Biologic activity of lipopolysaccharide of *Treponema hyodysenteriae*

by

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GENERAL INTRODUCTION

Treponema hyodysenteriae, a gram negative spirochete, is the etiologic agent of swine dysentery (64). Swine dysentery is a muco-hemorrhagic diarrhea of young feeder pigs causing morbidity and mortality (63). This disease has been estimated to cost the pork producer 100 million dollars annually (125). It was reported that the lipopolysaccharide (LPS), a component of the outer membrane of gram negative bacteria (121), of *T. hyodysenteriae* could be contributing to the pathogenesis of this disease (129). This molecule has been shown to cause a wide range of effects on the mammaliam system that resemble the pathophysiolgy of a gram negative septicemia (i.e., fever and inflammation) (120, 121, 141).

This research was performed to define the role of LPS and endotoxin of *T. hyodysenteriae* in the pathogenesis of swine dysentery. The first part of the study was to define the chemical content of the treponemal preparations (LPS and endotoxin) and to examine the classical biologic responses elicited. The second part of this study was to examine the biologic effects of treponemal LPS and endotoxin preparations on lymphoreticular cells.

Explanation of thesis format The alternative format was used in preparing this thesis. Two manuscripts were written for Infection and Immunity. The manuscripts are titled "Classic Biological Responses Induced by Lipopolysaccharide and Endotoxin of *Treponema hyodysenteriae*" and "Ability of *Treponema* Lipopolysaccharide and

Endotoxin to Induce Cytokine Release from Lymphoreticular Cells".

A general review of the literature is followed by the first manuscript with an overall summary following the second manuscript. Literature cited are referenced at the end of each manuscript. The literature cited in the general literature review and overall summary are cited in a literature cited section after the second manuscript.

LITERATURE REVIEW

Lipopolysaccharide (LPS) from the members of the *Enterobacteriaceae*, such as *Escherichia coli* and *Salmonella minnesota*, has been extensively studied, both chemically and biologically (29, 98, 120, 119). LPS is a heat resistant, amphipathic molecule ranging in molecular weight from 20,000 to 1,000,000 daltons (98). Structurally, classical LPS has three regions (92): 1) a polysaccharide region designated as the O-specific side chain (183). This hydrophilic portion is associated with the serologic classification of the organisms. Polyclonal rabbit serum, immunized with a variety of antigenic preparations, is used to differentiate strains due to similiar antigenic epitopes within the O-specific side chain (O-antigen) (58, 91, 90, 92). 2) The core region is made up of 2-keto-3-deoxyoctonate (KDO), L-glycero-D-mannoheptose phosphate, and other repeating hexose sugars that can vary in character and number between genera (92). 3) The lipid A region is composed of a galactosamine disaccharide backbone with six or seven saturated fatty acid groups connected through phosphoester and/or amide bonds (92, 98). The biologic activity (i.e., toxicity, pyrogenicity, lethality,...) of LPS has been shown to be associated with the lipid A region (57, 55, 181).

The LPS molecule can be prepared using different extraction procedures which give different characteristics to the product. The purest preparation of LPS is extracted by a hot phenol/water procedure of Westphal and Jann (180). This procedure removes virtually all of the

protein (precipitated in the phenol phase) leaving lipid and sugar moities as well as other water soluble materials in the aqueous phase. Rough or R mutant LPS is prepared with a phenol/chloroform/petroleum ether extraction as described by Galanos et al. (56). This procedure was developed to increase the recovery of LPS that had shorter carbohydrate side chains or proportionally more lipid thus changing the chemical characteristics. Other methods have been described that leave more protein in the preparation than do previous procedures. The trichloroacetic acid method (TCA) described by Boivin and Mesrobeanu (28), translated by Staub (159), and the butanol/water method of Morrison and Leive (118) leave large amounts of protein associated with the LPS. Historically, the TCA method was the first used to extract endotoxin; however, due to the acidic nature of the TCA procedure and the possible hydrolysis of the LPS, Morrison and Leive (118) devised the butanol/water method. The butanol/water method is more gentle than the phenol/water method, but it does not yield a purified LPS preparation. The LPS preparation extracted by the butanol/water procedure has the lipid-associated protein (LAP) attached to the LPS molecule. The LAP and LPS together are commonly referred to endotoxin. This constitutes a fourth region of the moiety as well as a molecular form which is more native to that found on the bacterium (121).

The list of biologic effects associated with LPS or endotoxin includes the stimulation of B cell mitogenicity, immunogenicity, polycolonal antibody induction, adjuvanticity, stimulation of macrophage (119),

effects on the non-specific immune system (i.e., complement) (120), as well as pathophysiologic conditions such as fever and disseminated intravascular coagulopathy (141). Due to the large number of cell types (i.e., lymphocytes, macrophages, platelets, neutrophils, endothelial cells) (98) affected by LPS, this molecule would appear to play a major role in alerting the body to a gram negative bacterial infection (113).

