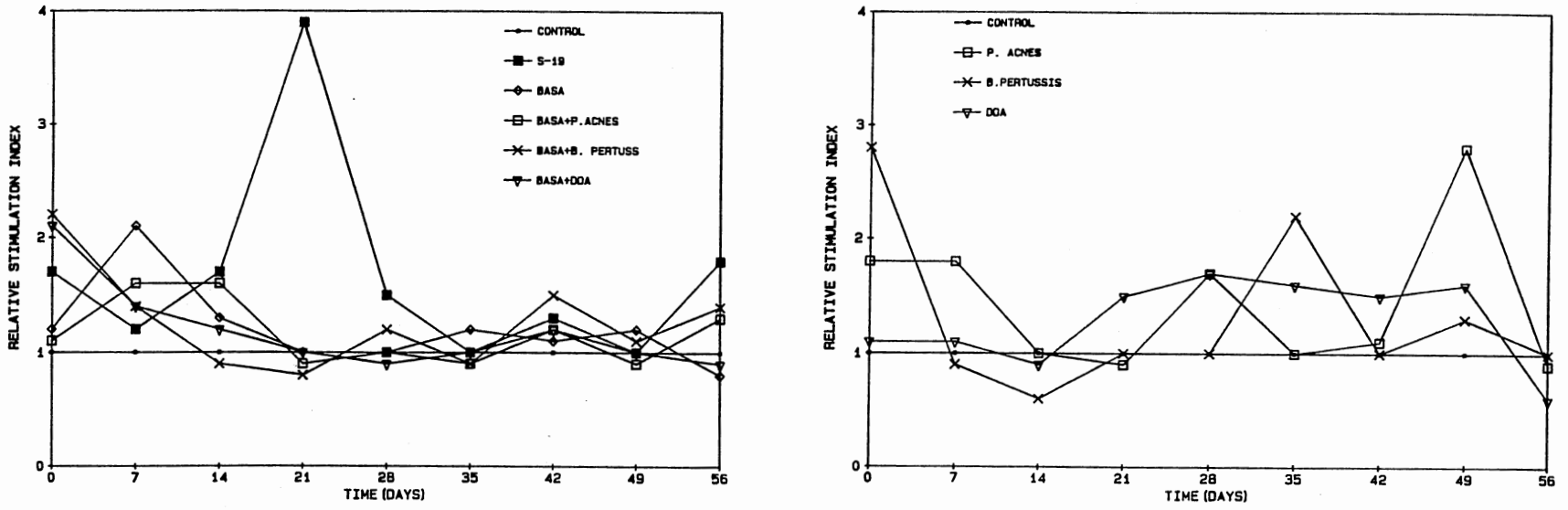
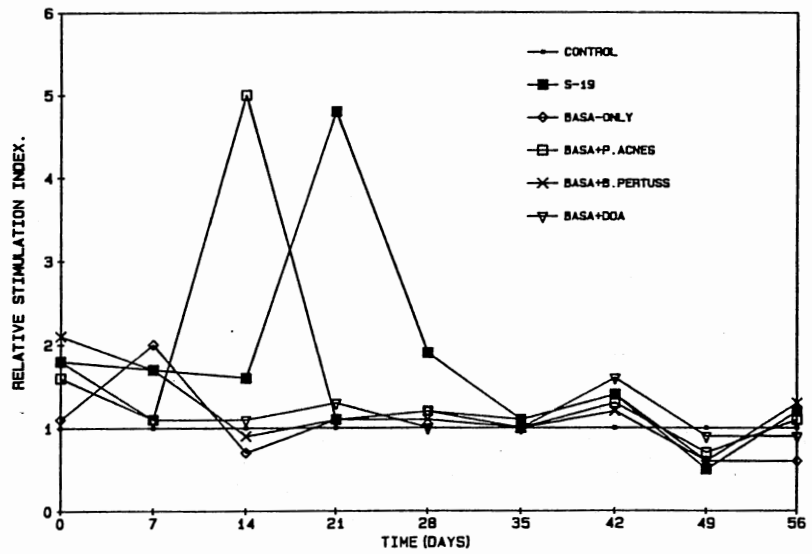


Figure 6A, B. Mean relative stimulation indices to heat killed B. abortus-induced IL-2 production in culture supernatant fluid. The vaccine and adjuvant preparations administered to each group of animals are shown in table 1.



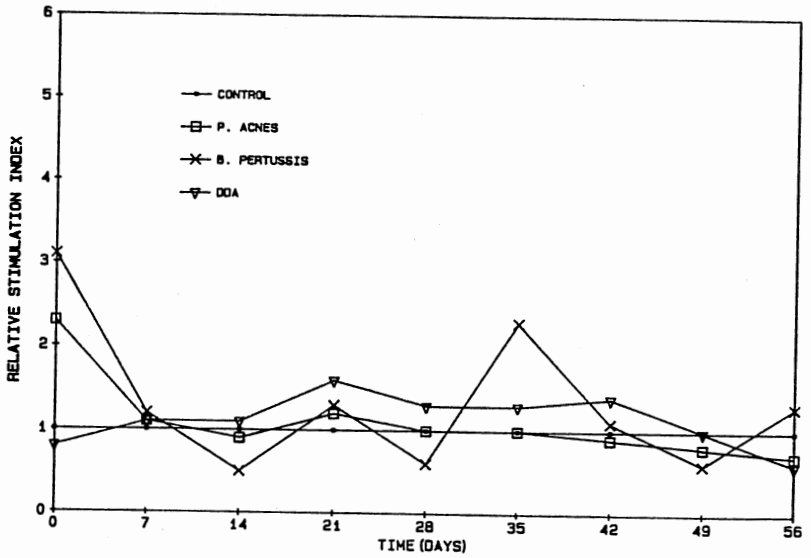


Figure 7A, B. Mean relative stimulation indices to gamma-irradiated B. abortus-induced IL-2 production in culture supernatant fluid. The vaccine and adjuvant preparations administered to each group of animals are shown in table 1.

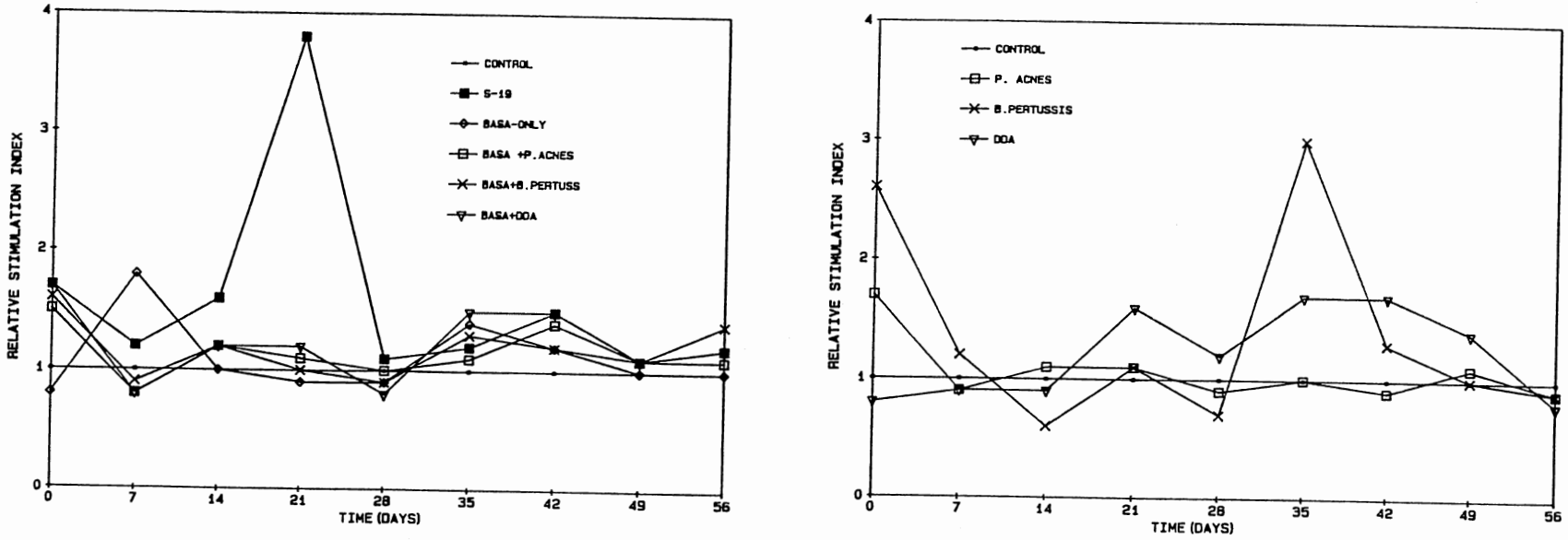
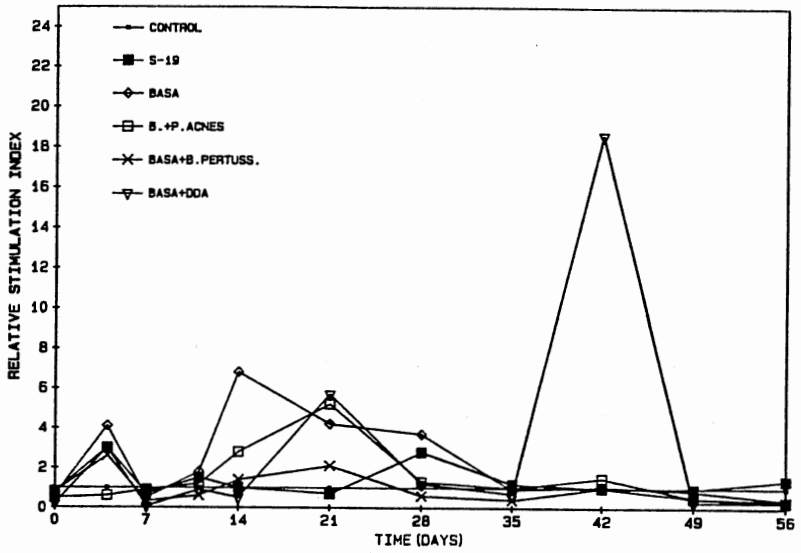


Figure 8A, B. Mean relative stimulation indices to BASA-induced IL-2 production in culture supernatant fluid. The vaccine and adjuvant preparations administered to each group of animals are shown in table 1.



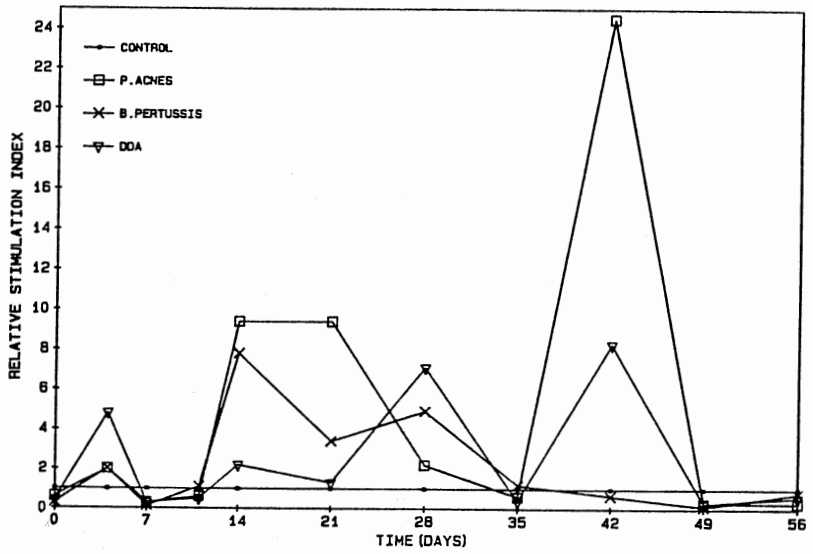
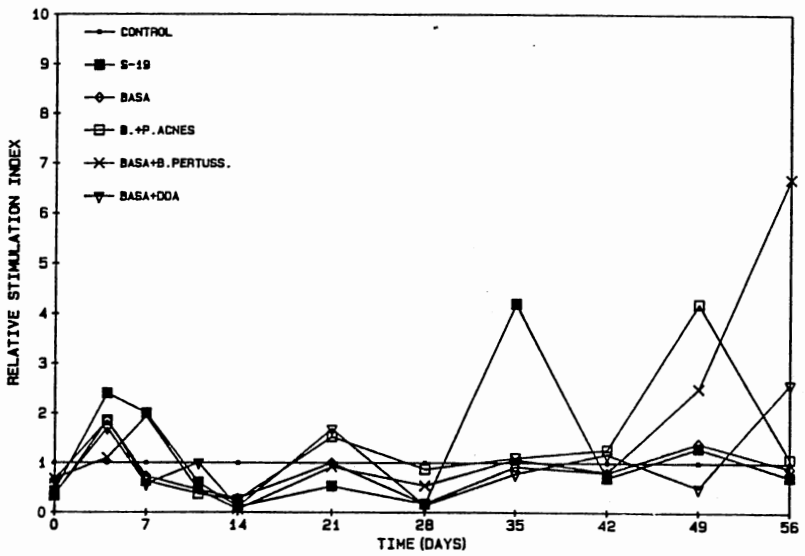


Figure 9A, B. Suppressive effects of culture fluids from pokeweed mitogen (4 ug/ml) stimulated lymphocytes on proliferation of normal PBMC.



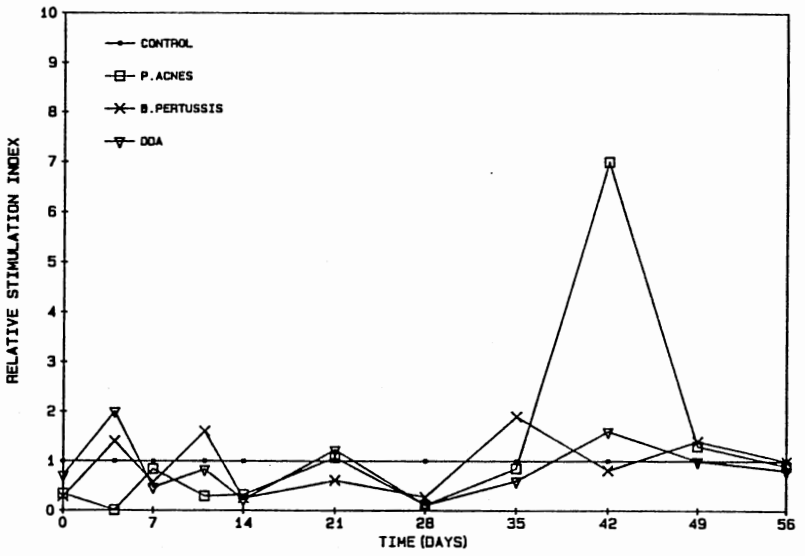


Figure 10A, B. Suppressive effects of culture fluids from heat-killed B. abortus (20 ug/ml) stimulated lymphocytes on proliferation of normal PBMC.

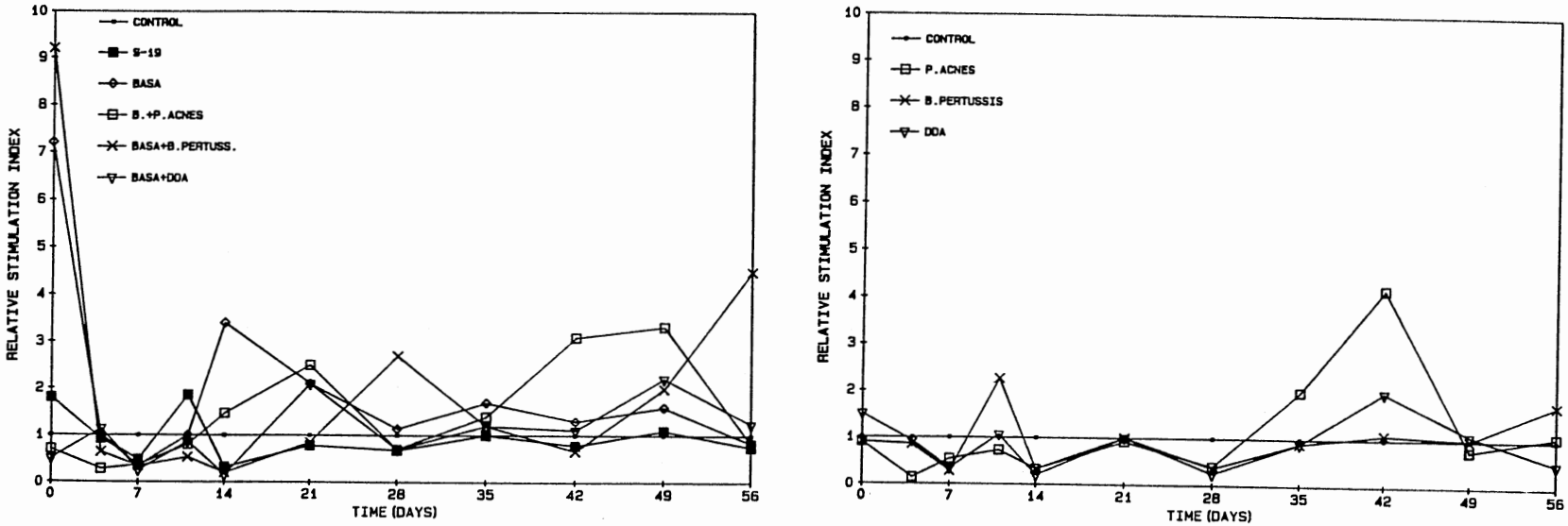


Figure 11A, B. Suppressive effects of culture fluids from gamma-irradiated B. abortus (20 ug/ml) stimulated lymphocytes on proliferation of normal PBMC.

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CHAPTER IV

IMMUNOPOTENTIATION OF CATTLE VACCINATED WITH A SOLUBLE *BRUCELLA ABORTUS* ANTIGEN WITH LOW LPS CONTENT: AN ANALYSIS OF CELLULAR AND HUMORAL IMMUNE RESPONSES

Introduction

Bovine brucellosis caused by *Brucella abortus* is an economically important disease associated with abortion and infertility resulting in substantial economic loss to cattle producers. Despite numerous methods of eradication, including vaccination, and test and slaughter, the disease has remained prevalent in many areas of the world (Sutherland, 1980; Matyas and Fujikura, 1984).

Vaccination of cattle with attenuated *B. abortus* strain 19 (S19) provides protection from abortion. However, S19 vaccine can cause some cattle to become seropositive, induce persistent infection and interfere with the interpretation of routine serologic diagnostic tests (Morgan, 1977; Sutherland et al., 1982). The problems currently associated with S19 vaccine have generated interest in the development of a subunit *B. abortus* vaccine which contains antigens essential only for stimulation of immunity.

Generally, non-living (subunit) vaccines can be weakly immunogenic and require adjuvants to enhance their immunogenicity.

Several researchers have examined the efficacy of vaccines to protect laboratory animals and cattle against experimental brucellosis (Woodard and Jasman, 1983; Winter et al., 1983; Montaraz and Winter, 1986; Confer et al., 1987; Winter et al., 1988).

We have previously reported that DDA stimulates increased CMI and low humoral immune responses in cattle when administered with BASA (Chapter II and III). The purposes of these studies reported herein were to attempt to enhance immune responses in cattle to a non-living B. abortus antigen preparation by a combination of different adjuvants and to evaluate the immunoenhancing effects of these adjuvants through analysis of serum antibody and cell-mediated immune (CMI) responses. Specific aspects of induced CMI responses investigated were macrophage activation (IL-1 production), T-helper cell (IL-2 production), and suppressor cell activity.

Materials and Methods

Cattle

Thirty-five weanling crossbred beef steers approximately 250 to 300 kg each were used. These steers were obtained from herds that had no clinical signs of brucellosis and were serologically negative for B. abortus. They were transported to the Livestock Health Research Center in Hugo, Oklahoma, maintained on a bermuda grass pasture and fed a protein supplement.

Antigen for Vaccines

The vaccine antigen (GBASA) was a soluble extract of gamma irradiated B. abortus 19 (GBA) and referred to as GBASA. GBASA was

used because it appeared to contain fewer denatured protein antigens than BASA (J.H. Wyckoff, unpublished data). Briefly, 1 gm of sterile, lyophilized GBA was rehydrated with 10 ml of distilled water and further diluted with 10 ml PBS (0.01M PO_4, 0.15M NaCl). 5 ml portions of the bacterial suspension was run through a French pressure cell for 10 minutes at 20,000 PSI. Supernatant aliquots were pooled and ultra centrifuged (30,000 g, 1 hr.) at 4° C. Proteins were precipitated from the supernatant with 70% saturated ammonium sulfate stirred overnight at 4° C and then washed with 70% saturated ammonium sulfate with centrifugation (30,000 g, 1 hr.) at 4° C (Sorval RC5C, Dupont). The precipitate was solubilized in PBS and dialyzed against PBS (5 changes of PBS) with a 2000 kDa exclusion dialysis membrane. GBASA contained (2.78 mg/ml of protein) (Bradford, Bio-Rad) and 0.147 ug of 2-keto-3 deoxyoctulosonic acid (KDO) per mg of protein (Warren, 1956).