Lipopolysaccharide is described as a T-independent antigen, inducing B cell proliferation (5, 110). Andersson et al. (7) reported that a majority of B cells respond to LPS but only a small portion of that population were specific for O antigen. The following year, lipid A was isolated and shown to induce the B cell responses associated with LPS (6, 36, 135, 143).

Conversely, LPS was shown to have no effect on T cell proliferation (8, 109, 135). However, there is evidence which indicated LPS can enhance the proliferation of T cells in the presence of T-dependent lectins (53, 132, 151).

As an immunogen LPS has been shown to induce exceptionally high serum antibody responses when administered at a low dosage. This was shown by stimulating a primary immune response to the O-specific side chain in the popliteal lymph node of rabbits following immunization with only 1000 molecules of purified endotoxin (83). Rudbach reported that when mice were primed with a few hundred molecules of LPS, an enhanced secondary response to endotoxin immunization was seen (144).

Previous reports have established LPS as a T-independent antigen, however, there have been conflicting reports suggesting T cell and/or macrophage regulation upon endotoxin-induced B cell proliferation (111, 117, 119). Travniczek et al. (170) and Yoshinaga et al. (185) reported that macrophage could exert suppressive effects upon LPS-induced B cell proliferation. Smith and Eaton demonstrated the presence of a subset of T cells that could inhibit LPS-induced B lymphocyte responses (157). Corbel and Melchers reported on a need for the combination of T cells and macrophages as accessory cells for for LPS-induced B cell stimulation (43).

Lipopolysaccharide can also serve as an adjuvant for a humoral immune response (131). An adjuvant serves to potentiate the immune response to an antigen when both are administered simultaneously. The adjuvant response of LPS was first described by Johnson et al. (72) in 1956 and then localized to the lipid A region of the molecule (36, 124, 127). The adjuvant activity, as with B cell stimulation, was reported to be a T cell-independent response (109, 155). Conversely McGhee et al. (99) showed that the transfer of T cells from LPS responsive mice to the LPS hyporesponsive C3H/HeJ mice resulted in a good adjuvant response. Thus, implicating T cells in some of the biologic responses induced by LPS. Additionally, LPS given with a toleragenic dose of antigen induced an immune response to the antigen (5, 38).

The classes of antibodies to LPS produced by B lymphocytes are IgM, IgG, and IgA (119). The primary response seen was IgM and IgG (7, 76). The class of antibody was shown to vary according to the amount

and type of LPS given, as well as the immunization procedure (i.e., route) (119).

Suppression of the immune response has been associated with the administration of LPS and was found to be dependent upon the time difference between LPS administration and immunization with antigen (88, 136, 171). For example, LPS administered with antigen will act as an adjuvant, however, if LPS is given 24 hours prior to antigen, suppression of the immune response to that antigen will be seen (171). There are two possible hypotheses suggested for this suppression: 1.) a suppressor B cell population is generated (136); or 2.) a suppressive T cell response is stimulated by the LPS (170). McGhee et al. (100) indicated the involvement of T cells in the suppression of the mitogenic and immunogenic responses of B cells in conventional mice but not in germ free mice stimulated by LPS.

An aspect that has not been addressed is the biologic activity of LAP and other outer membrane proteins, specifically porins, which are co-extracted with LPS. Porins are trimeric complexes that serve as channels through the outer membrane of gram negative bacteria (17, 139, 149). The porins are contaminants extracted with the endotoxin in the butanol/water procedure. They also stimulated B cell proliferation and polyclonal activity (20, 161, 175, 176). Skidmore et al. (155, 156) reported that there were differences between extraction procedures when using LPS to stimulate B cell proliferation of C3H/HeJ mice. Morrison et al. (115) and Sultzer and Goodman (162) separated a protein-rich portion from the phenol phase of endotoxin and tested the

fraction for stimulatory properties. They reported that the fraction induced B cell proliferation and acted as a polyclonal activator (115, 162). These reports suggest that LAP stimulated B cells without T cell or macrophage involvement (61, 102,162, 163). Hogan and Vogel reported that the LAP stimulated tumoricidal activity via interferon primed macrophages in C3H/HeJ mice in the absence of a response to LPS (68). Additionally, the presence of T cells stimulated by LAP may play a role in establishing an IgG class of antibody produced by B cells to LPS (65). Other cell types such as fibroblasts and mast cells have also been shown to respond to LAP (114, 115,161).

Expression of Ia antigen has been shown to be essential for the induction of an immune response (14). Ia antigens are class II major histocompatibility antigens expressed on the surface of antigen presenting cells (APC) such as macrophage, T cells, and B cells (15, 173, 172). Unless antigen is presented to B cells in conjunction with Ia, no immune response to the antigen developes (15, 173, 172). Endotoxins have been reported to activate Ia expression on B cells and macrophage through a T-independent mechanism (15, 16, 179, 186). This information indicates that the basis for the immunomodulation following treatment with LPS involves differential expression of cell surface markers without direct involvement from T cells.