Lymphoproliferation (LP) Assay

The LP assay, using peripheral blood mononuclear cells (PBMC) separated on Ficoll-hypaque, was essentially the same as previously described (Chapter II). Antigens used were heat-killed B. abortus (HKA, 2 ug/well) and GBA (2 ug/well). Pokeweed mitogen (PWM) was used as a positive stimulation control.

Serologic Tests

The standard serologic tests used were the brucellosis card test (CARD), rivanol precipitation-plate agglutination test (RIV) and a microtiter complement fixation test (CF) (Alton, et al, 1975).

A quantitative fluorometric immunoassay [Fluorescent Immunoassay

System (FIAX) Whittaker M.A. Biologics] was performed as previously described (Hall et al., 1984).

The enzyme-linked immunosorbent assay (ELISA) was performed as previously described (Confer et al., 1985) except that the antigen was GBASA (6 ug/ml).

Delayed-type Hypersensitivity

As an in vivo correlate of CMI, the delayed-type hypersensitivity (DTH) to GBASA was performed 35 days after vaccination, as previously described (Chapter II) except that increases in skin fold thickness were measured 24 hours after the initial injection of antigen or saline. An increase in double skin fold thickness of 2 mm or greater than the initial measurement prior to injection of antigen was considered as a positive reaction (Nicolleti, 1983).

Assays for Interleukin-1 (IL-1), Interleukin-2 (IL-2), and Other Immunomodulatory Factors

Collection of culture supernatant fluids and assays for IL-1 and IL-2, and immunomodulatory factors were performed as previously described (Chapter III). For the suppressor factor assay, a suboptimal dose of Con-A at 1 ug/ml was used as determined from a dose-response curve.

Adjuvants

The adjuvants used were trehalose dimycolate (TDM, Ribi Immunochem Research, Inc., Hamilton, Montana), muramyl dipeptide (MDP, Sigma Chemical Co., St. Louis, MO), and dimethyl dioctadecyl ammonium

bromide (DDA) (Prolong, kindly provided by Diamond Scientific Co., Des Moines, IA).

Experimental Design

Two mg of GBASA (2 mg protein/ml) was mixed with an equal volume of the relevant adjuvant or sterile PBS. A single vaccine was injected subcutaneously in the cervical region anterior to the scapula on day 0. The 35 steers were randomly allotted into nine groups (Table 1). Group 1 (control, n=5) was not vaccinated. Group 2 (n=4) was vaccinated with the standard reduced dose of S19, 1 x 10^9 colony forming units (CFU). Group 3 (n=5) received GBASA only (1 ml per dose). Group 4 (n=5) received GBASA and DDA. Group 5 (n=5) received GBASA + DDA + MDP (2 mg/dose). Group 6 (n=5) received GBASA + DDA + TDM (2 mg/dose). Group 7 (n=2) received DDA + MDP (2 mg/dose). Groups 9 (n=2) received DDA + TDM (2 mg/dose). Jugular blood samples were collected from all steers at the time of vaccination (day 0) and sequentially thereafter for serological assays (days 7, 14, 21, 28, 35, 42, 49, 56), LP and suppressor factor (days 3, 7, 10, 14, 21, 28, 35, 42, 49, 56) and for interleukin 1 and 2 assays (days 7, 14, 28, 56).

Data for the LP assay, IL-1, IL-2, and suppressor factor production are shown as relative stimulation indices (RSI) and are expressed relative to the responses in the control steers. RSI = stimulation index of experimental group/stimulation index of control group. For the suppressor factor assay RSI values below group 1 values indicate suppression.

Statistical Analysis

All data, except DTH responses, were compared among groups using the least significant difference (LSD) method (SAS, 1982). The means for DTH responses were analyzed by Students t-tests (Bailey, 1981). Statistically significant differences were considered where $P < 0.05$.

Results

Serology

The number of seropositive steers as determined by the CARD test reached a maximum on day 14 and declined thereafter except for S19 vaccinates (Table 1). The mean antibody responses measured by all four serological tests are shown in Figs. 1-4. Unvaccinated controls and steers receiving DDA, DDA + MDP, or DDA + TDM remained seronegative by all four serological tests throughout the experiment. In general, the highest mean titers for the GBASA vaccinated groups as measured by all serologic assays was on day 14 (Figs. 1-4). By all four serological tests, S19-vaccinated steers consistently had significantly higher titers ($P < 0.05$) than all treatment groups between days 14 and 56. As measured by the FIAX and ELISA, GBASA + DDA, GBASA + DDA + MDP, and GBASA + DDA + TDM vaccinates had significantly elevated ($P < 0.05$) antibody titers over control steers on days 21 and 28. There were no significant differences in antibody titers among GBASA + DDA, GBASA + DDA + MDP, and GBASA + DDA + TDM vaccinates. As measured by the RIV, antibody titers for GBASA + DDA and GBASA + DDA + TDM vaccinates were significantly higher ($P < 0.05$) than for steers vaccinated with GBASA alone on days 14 and 21. On day

14 as measured respectively by the ELISA and FIAX, antibody titers for GBASA + DDA + TDM vaccinates and GBASA + DDA + MDP vaccinates were significantly higher ($P < 0.05$) than for steers receiving GBASA alone.

Lymphoproliferation assay (LP)

The LP responses to HKA and GBA were highly variable with no consistent pattern in any of the groups (Figs. 5 and 6). LP responses to HKA in all groups were greater than the control responses on days 0, 7, 21, and 35. With the exception of DDA + MDP vaccinates, LP responses to HKA for GBASA + DDA + TDM vaccinates were significantly elevated ($P < 0.05$) over all treatment groups on day 35. Overall, LP responses to HKA and GBA were most pronounced in GBASA + DDA + TDM vaccinates. With the exception of days 28 and 42, LP responses to GBA for all treatment groups were generally greater than the control responses. With the exception of GBASA and GBASA + DDA vaccinates, LP responses to GBA gradually increased above the control responses after day 42 (Fig. 6). LP responses to GBA for steers receiving DDA alone were significantly different ($P < 0.05$) from the control responses on day 35.

Delayed Type Hypersensitivity

On day 35 two steers in S19 and GBASA + DDA + TDM, and 1 steer in GBASA, GBASA + DDA, and GBASA + DDA + MDP vaccinated steers had a positive reaction to GBASA by the DTH test. Also the mean DTH responses for S19, GBASA, GBASA + DDA + MDP and GBASA + DDA + TDM vaccinates were significantly higher ($P < 0.05$) than the control

(group 1) responses. However, the responses for S19, GBASA + DDA + MDP, and GBASA + DDA + TDM vaccinates were not significantly different from responses for steers receiving GBASA alone. Overall, steers receiving S19 had the highest mean DTH response (Fig. 7).

IL-1 and IL-2 Production

The IL-1 levels in culture supernatants for all groups were insignificantly elevated over the control levels (group 1) on day 7 (Figs. 8-10). This was followed by a decline and insignificant sporadic increases in group means. On day 7, S19 appeared to provide the greatest stimulus for IL-1 production. IL-1 levels for the S19 group were consistently lower than group 1 levels on day 14 followed by a gradual increase in IL-1 activity. There was a marked variability in IL-1 activity in culture supernatants from steers receiving adjuvants alone. However, IL-1 activity appeared most pronounced in culture supernatant from DDA + TDM vaccinates. Con A-induced IL-1 production for S19, GBASA + DDA, and GBASA + DDA + TDM was significantly higher ($P < 0.05$) than IL-1 production in group 1 steers in day 56. Overall, IL-1 production was greatest in S19 and GBASA + DDA vaccinates.

Con A-, HKA-, and GBA-induced IL-2 production was highly variable with no consistent pattern in any of the groups. There was an initial rise in IL-2 levels over the unvaccinated controls (group 1) on day 0, which generally declined below control levels on days 7 and 14, and thereafter gradually increased above the control levels to day 56 (Figs. 11-13). As measured by relative stimulation indexes, Con A-induced IL-2 activity peaked on day 28. At that time, IL-2 activity

in groups 2-7 steers was significantly greater ($P < 0.05$) than the IL-2 activity for controls (group 1). HKA- and GBA-stimulated cultures induced minimal levels of IL-2 activity in that the RSI for all treatment groups were substantially below day 0 values (Figs. 12 and 13). However, there was a gradual non-significant rise in IL-2 levels above the control levels after day 14. Overall, the greatest IL-2 levels were observed in S19 and GBASA + DDA + TDM vaccinates.

Production of Immunomodulatory Factor(s)

Culture supernatant fluid from HKA- and GBA-stimulated lymphocytes did not appear to inhibit Con A-induced blastogenesis of normal PBMC as the mean RSI for all groups were generally greater than the mean RSI of the controls (Figs. 22-25). The mean proliferative responses for groups 2-6 to HKA-stimulated supernatant fluid were significantly greater ($P < 0.05$) than the control responses on day 3. On day 21, the mean RSI for proliferative responses in GBA-generated culture supernatant for group 2 was significantly higher ($P < 0.05$) than the control responses. Stimulation of PBMC from group 6 steers with GBA induced suppressive supernatants on days 3 and 56.

Discussion

The development of a subunit vaccine for bovine brucellosis for replacement of S19 is an important research objective in several laboratories worldwide. The success of a subunit B. abortus vaccine will depend on the ability of the vaccine components to stimulate an effective host immune response. Several investigators have examined

the immunogenicity of surface proteins of B. abortus (Winter et al., 1983; Confer et al., 1987; Winter et al., 1988; Winter and Rowe, 1988). However, the composition of a potential subunit vaccine has yet to be determined (Winter et al, 1983).

In the present study, we evaluated the serum antibody and CMI responses to GBASA given in combination with various adjuvants. A single administration of antigen was used to study the primary response in comparison to the single inoculation with S19 as a standard. Adjuvants were selected which enhance cell-mediated as well as humoral immune responses (Winter et al., 1983; Snippe et al., 1982) and the combination of TDM and MDP (Woodard, 1981; Winter et al., 1983) induced cell-mediated immune responses to B. abortus antigens in cattle. Previously, we demonstrated that DDA + BASA stimulates CMI responses in cattle. We, therefore attempted to further enhance this response by the addition of MDP or TDM (known potentiators of CMI responses) to DDA.

GBASA contains S-LPS (but a reduced content compared to BASA) as evidenced by the development of positive standard serological tests. The highest and most sustained serum antibody responses to B. abortus were elicited by S19 vaccine. Steers receiving GBASA adjuvanted vaccines had antibody responses of a magnitude intermediate between S19-vaccinated and control steers. Humoral responses were lowest when no adjuvant was included with the antigen. A combination of DDA + TDM with GBASA induced higher serum antibody titers than DDA + GBASA alone. However, titers declined markedly by day 28, therefore suggesting that a mere transient response to LPS can be induced by a subunit of B. abortus in combination with adjuvants.

The CMI response as determined by the LP and DTH was highest among GBASA + DDA + TDM and S19 vaccinates. This indicates that the addition of TDM to DDA enhanced classical CMI responses. A rise in LP responses to PWM and GBA was observed in all groups after the DTH test, suggesting an anamnestic CMI response to the previous antigenic stimulation.

Cells from S19 vaccinated steers had the greatest IL-1 production. For groups 4, 5, and 6, IL-1 production was most evident in the GBASA + DDA group. IL-2 production was most evident in S19 and GBASA + DDA + TDM vaccinated steers similar to that of the LP and DTH responses. Lymphocyte cultures from group 6 steers stimulated with GBA induced suppressive supernates on days 3 and 56.

In the present study, DDA and a combination of DDA + TDM proved to be most effective with respect to enhancing CMI responses to GBASA, i.e. increased IL-1 production (GBASA + DDA) and increased LP and DTH responses and IL-2 production (GBASA + DDA + TDM). Within groups, there were marked variations in LP responses to HKA and GBA. Other researchers have (Rossi et al., 1978; Jones, 1981) demonstrated inconsistency in lymphocyte reactivity to B. abortus antigen preparations. IL-1 production in HKA and GBA-stimulated cultures were generally low even in S19 vaccinates compared to the results obtained in the previous study (Chapter III). Recently, Splitter and Everlith (1989) demonstrated that in macrophages B. abortus induces the production of both membrane IL-1 and secreted IL-1. It is therefore possible that cells used in the lymphocyte cultures preferentially express membrane IL-1 rather than secreted IL-1, the latter which was measured by our assay system. HKA- and GBA-induced IL-2 production

were substantially below day 0 values. The reason for this is not known. Perhaps, the presence of an inhibitory factor in culture supernatants could have blocked IL-2 activity in the culture fluids (Lafferty et al., 1980). Generally, soluble suppressor factor(s) was not detected in lymphocyte cultures from cattle immunized with GBASA adjuvanted vaccines. This could be due to the low content of LPS in GBASA; LPS has been shown to down regulate lymphokine production and T-cell responses (Steeg et al., 1982).

DDA enhances macrophage function and T-lymphocyte functions and reduces suppressor T-cell function (Gordon et al., 1980; Kraaijeveld et al., 1982; Prager, 1985). The stimulatory effect of DDA on macrophages may be particularly important because B. abortus is an intracellular pathogen and macrophages are believed to be important in host defense in bovine brucellosis (Splitter and Everlith, 1986). TDM increased the immunogenic potency of non-viable vaccines against a variety of killed microorganisms such as B. abortus (Woodard et al., 1980). In vitro, TDM can stimulate proliferation of T-cells (Kierszenbaum et al., 1981). The observed effects of many adjuvants are secondary to their action on macrophages as a target and mediator cell (Unanue et al., 1969; Allison, 1979; Osebold, 1982). Many agents exert their adjuvant effects by inducing the release of IL-1 from macrophages (Genounou et al., 1985; Staruch and Wood, 1983; Oppenheim et al., 1980). Adjuvants also stimulate the proliferation and differentiation of immunocompetent cells. IL-1 released from macrophages, enhances the release of IL-2 from T-cells; IL-2 would in turn enhance the proliferation of those T-helper cells that recognize bacterial antigens (Dinarello, 1984).

These studies demonstrated that a combination of DDA + TDM potentiates CMI responses to a nonliving, soluble B. abortus antigen preparation and may be useful as adjuvants for future vaccines, particularly subunit vaccines.

TABLE 1. Numbers of serologically positive cattle as detected by the *Brucella abortus* CARD test.

Group	(n)	Vaccine	Days after vaccination (No. seropositive)								
			0	7	14	21	28	35	42	49	56
1	(5)	None (control)	0	0	0	0	0	0	0	0	0
2	(4)	S19	0	4	4	4	4	4	4	4	4
3	(5)	GBASA	0	3	3	0	0	0	0	0	0
4	(5)	GBASA + DDA	0	1	4	4	2	3	1	1	1
5	(5)	GBASA + DDA + MDP	0	2	4	4	2	4	1	1	1
6	(5)	GBASA + DDA + TDM	0	4	5	5	4	5	0	1	0
7	(2)	DDA only	0	0	0	0	0	0	0	0	0
8	(2)	DDA + MDP (only)	0	0	0	0	0	0	0	0	0
9	(2)	DDA + TDM (only)	0	0	0	0	0	0	0	0	0

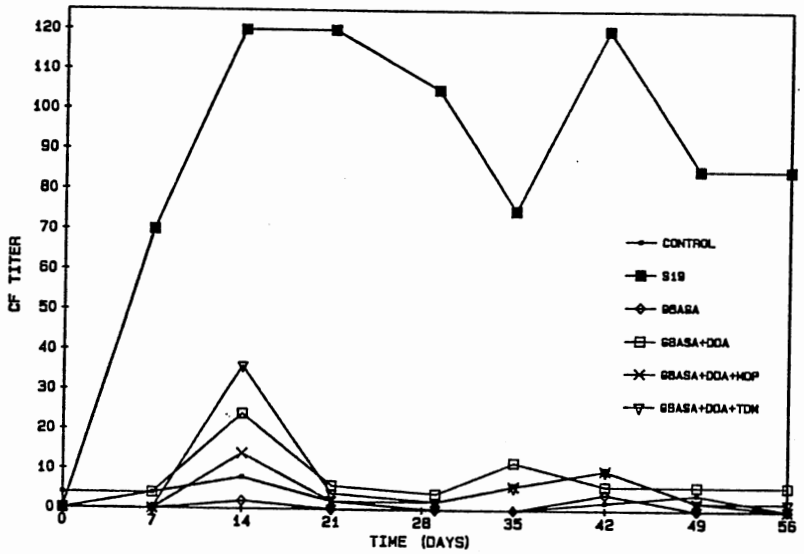


Figure 1. Mean antibody responses to B. abortus as measured by complement fixation (CF). Antibody responses for steers receiving DDA, DDA + MDP and DDA + TDM alone were similar to responses for steers in the control group.

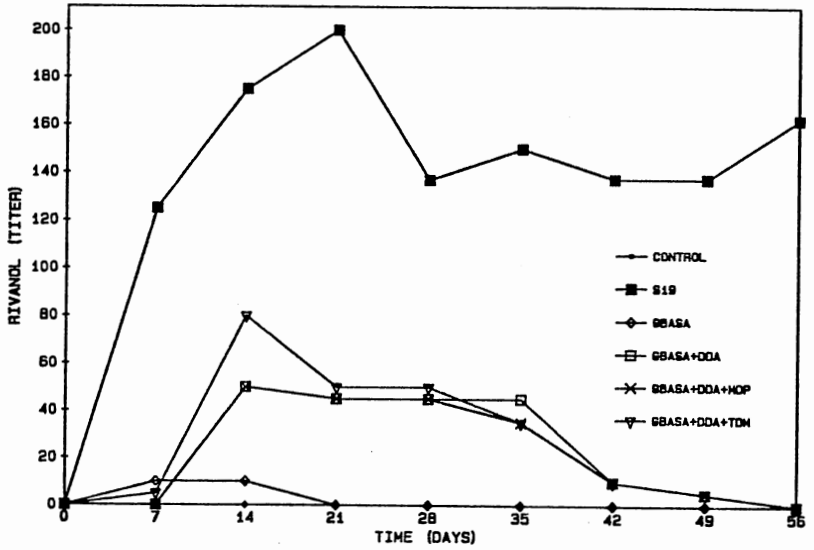


Figure 2. Mean antibody responses to B. abortus as measured by Rivanol agglutination (RIV). Antibody responses for steers receiving DDA, DDA + MDP and DDA + TDM alone were similar to responses for steers in the control group.

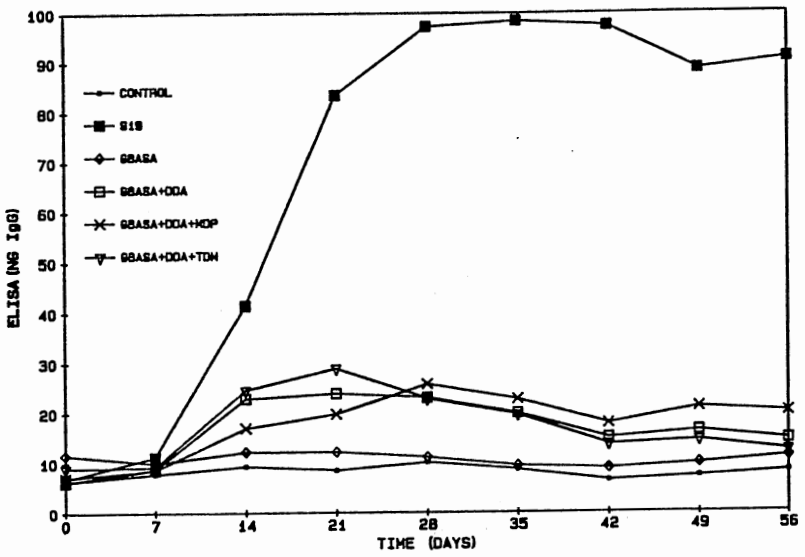


Figure 3. Mean antibody responses to B. abortus as measured by an enzyme-linked immunosorbent assay (ELISA). Antibody responses for steers receiving DDA, DDA + MDP and DDA + TDM alone were similar to responses for steers in the control group.