Activated macrophages are APCs which process antigens for delivery to lymphocytes, but they also function to stimulate lymphoblast formation via cytokine release (172). Macrophages stimulated with LPS synthesize and release cytokines such as interleukin 1 (IL1), colony stimulating

factor, interferon (IFN), glucocorticoid antagonizing factor, and tumor necrosis factor (TNF) or cachectin (98). There are many other products such as arachidonic acid metabolites and complement components that are also produced by macrophage (1). Many of these monokines have been shown to augment immune responses.

Interleukin 1 (IL 1) is a hormone-like protein (15,000 daltons) that is primarily secreted by macrophage, but may also be produced by B lymphocytes, fibroblasts, endothelial cells, glioma cells, astrocytes, corneal epithilium, and large granular lymphocytes (49). This cytokine is made in response to a wide variety of stimulants including immunologic interactions (i.e., T cell-macrophage interactions) (102, 104), immune complexes, C5a, microbial stimulants (i.e., lipid A of gram negative bacteria, gram positive bacterial exotoxins, muramyl dipeptide, yeast cell walls, virus hemagglutinins, and double-stranded RNA) (3, 49), and chemical stimulants (i.e., silica crystals, urate crystals and phorbol myristate acetate) (49).

Interleukin 1 induces numerous biologic effects, one of which is important to the humoral response. This monokine serves as a stimulant for the T and B lymphocyte blastogenesis (108). When T cells are stimulated with IL 1, they produce IL 2 which causes T cell proliferation (49). This T cell population thus enhances the immune response to an antigen. Interleukin 1 has been reported as an endogenous pyrogen as well as a mediator of inflammation (49). Interleukin 1 stimulates macrophage, neutrophils, fibroblasts, synovium, endothelium, and hepatocytes to release prostaglandins, collagenases, and acute-phase

reactants which lead to an inflammatory state (19, 44). An example of this type of phenomenon can be demonstrated by the local Shwartzman reaction (13).

Tumor necrosis factor is a hormone secreted by macrophages activated by stimulants such as LPS (25, 93, 95, 96, 97). This molecule is a dimeric polypeptide with a monomeric molecular weight of 17,000 daltons (22). When secreted by cell lines or macrophages, TNF can account for 1-2% of the total protein produced in vitro (25).

As the name implies, TNF causes hemorrhagic necrosis of tumors (24). This was first exploited by injecting bacterial culture broth into patients with cancer (39, 40, 126). The hope was to induce tumor regression, however mixed results were observed (39, 40, 126). The mechanism of TNF activity on tumors is unknown.

When TNF has been induced by exogenous stimulants, such as LPS, a general wasting or cachexic state of the host was observed. In 1985, Cerami et al. (34) reported that TNF given to endotoxin-resistant C3H/HeJ mice caused anorexia and weight loss, and if enough TNF was given, death occurred. The condition was described as metabolic acidosis with changes in plasma glucose levels, and inflammatory lesions in the lungs, gastrointestinal tract, kidneys, adrenal glands, and pancreas (168).

Tumor necrosis factor stimulation can induce biosynthesis and/or release of specific proteins. These proteins vary in nature from inflammatory agents such as prostaglandin E_2, leukotrienes, platelet activating factor, and collagenase (24, 48), to class I major

histocompatibility antigens (42), and IL 1 (44). Induction of IL 1 resulted in fever, and, thus allowing TNF to be referred to as an endogenous pyrogen (45).

Numerous studies suggest that TNF is a major contributor to the effects caused by LPS (23, 25, 52, 168, 169). Beutler et al. (23) showed that mice passively immunized with anti-TNF antibody were protected from a normally lethal dose of LPS. In 1986, Tracey et al. (168) reported that treatment of rats with recombinant TNF induced lesions consistent with those caused by lethal doses of LPS. Subsequently, Tracey et al. (169) reported that anti-TNF antibody would protect baboons from a lethal bacteremic shock induced by a LD_{100} dose of live *E. coli*. This supported evidence that TNF was a mediator of endotoxin induced lethality (85, 169).

Macrophages stimulated with LPS have also been shown to secrete interferon (66). It should be noted that lymphocytes (T cells) and leukocytes are the primary sources of interferon (80, 84). Interferon is a 15,000-17,000 KDa glycoprotein that functions as an anti-viral agent, slows the growth of tumors, activates macrophages and neutrophils, enhances the activity of natural killer cells (46), and induces enhanced expression of Ia antigens (69, 184). Interferon was also reported to prime macrophage for the induction of TNF and IL 1 by LPS (26, 41, 59). Billiau suggested that interferon was very important in the Shwartzman reaction (27, 154). It has been shown that IL 1 and TNF without interferon could cause portions of the Shwartzman reaction but not the complete syndrome (27).