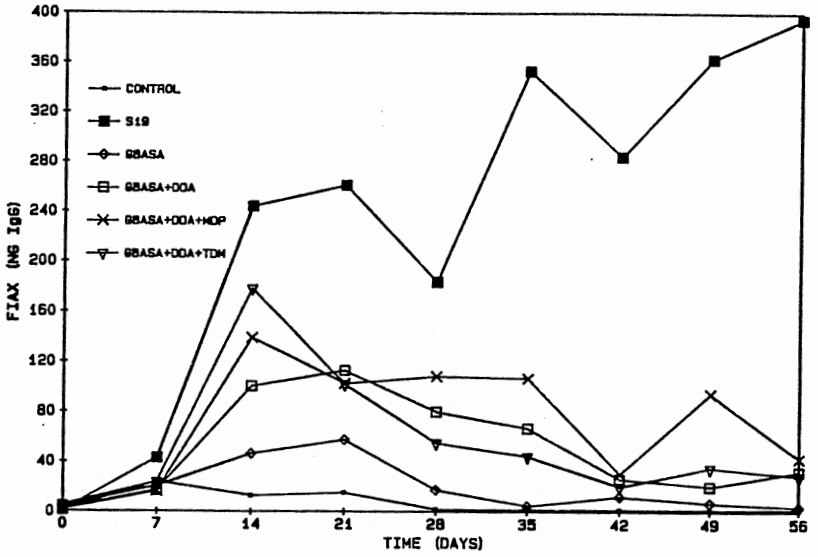


Figure 4. Mean antibody responses to B. abortus as measured by a fluorometric immunoassay (FIAX). Antibody responses for steers receiving DDA, DDA + MDP and DDA + TDM alone were similar to responses for steers in the control group.

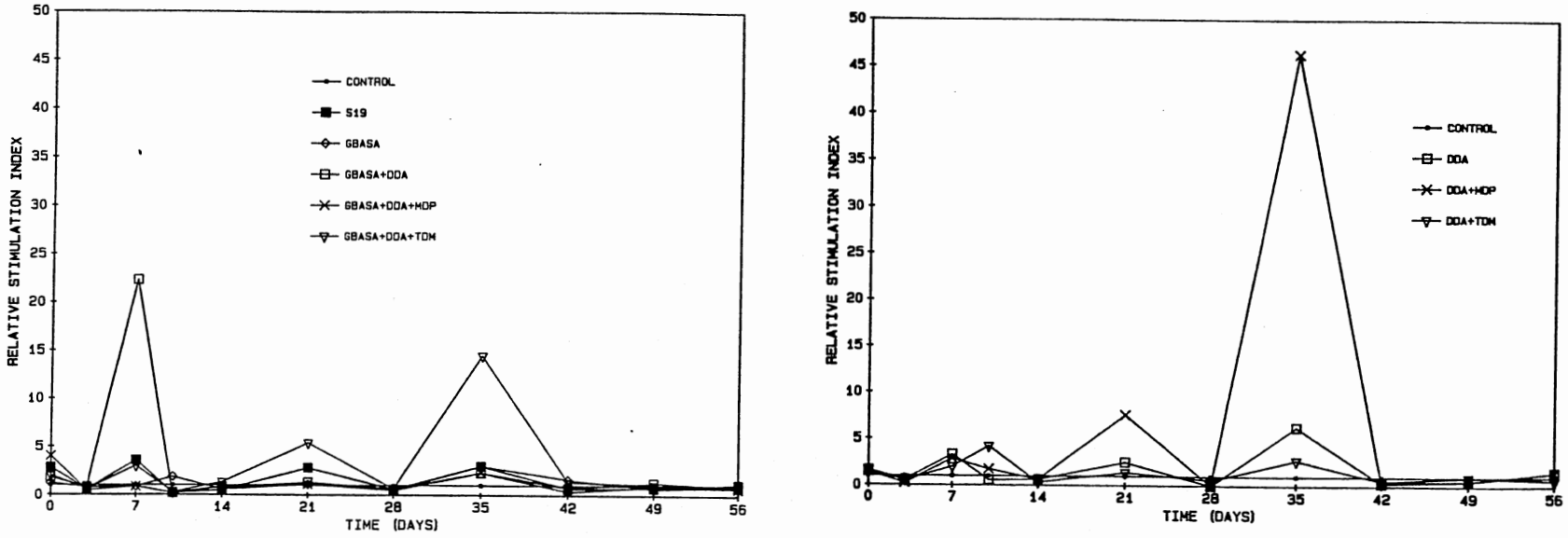
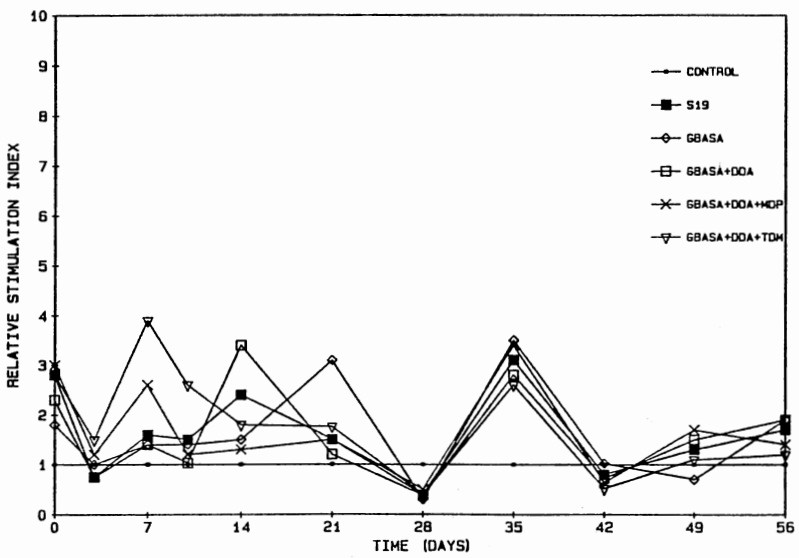


Figure 5A, B. Mean relative stimulation indices to heat-killed B. abortus as determined by LP assay.



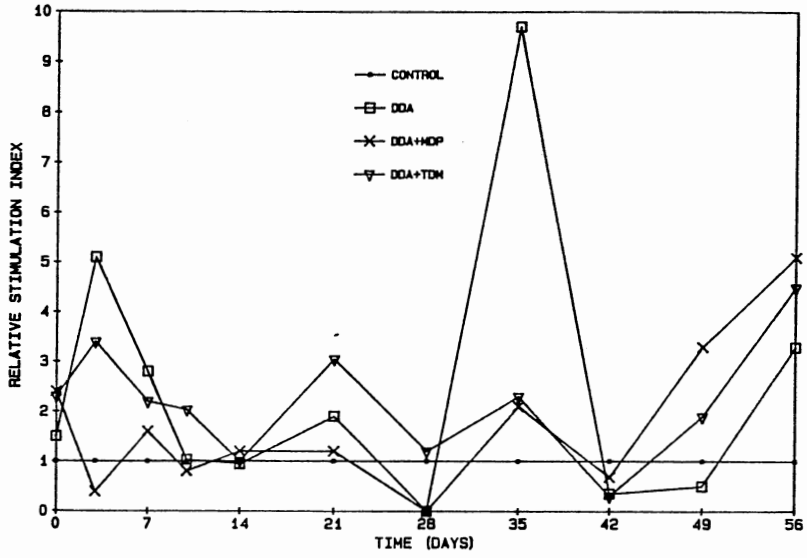


Figure 6A, B. Mean relative stimulation indices to gamma-irradiated B. abortus as determined by LP assay.

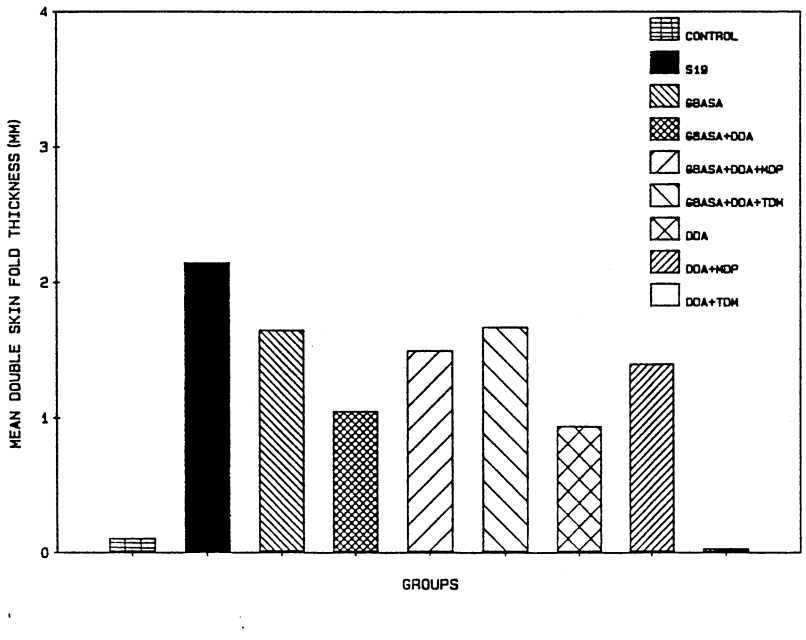


Figure 7. Mean delayed-type hypersensitivity (DTH) responses to GBASA.

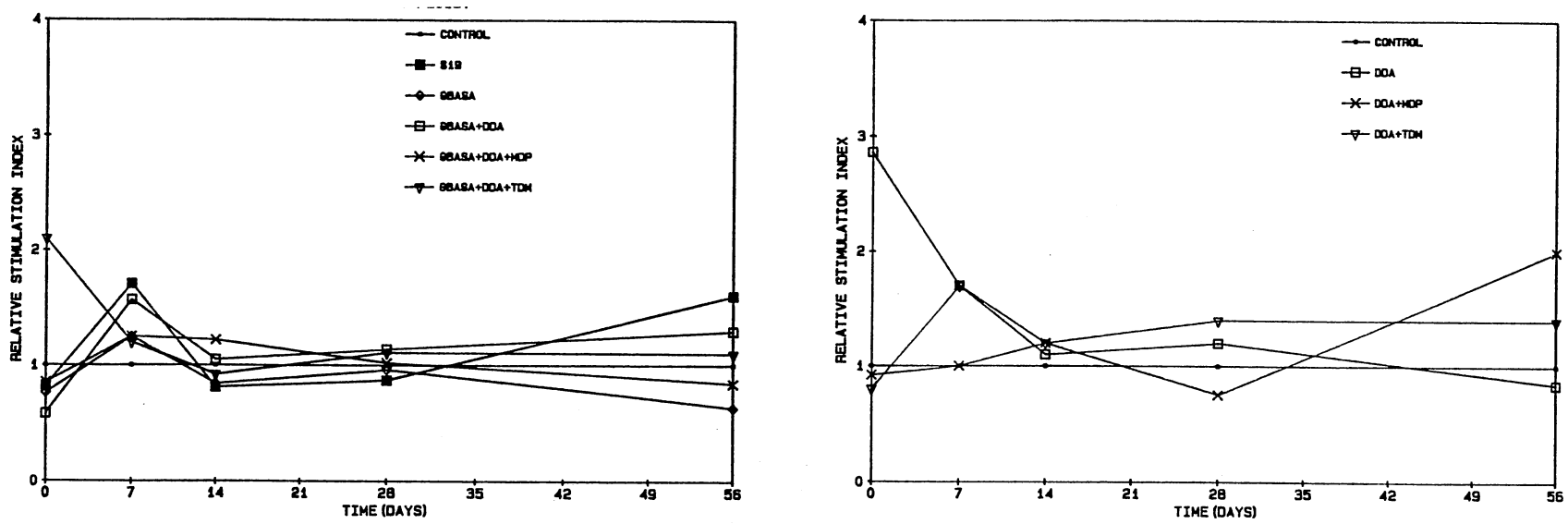


Figure 8A, B. Mean relative stimulation indices to Con A-induced IL-1 production in culture supernatant fluid. The vaccine and adjuvant preparations administered to each group of animals as shown in table 1.

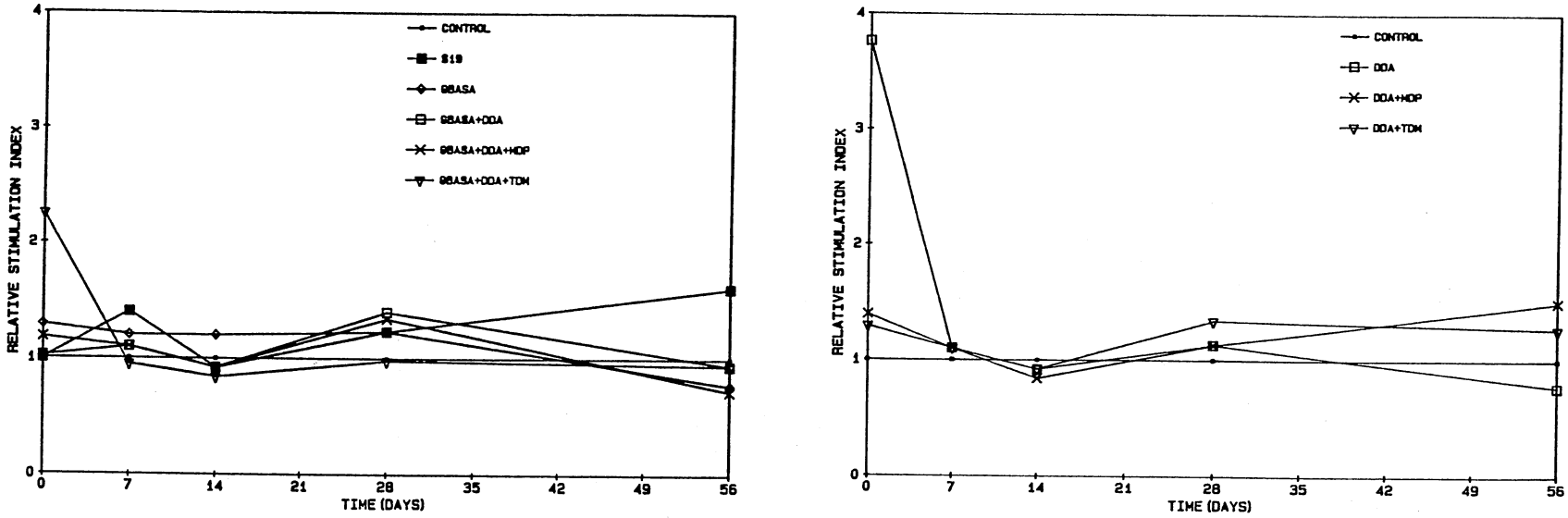
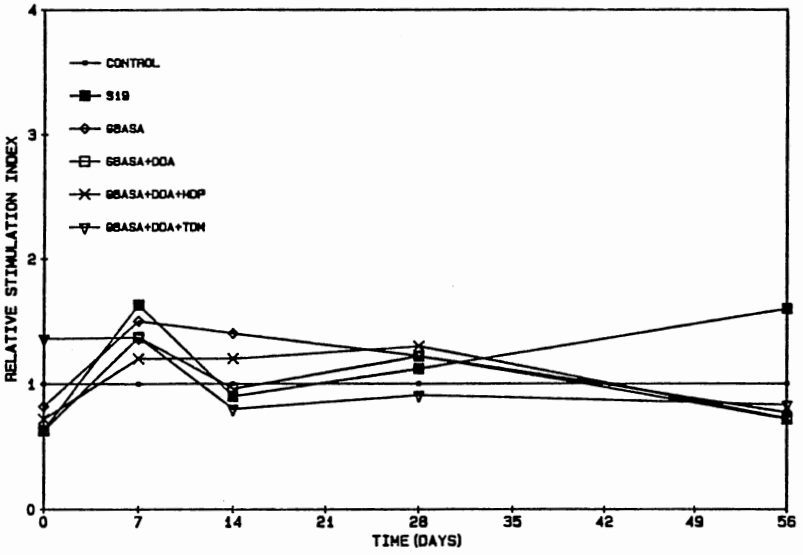


Figure 9A, B. Mean relative stimulation indices to heat-killed B. abortus-induced IL-1 production in culture supernatant fluid. The vaccine and adjuvant preparations administered to each group of animals are shown in table 1.



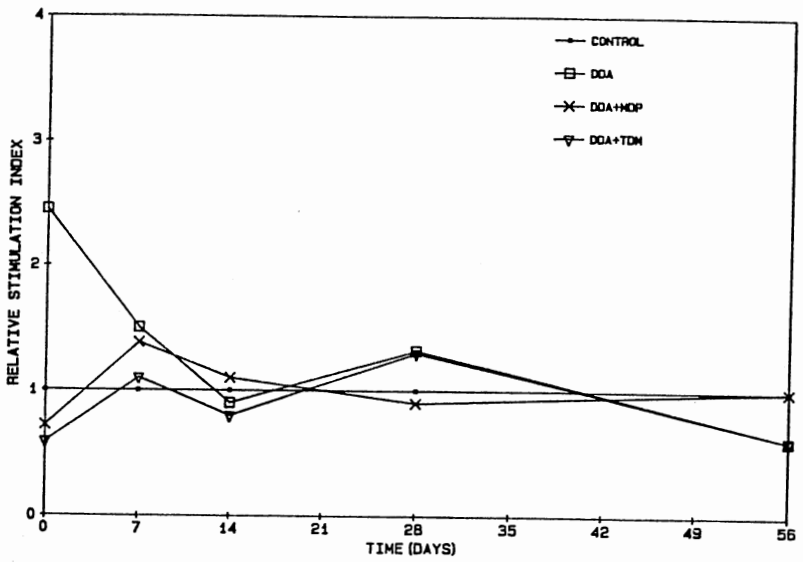
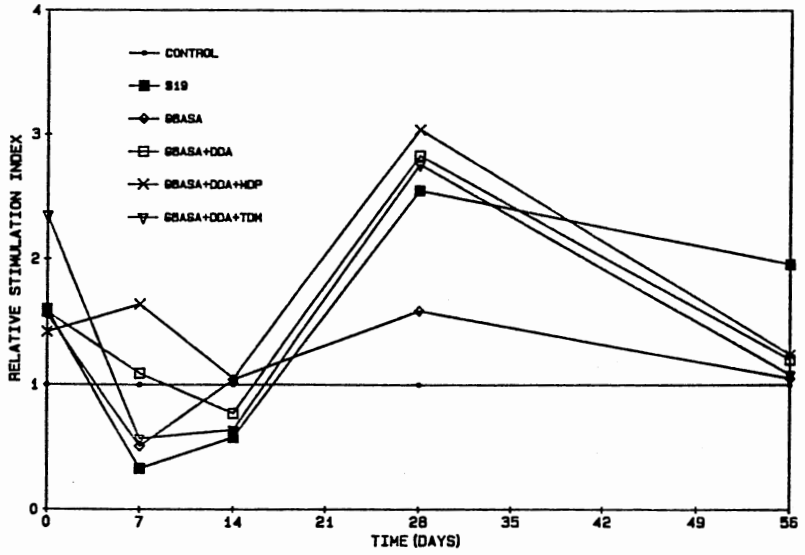


Figure 10A, B. Mean relative stimulation indices to gamma-irradiated B. abortus-induced IL-1 production in culture supernatant fluid. The vaccine and adjuvant preparations administered to each group of animals are shown in table 1.



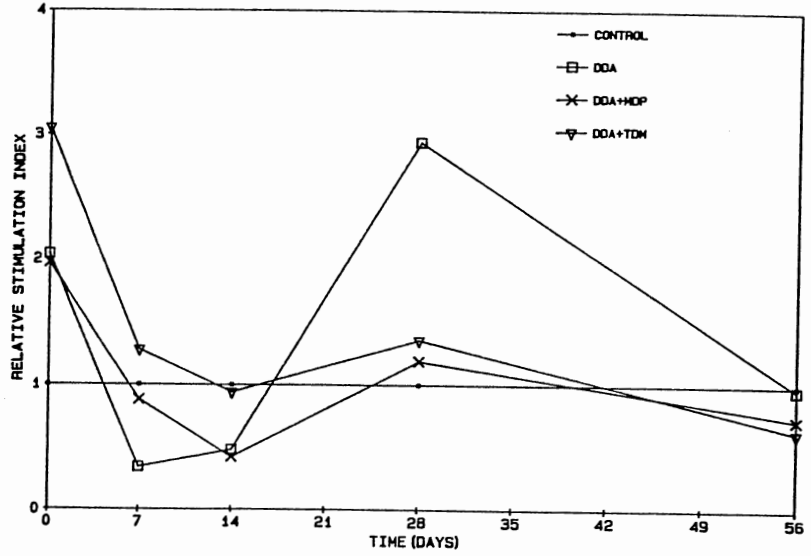


Figure 11A, B. Mean relative stimulation indices to Con A-induced IL-2 production in culture supernatant fluid. The vaccine and adjuvant preparations administered to each group of animals are shown in table 1.