Macrophages can function as effector cells against tumor cell targets when stimulated with LPS (3, 133). The tumoricidal activity was observed without T or B cell help (47, 145, 178). It has been established that macrophage can kill tumor cells when primed with interferon and then given a second signal, namely LPS (51, 103, 134). There has been some controversy as to whether the tumoricidal activity was caused by the macrophage directly (i.e., cell-cell contact) or by a released toxic factor (i.e., cytotoxin or TNF) (97, 164). Suzuki et al. (164) reported on the gamma-interferon induced anti-tumor cytotoxic activity against a TNF-sensitive cell line suggesting that activated macrophages have tumoricidal activity of their own. Subsequently, a report from that laboratory indicated a synergistic effect caused by interferon, TNF and IL 1 to induce the macrophage tumoricidal activity (35).

Other cell types besides macrophages, B cells, and T cells are affected by LPS. As mentioned above, natural killer cells are activated to be cytotoxic (46). Endothelial cells have the capacity to produce prostaglandin mediators, leading to shock and disseminated intravascular coagulopathy (120). Lipopolysaccharide has been shown to bind to neutrophils stimulating enhanced phagocytic activity, glycolysis, and an increased reduction of nitroblue tetrazolium (120). After administration of LPS a leukopenia is observed followed by a leukocytosis showing the change in neutrophil populations (10, 101). Platelets have a high affinity for LPS as shown by the the uptake of labeled endotoxin when injected in vivo (33, 50). In a review by McCartney and Wardlaw

(98), it was suggested that platelets could play a large role in endotoxemia due to release of platelet contents by LPS stimulation.

Lipopolysaccharide has been shown to interact with serum proteins, in particular the proteins of the complement system (120). Lipopolysaccharide can activate the complement cascade by either the classical or by the alternative pathway (122). C3 and other components of the complement system have been shown to mediate some of the pathology and toxicity associated with endotoxemia (120). For example, complement-LPS interactions lead to generation of anaphylotoxins (C3a, C4a, C5a), chemotactic factors, and activation of the coagulation system (105, 107).

The coagulation system has an intrinsic and extrinsic pathway for activation (120). Lipopolysaccharide has been shown to activate Hageman Factor (116), the first component of the intrinsic pathway, as well as kallikrein, which can initiate fibrinolysis (140). The extrinsic pathway is activated by secretory products of monocytes, which as described above are very receptive to LPS stimulation (98). In severe cases of endotoxemia, disseminated intravascular coagulation (DIC) occurs leading to shock and ultimately death.

The evolution of the clotting system from the lower animals to the higher animals has some similarities. The clotting system is a containment mechanism for infection. Levin and Bang (87) developed a clotting system to measure LPS activity using the cytosol of amebocytes of *Limulus polyphemus*, a horseshoe crab. In the presence of Ca^{++}, LPS acts on a proenzyme to initiate the gelation of the substrate (98). This

assay was reported to be sensitive to 0.1 ng/ml LPS and is available as a chromogenic assay (123, 152).

Endotoxin-induced lethality of mice is a common assay used when characterizing LPS (54). The relative toxicity of LPS is often measured in vivo by the determination of the mean lethal dose (LD_{50}) for mice. Various models have been developed using chemicals such as lead acetate (153), actinomycin D (18), and D-galactosamine (54) to increase the sensitivity of animals to LPS several thousand fold (29). In 1979, Bradley suggested a mechanism to explain LPS toxicity (29). Lipopolysaccharide is endocytosed by the phagocyte and transported to the mitochondria where LPS affects the proton gradient causing ADP and NAPH to pool in the cytosol of the cell. The accumulation of these compounds causes an increased glycolysis inducing lysosomal release and proteolysis which leads to cell destruction. With the release of the cellular contents (i.e., lysosomal hydrolases, prostaglandins) symptoms of endotoxemia will develop leading to shock, DIC, and death unless the body can recover from the ensuing damage (29).

There are two *Treponema* species which colonize the colon and cecum of swine. These spirochetes are gram negative, ß hemolytic, anaerobic organisms (64, 63) that have a DNA G+C ratio of 25-28% (106). One of these species,*T. hyodysenteriae*, was shown to be pathogenic for swine (64, 167) and the other species, *T. innocens*, was reported as a non-pathogenic organism isolated from normal swine colon (79). The differentiation between the two species is based on the strong ß hemolysis produced by *T. hyodysenteriae* versus the weak beta-

hemolysis seen with *T. innocens* (78). In addition, biochemical identification by hydrolase activity, using an API-ZYME kit, has been reported (70). Enteropathogenicity in mice is a third criterion used to distinguish pathogenic from non-pathogenic species (130).

Swine dysentery was first described in 1921 by Whiting et al. (182); however, it was 50 years later that Taylor and Alexander (167) and Harris et al. (64) described *T. hyodysenteriae* as the causitive agent of swine dysentery. Swine dysentery is characterized as a muco-hemorrhagic diarrhea of 6-20 week old feeder pigs. Incubation periods ranging from 2 days to 3 months in naturally infected pigs (62). The mortality and morbidity due to swine dysentery can be high (i.e., greater than 50%) in untreated herds (125).

The lesions of swine dysentery are described as a catarrhal, necrotic, hemorrhagic colitis confined to the cecum and large intestine of the pig (60). In addition to the presence of blood and excess mucus, fibrin and denuded epithelium are often seen on the colonic surface (2, 60).

Swine dysentery is not a secretory diarrhea but an absorptive defect where water and ions from the colonic contents are not adsorbed leading to dehydration. The blood-to-lumen flux of Na+ ions and Cl- ions are not changed following infection (9, 150). This differs from diarrhea caused by *E. coli* where the blood-to-lumen flow of fluids and ions far exceeds the absorbtive capacity of the colon (9).

The two virulence factors attributed to *T. hyodysenteriae* are the hemolysin and the lipopolysaccharide (LPS). The hemolysin was

characterized as a lipoprotein with a molecular weight of 19,000 daltons (77), oxygen resistant, heat labile at 60°C, and was stable over a large range of pH values (148). Both *T. hyodysenteriae* and *T. innocens* has hemolysin. The two hemolysins differ due to sensitivity of *T. innocens* hemolysin to cardiolipin (147) *T. hyodysenteriae* hemolysin had no cytolytic action on either prokaryotic or eukaryotic cells. The hemolysin lacked any mitogenic activity but decreased the mitogenic response of concanavalin A or *E. coli* LPS when combined in the assay (146). Saheb et al. (146) suggested this decrease in the mitogenic response may have been due to inactivation of macrophage or inhibition of the mitogens.

Kent and Lemcke (77) injected rat ileal loops with the hemolysin from *T. hyodysenteriae* and induced fluid accumulation, desquamation of epithelial cells, hemorrhage, and neutrophil accumulation. These authors suggested that the hemolysin may contribute to the pathogenicity of *T. hyodysenteriae* (77). When the hemolysin of *T. hyodysenteriae* was added to various porcine cells, fibroblasts were affected more than epithelial cells and porcine lymphocytes were more sensitive to the cytotoxic effects than porcine macrophages and neutrophils (77).

Lipopolysaccharide has also been described as a virulence attribute of *T. hyodysenteriae* (130). Nuessen et al. (128) reported that the treponemal LPS was toxic for murine peritoneal macrophages and enhanced both complement (C3) and immunoglobulin G-Fc receptor-mediated phagocytosis. It was also shown that the treponemal LPS was mitogenic for murine spleen cells, and stimulated chemotaxis of porcine

leukocytes (128). Additionally, Nuessen et al. (130) reported that treponemal LPS was lethal for actinomycin D-treated C3HeB/FeJ mice but not for LPS-hyporesponsive C3H/HeJ mice. Following infection with *T. hyodysenteriae*, it was shown that the LPS-hyporesponsive C3H/HeJ mice failed to develop lesions, suggesting that responsiveness to LPS was required for pathogenesis to occur (130). Nuessen and Joens (129) demonstrated serotype specific opsinization of the treponemal organism with convalescent serum and suggested that LPS could serve as a protective antigen.

T. hyodysenteriae has been subdivided into seven serotypes based on the presence of lipopolysaccharide (11, 94). The serotypes are represented by the following strains of *T. hyodysenteriae*: B234, serotype 1; B204, serotype 2; B169, serotype 3; A-1, serotype 4; B8044, serotype 5; B6933, serotype 6; Ack 300/8, serotype 7. Lemke and Bew (86) have also described three additional serogroups, which have not been compared to the previously listed serogroups.

As mentioned earlier, most of the work in LPS research has been with *Enterobacteriacae* LPS. There are numerous reports that show a wide variety of chemical forms and biologic activities between genera and species of LPS (98).

Coxiella burnetii, the etiologic agent of Q fever, has a host-controlled phase variation that regulates the virulent and nonvirulent form of the organism (160). The virulent phase 1 organism has a smooth LPS that was 100 to 1000 times less toxic than *E. coli* LPS or *Salmonella typhi* LPS (4). The endotoxin toxicity was associated with the Lipid A portion

of the *C. burnetii* LPS (4). Joshi and Banerjee showed the phase I LPS was pyrogenic, caused weight loss, as well as many other biologic effects associated with LPS activity (74). The LPS-induced symptoms were similar to the bacterial infection; however, though the LPS-induced condition was accelerated, it was of shorter duration (74).