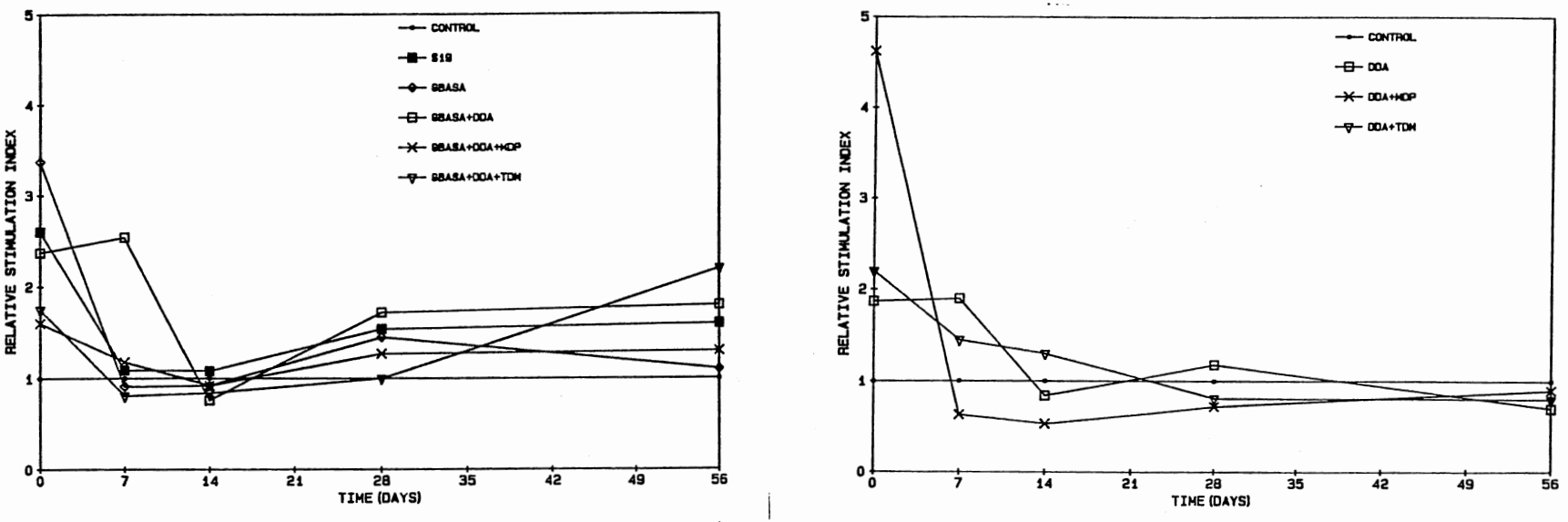


Figure 12A, B. Mean relative stimulation indices to heat killed B. abortus-induced IL-2 production in culture supernatant fluid. The vaccine and adjuvant preparations administered to each group of animals are shown in table 1.

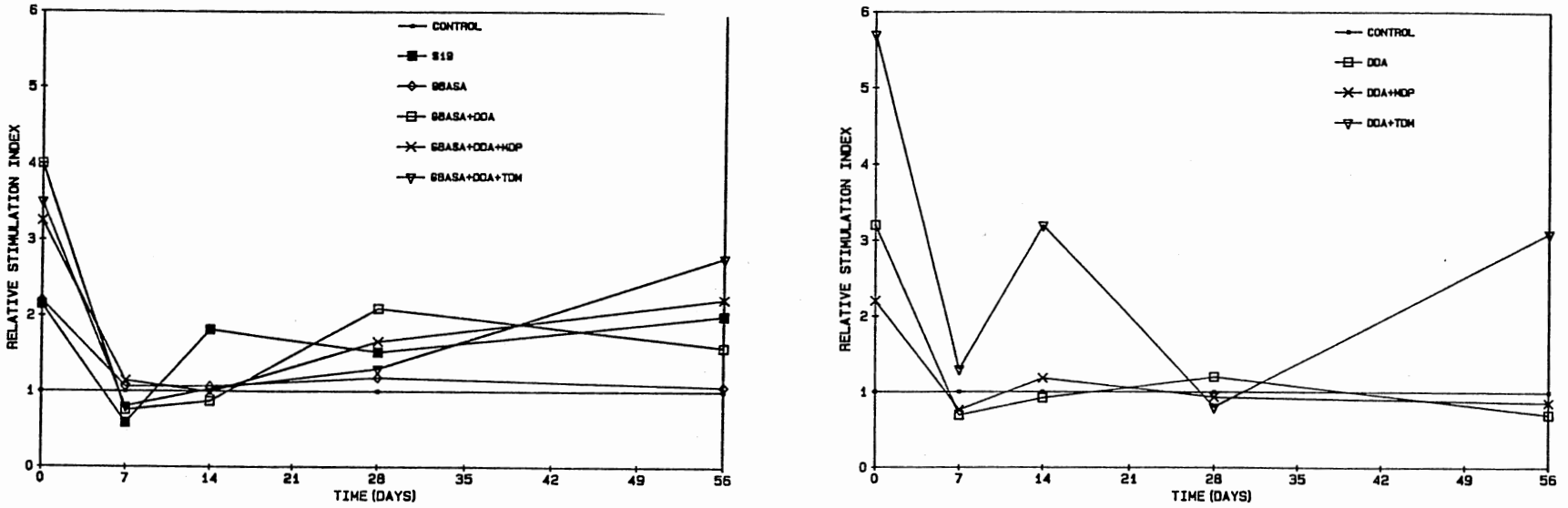


Figure 13A, B. Mean relative stimulation indices to gamma-irradiated B. abortus-induced IL-2 production in culture supernatant fluid. The vaccine and adjuvant preparations administered to each group of animals are shown in table 1.

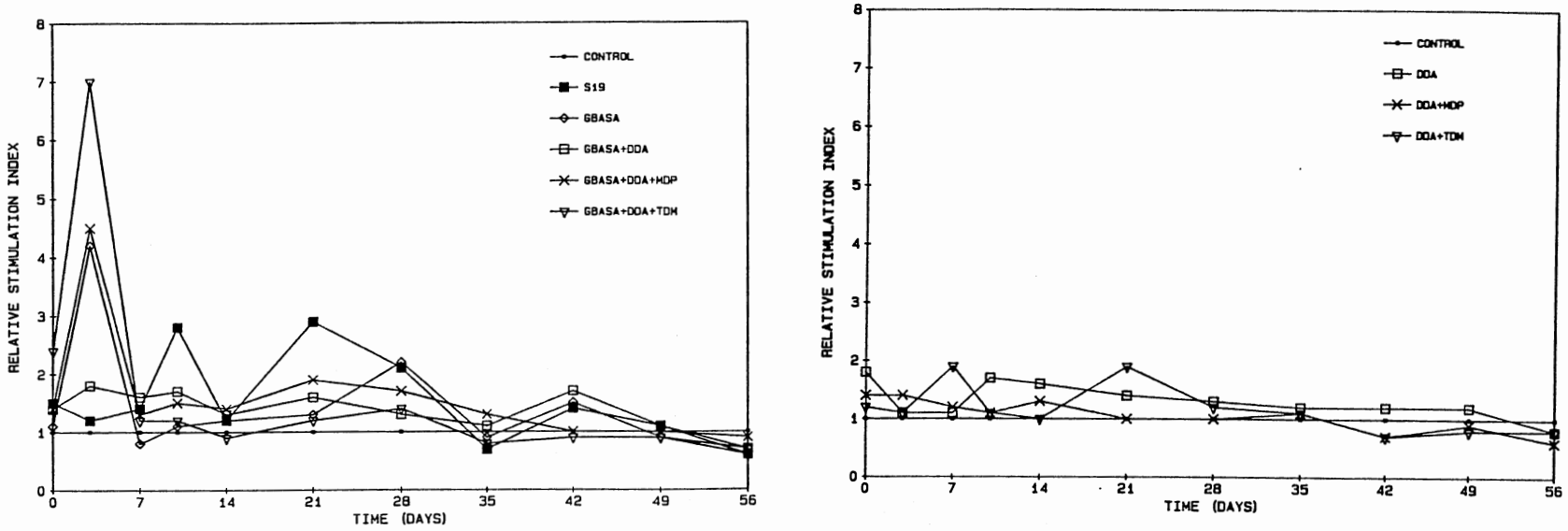
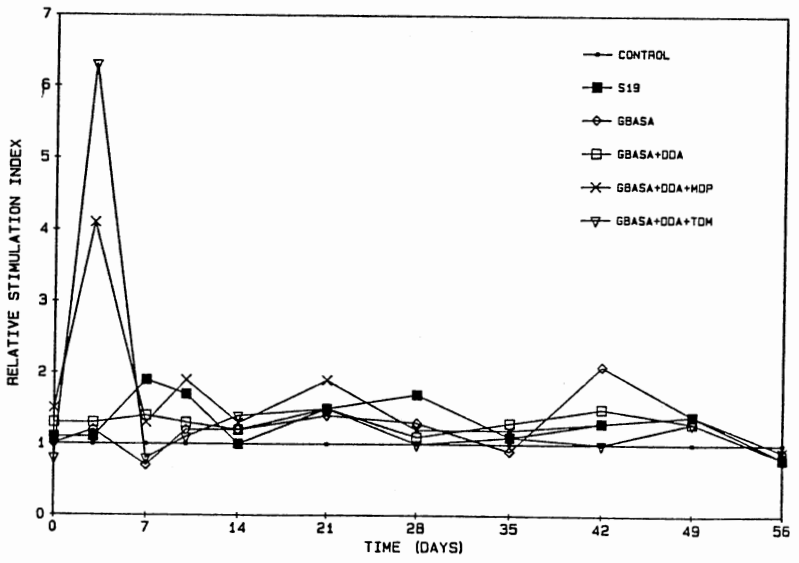


Figure 14A, B. Immunomodulatory effects of culture fluids from pokeweed mitogen (4 ug/ml) stimulated lymphocytes on proliferation of normal PBMC.