Chlamydia psittaci, an obligate intracellular parasite, has a rough LPS that was biologically inactive in comparison to *E. coli* LPS (toxicity, pyrogenicity and local Shwartzman reaction), but was mitogenic and stimulated prostaglandin E_2 from peritoneal exudate cells (31, 32).

Brucella abortus, a bovine pathogen, has two types of LPS, a smooth LPS that extracts in the phenol phase, and a rough LPS found in the aqueous phase of a phenol/water extraction procedure (81). The lipid A is unique due to its presence in both the aqueous and phenol phase. This LPS was reported to be mitogenic in spleen cells from athymic nude mice as well as LPS-hyporesponsive C3H/HeJ mice (112, 158).

Pseudomonas aeruginosa LPS has been reported as mitogenic, immunogenic, toxic for macrophage, but not lethal for LPS-hyporesponsive C3H/HeJ mice (137). Reports of small structural differences between the LPS of *P. aeruginosa* and *E. coli* have been suggested as the cause of different biologic activities in the LPS-hyporesponsive mice (137, 142).

Bacteroides, *Fusobacterium*, and *Veilonella* species have LPS that are quite different from that of *E. coli* LPS (165). There is a loose association of lipid to polysaccharide with no detectable KDO or heptose sugars (67, 165). *B. fragilis* LPS was not lethal to chick embryos at

doses >200 μg while *Neisseria meningititdis* LPS was lethal at a dose of 1.2 μg. A local Shwartzman reaction was not induced by *B. fragilis* LPS when 1000 μg was injected while *Salmonella typhi* LPS showed a good response at 3 μg (75). The lethality and pyrogenicity of the *B. fragilis* LPS were established at 1% that of *Salmonella* lipid A (165). *B. fragilis* LPS was shown to be mitogenic for LPS-hyporesponsive C3H/HeJ mice (73) as well as capable of stimulating murine splenocyte mitogenesis and polyclonal activity in LPS responsive mice (73, 177).

Several spirochetes have also been shown to contain LPS with varying biologic activity. Vinh et al. (174) reported that *Leptospira interrogans* serovar*copenhageni* LPS had similar morphological and chemical characteristics to *Enterobacteriacae* LPS except for the absence of KDO. Cinco et al. (37) compared *L. interrogans* and *L. biflexa* and observed a rough LPS containing KDO with similar SDS-PAGE profiles. Isogai et al. (71) compared the biologic activity of *L. interrogans* serovar *copenhageni* to *E. coli* LPS. They reported that the *Leptospira* LPS was less toxic for mice, was pyrogenic (without the biphasic curve seen with *E. coli* LPS), acted as an adjuvant, and capable of clotting the Limulus amebocyte lysate test (71).

The presence of LPS in *Borrelia burgdorferi* has not been fully established due to conflicting reports (12, 166). In 1985 Beck et al. (12) reported that LPS constituted 1.5% of the total dry weight of *B. burgdorferi* and that the LPS had classical biologic activity (pyrogenicity, mitogenicity, clots Limulus amebocyte lysate, and was cytotoxic for murine macrophages). Habicht et al. (62) reported that a *B. borgdorferi*

infection stimulated large quantities of IL 1 suggesting that the LPS could be the mediator for the cytokine production as well as playing an important role in the pathogenesis of Lyme disease.

Takayama et al. (166) reported on the absence of a LPS from *B. burgdorferi*. They used a phenol/water extraction as well as a phenol-chloroform-petroleum ether extraction to determine the absence of KDO, glucosamine, and hydroxy fatty acids by gas-liquid chromatography. They also studied prostaglandin E_2 production and the formation of a clot by the Limulus amebocyte lysate showing no response from the two preparations suggesting that there was no classic LPS in *B. burgdorferi* (166).

Reports to date suggest that the LPS of *T. hyodysenteriae* plays a role in the pathogenicity of swine dysentery. The following reports examine the biologic activity of treponemal LPS in more detail and discuss the role of LPS in the pathogenesis of swine disease.

SECTION I. CLASSIC BIOLOGICAL RESPONSES INDUCED BY LPS AND ENDOTOXIN OF *TREPONEMA HYODYSENTERIAE*

ABSTRACT

The chemical composition and classical biologic activities of lipopolysaccharide (LPS, phenol/water) and endotoxin (butanol/water) preparations from virulent *Treponema hyodysenteriae* and avirulent *T. innocens* were examined. The LPS and endotoxin preparations from *T. hyodysenteriae* strain B204 contained approximately 76% and 34% hexose, 0.12% and 0.45% 2-keto-3-deoxyoctanate (KDO), and 0.1% and 8% protein, respectively. The LPS and endotoxin preparations of *T. innocens* strain B1555a contained approximately 47% and 38% hexose, 0.45% and 0.4% KDO, and 0.1% and 26% protein, respectively. A silver stained 7.5 - 15% sodium dodecyl sulphate polyacrylamide gel showed approximatey 5 bands for the *T. hyodysenteriae* preparations while the *T. innocens* preparations failed to develop discrete bands upon electrophoresis. The Limulus amebocyte lysate assay determined that the treponemal preparations had comparable amounts of endotoxin activity when *E. coli* LPS was used as a standard. The LD_{50} of LPS and endotoxin from *T. hyodysenteriae* for BALB/cByJ mice was 380 μg and 80 μg, respectively. The adjuvant activity, ability to induce a dermal Shwartzman reaction, and pyrogenicity of the treponemal preparations were very weak when compared to *E. coli* LPS. The treponemal LPS preparations were not mitogenic for murine spleen cells but the endotoxin preparations were very mitogenic. The results indicate no major difference in the biologic activity from the LPS and endotoxin of these *Treponema* species.

INTRODUCTION

Lipopolysaccharide (LPS) is a molecule found in the outer membrane of gram negative bacteria and is associated with numerous biologic effects on the mammalian immune system (30, 35). These responses include B cell mitogenicity (12), polyclonal antibody induction (40), adjuvanticity (16), macrophage activation (1, 45, 46), immunogenicity (21), pyrogenicity (53), lethality (53), induction of tolerance (7, 23), and inflammatory reactions (47).

Different extraction methods for LPS have been reported. A relatively pure LPS is extracted with the hot phenol/water method of Westphal and Jann (60). Endotoxin (LPS with the lipid-associated protein (LAP)) is extracted using either the trichloroacetic acid method of Boivin and Mesrobeanu (6), translated by Staub (50), or a butanol/water method of Morrison and Leive (33). Endotoxin possesses all of the aforementioned biologic activities, including stimulation of the LPS-hyporesponsive C3H/HeJ mouse strain (14, 59). The ability to stimulate C3H/HeJ mice has been shown to reside with the LAP of endotoxin (5, 14, 32, 48).

In contrast to the *Enterobacteriaceae*, there are numerous gram negative organisms which have LPS with varying chemical and biologic characteristics (17, 31, 42, 49). For example, the LPS of *Bacteroides fragilis* was previously reported not to contain 2-keto-3-deoxyoctanate (KDO) (13), however, development of more sensitive tests has shown the presence of KDO (44). Additionally, little carbohydrate has been

detected in *B. fragilis*, but a long fatty acid composition that was quite divergent when compared to *E. coli* LPS (61). This LPS has the distinct difference from classical LPS in that it stimulates spleen cell mitogenesis from the LPS-hyporesponsive C3H/HeJ mouse strain (17, 59).

The gram negative spirochetes *Leptospira interrogans* and *Borrelia burgdorferi* possess rough types of LPS and have varying biologic effects on the host system when compared to those established for classical LPS (4, 8, 56). *Treponema pallidum*, the causitive agent of syphilis, was reported to contain LPS, but this molecule failed to elicit pyrogenic activity (41).

Previous investigators have indicated that the LPS of *T. hyodysenteriae* is involved in the pathogenesis of swine dysentery (39). The LPS has been used to show serotype specificity for *T. hyodysenteriae* strains (3, 25). Nuessen and Joens (37) reported that trepomenal LPS was stimulatory for murine splenocyte mitogenesis, was toxic for murine macrophage, and was chemotactic for porcine neutrophils. To date, there have been no published studies comparing the LPS from *T. hyodysenteriae* and the LPS from *T. innocens* to determine the role of this molecule in virulence.

Therefore, the objectives of the current study were to compare the biologic activities of LPS and endotoxin from *T. hyodysenteriae* and *T. innocens*. These activities were compared with those of classical *E. coli* LPS. The results indicate that the treponemal LPS preparations are much less active than *E. coli* LPS and that virulence of the two

treponemal species examined is not associated with the biologic function of LPS or endotoxin.

MATERIALS AND METHODS

Animals Original C3H/HeJ and BALB/cByJ breeder mice were obtained from Jackson Laboratory (Bar Harbor, ME), and C3H/HeN breeder pairs were obtained from Harlan Sprague Dawley (Madison, WI). Mice were housed at the Laboratory Animal Resources (LAR) facility at the College of Veterinary Medicine, Iowa State University (ISU), Ames, IA. The mice were given autoclave sterilized water and feed (Purina Lab Chow #5010, Purina Mills, Inc., St. Louis, MO) *ad libitum*. New Zealand White rabbits were obtained from Small Stock Industries (Pearidge, AR) and housed at LAR, ISU.