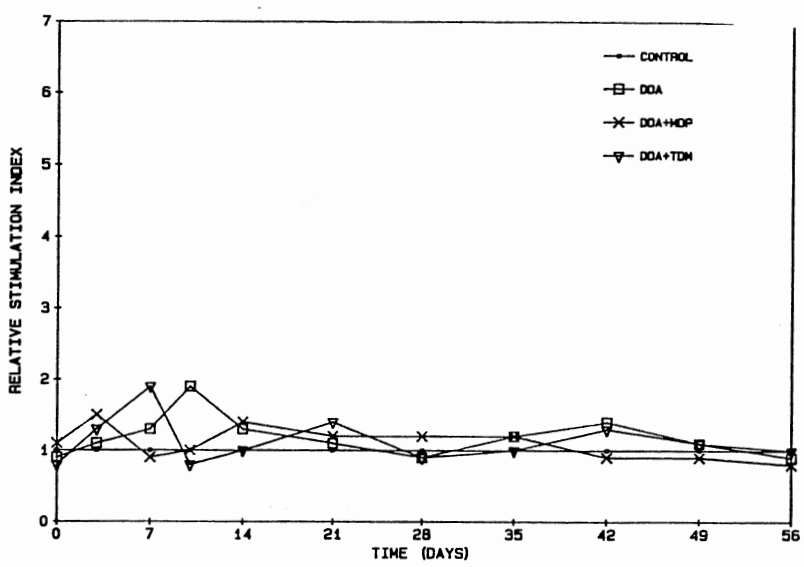


Figure 15A, B. Immunomodulatory effects of culture fluids from heat-killed B. abortus (20 ug/ml) stimulated lymphocytes on proliferation of normal PBMC.

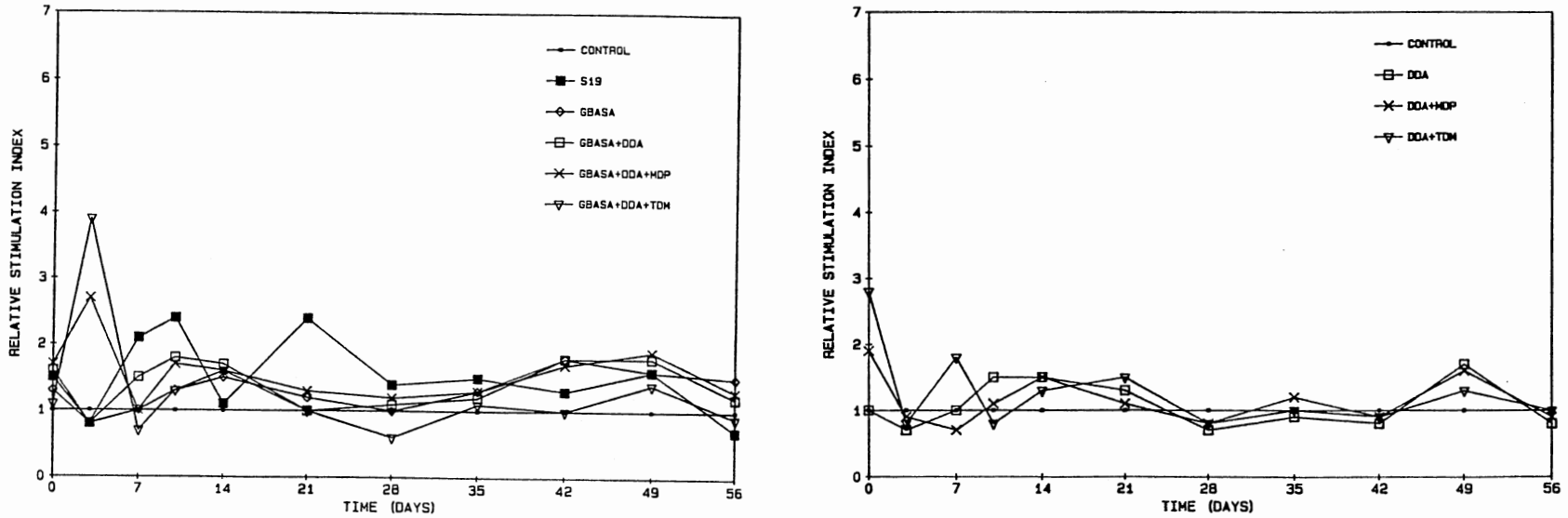


Figure 16A, B. Immunomodulatory effects of culture fluids from gamma-irradiated B. abortus (20 ug/ml) stimulated lymphocytes on proliferation of normal PBMC.

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CHAPTER V

SUMMARY AND CONCLUSIONS

Bovine brucellosis caused by Brucella abortus is an economically important disease associated with abortion and infertility. Despite numerous methods of eradication including vaccination, and test and slaughter, the disease has remained prevalent in many areas of the world.

The use of B. abortus strain 19 (S19) vaccine in cattle does provide protection from abortion but its use can cause cattle to become seropositive or even develop persistent infections with S19. Furthermore, S19, although attenuated for cattle is virulent for humans and thus constitutes a human health hazard. Some of the problems associated with S19 have been decreased but not eliminated by using a reduced vaccinal dose. The problems currently associated with S19 vaccine have generated interest in the development of a non-living (subunit) B. abortus vaccine.

However, non-living (subunit) vaccines are often weakly immunogenic and require the use of adjuvants to enhance their immunogenic potential. By augmenting the efficacy of vaccination, an appropriate adjuvant could induce long-lasting immunity using a low dose of antigen and thus minimizing side effects of vaccination. Numerous investigators have tested various non-living (subunit) vaccines in experimental bovine brucellosis. Variable degrees of

success were seen.

The studies in this dissertation were conducted to evaluate in cattle, the immunopotentiating effects of various adjuvants for use with non-living B. abortus antigens through comparison of serum antibody and CMI responses induced by these experimental vaccines. Additionally, the CMI responses were assessed with regard to functional lymphocyte and mononuclear phagocyte activities from the peripheral blood of calves vaccinated with S19 or non-living (subunit) vaccines.

Cattle were immunized with B. abortus soluble antigen (BASA) or a soluble extract of gamma-irradiated B. abortus (GBASA) in combination with various adjuvant preparations. GBASA had a reduced LPS content as compared to BASA. The humoral immune responses were measured using standard serologic tests and immunoassays. The CMI responses were evaluated using the lymphoproliferative assay and cutaneous DTH responses. Specific aspects of induced CMI responses investigated were macrophage activation (IL-1 production), T-helper cell (IL-2 production), and suppressor cell activity.

The findings reported in this work demonstrated a marked variability in the ability of different adjuvants to augment both humoral and CMI responses when administered with BASA and GBASA. A single administration of antigen was used to study the primary response in comparison to the single inoculation with S19 as a standard. Humoral and CMI responses were lowest when no adjuvant was included with the antigen.

In experiment 1, FIA provided the greatest stimulus for antibody production, whereas in Experiments 2 and 3, the largest antibody

titers were evident in the S19 group. GBASA-adjuvanted vaccines produced lower antibody titers than BASA-adjuvanted vaccines. These may be due to the low LPS content in GBASA as compared to BASA. Cell-mediated immune responses as determined by LP assay and DTH tests were most evident in S19, BASA + DDA and GBASA + DDA + TDM vaccinates, with the highest responses occurring in S19 vaccinates.

The ability of an individual's lymphocytes to produce and respond to IL-2 may provide a quantitative measure of CMI responses to specific antigens. In the present study, S19 and DDA provided the greatest stimulus for IL-2 production, which in most instances corresponded with the LP responses. IL-1 modulates many of the responses involved in the process of host defense against infection. Many agents exert their adjuvant effects by inducing the release of IL-1 from macrophages. In the present study DDA provided the strongest stimulus for IL-1 production. The stimulatory effect of DDA on macrophages may be particularly important because B. abortus is an intracellular pathogen and macrophages may be important in host defense in bovine brucellosis.

Inhibition of the LP response to several mitogens was described in experimental murine brucellosis. In the present study soluble suppressor factors were demonstrated in the culture fluids of B. abortus antigen-stimulated PBMC from cattle immunized with BASA adjuvanted vaccines. Generally, soluble suppressor factor(s) was not detected in lymphocyte cultures from cattle immunized with GBASA-adjuvanted vaccines. This could be due to the low content of LPS in GBASA as compared to BASA. LPS has been shown to down regulate lymphokine production and T-cell responses.

Development of an improved and effective non-living B. abortus vaccine would constitute major progress toward the goal of eradicating bovine brucellosis in the United States. However, successful application of adjuvants to subunit vaccines will depend on the understanding of the mechanisms of action of the adjuvant and the appropriate factors and segments of the immune system that must be stimulated. The studies reported herein demonstrated that DDA potentiates CMI responses to non-living, soluble B. abortus antigens and may be useful as an adjuvant for future vaccines, particularly subunit vaccines.

VITA 2

Gladstone Kwabla Dzata

Candidate for the Degree of

Doctor of Philosophy

Thesis: IMMUNOPOTENTIATING EFFECTS OF ADJUVANTS ON BOVINE HUMORAL AND CELLULAR IMMUNE RESPONSES TO BRUCELLA ABORTUS VACCINES

Major Field: Veterinary Pathology

Biographical:

Personal Data: Born in Worawora, Ghana, October 6, 1953, the son of Emmanuel and Elizabeth Dzata. Married to Angela Maria Igbinadolor on December 22, 1984. Children, Emmanuel Nosa, 1985 and Bernard Wola, 1987.

Education: Completed General Certificate of Education, Ordinary Level, 1973, and Higher School Certificate, 1975 at Bishop Herman Secondary School, Kpando, Ghana; received Doctor of Veterinary Medicine from the University of Zagreb in January, 1982; received Master of Science degree from Sam Houston State University in May, 1984; completed requirements for Doctor of Philosophy degree at Oklahoma State University in July, 1989.

Professional Experience: Field Veterinarian, Veterinary Station, Dvor, Yugoslavia, 1982 to 1983; Research Assistant (Integrated Tropical Farm Project), Office of International Programs, Sam Houston State University, 1983 to 1984; Resident, Department of Veterinary Pathology, Oklahoma State University, 1985 to 1989.

Professional Organizations and Honoraries: Member of American Veterinary Medical Association; C.L. Davis Foundation for the Advancement of Veterinary Pathology.