Bacterial strains *Treponema hyodysenteriae* strain B204 and *T. innocens* strain B1555a were obtained from ISU. *Escherichia coli* strain K235 was obtained from Dr. Suzanne Michalek, Department of Microbiology, University of Alabama in Birmingham. The *Treponema* species were grown in trypticase soy broth containing dextrose (BBL #11768, Cockeysville, MD) and supplemented with 5 g/l yeast extract (BBL), 20 ml of each VPI A and B salt solutions (stock A - 0.4 g $CaCl_2$, 0.4 g $MgSO_4$, 1 liter H_2O; stock B - 2.0 g K_2HPO_4, 2.0 g KH_2PO_4, 20.0 g $NaHCO_3$, 1 liter H_2O), and 0.5 g/l L-cysteine. Medium was adjusted to pH 7.3. After autoclave sterilization, 50 ml/l horse serum (Hyclone Laboratories, Inc., Logan, UT) was added and cultures grown under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2) for 18 - 24 hours. The cells were harvested by centrifugation at 10,000 x g for 20 minutes, washed twice in phosphate buffered saline (PBS - 8 g NaCl,

1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, to 1 liter H_2O, pH 7.2) and once in distilled water. Whole cells were frozen at -20°C until the LPS or endotoxin extraction was performed.

LPS and endotoxin extraction Lipopolysaccharide was extracted by a modified hot phenol/water extraction procedure (3, 60). Briefly, 200 mg of lyophilized whole cells were suspended in 10 ml water and mixed with an equal volume of liquified phenol at 68°C. The mixture was stirred for 15 minutes at 68°C. After cooling, the mixture was centrifuged at 200 x g to separate the two phases. The aqueous phase was collected and saved and the phenol phase was extracted twice with half the original volume of water. The aqueous phases were pooled and dialyzed exhaustively against distilled water. The LPS containing material was concentrated and then centrifuged at 100,000 x g for four hours. The pellet was resuspended in pyrogen-free water and the centrifugation was repeated two times. After the last centrifugation, the pellet was resuspended in pyrogen-free water and lyophylized. The LPS was stored at -20°C until used. *Esherichia coli* strain K235 LPS was prepared by the Westphal and Jann method (60).

Endotoxin was prepared by the butanol/water extraction procedure (33). Briefly, 250 mg wet weight of whole cell paste was suspended in 1 ml water and then 1 ml of butanol added. The endotoxin partitioned to the aqueous phase below the butanol. The butanol phase was extracted with half the original volume of water three times. The pooled aqueous phase was digested with Pronase (20 μg/ml final concentration) in 0.2 M Na_3PO_4 buffer (pH 7.0) overnight at 37°C. The

digested endotoxin preparation was centrifuged at 10,000 x g for 40 minutes and the precipitated interphase removed. The endotoxin solution was exhaustively dialyzed, concentrated, lyophylized, and stored at -20°C until assays were performed.

The LPS and endotoxin preparations were dissolved in pyrogen-free saline and sterilized by heating to 100°C for 10 minutes. These solutions were stored at 4°C until use. The preparations were heated for 1 - 2 minutes at 100°C before use in assays.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) LPS and endotoxin were analyzed by SDS-PAGE as described by Laemmli (20), using a 1 mm, 7.5 - 15% acrylamide separating gel with a 4% stacking gel. The gel was electrophoresed at 35 mA per gel (Model SE600, Hoeffer Sientific Instruments. San Francisco, CA) for 2 - 3 hours until the dye front was 1 cm from the bottom of the gel. The LPS and endotoxin samples were visualized by the silver stain procedure of Tsai and Frasch (55) or a Coomaisse blue stain (Kodak R-250) (9).

Chemical determination The protein content of the LPS and endotoxins were measured as described by Lowry et al. (24) with bovine serum albumin as a standard.

Carbohydrate content was determined by phenol-H_2SO_4 method (2). Briefly, the aliquots of LPS were placed into acid cleaned 13 x 100 mm glass tubes with 50 µl of 80% phenol added to each tube. Five ml of H_2SO_4 was added to each tube. The tubes were incubated 30

minutes at room temperature before reading adsorbance at 485 nm. D-glucose was used as the carbohydrate standard.

2-keto-3-deoxyoctonate (KDO) content was determined by the thiobarbituric acid procedure of Karkhanis et al. (18).

Mitogenesis Mitogenesis was performed by inoculating 5 x 105 murine splenocytes/well of a 96 well microtiter plate (Costar #3799, St. Louis, MO) in RPMI 1640 supplemented with 10 mM HEPES, 25 units/ml penicillin, 25 μg/ml streptomycin, and 20 mM L-glutamine. Various concentrations of LPS or endotoxin were added to the appropriate wells. Cultures were incubated for 48 hours at 37°C in 5% CO_2 in air. Cultures were pulsed with 0.5μC 3H-methyl-thymidine (Amersham Corp., Arlington Heights, IL) during the final 8 hours of incubation. Cells were harvested onto filter paper discs using a microharvester (Bellco, Vineland, NJ), dried and counted using liquid scintillation. Cultures were performed in triplicate and results are expressed as the stimulation index (experimental CPM/control CPM) (59).

Lethality BALB/cByJ mice sensitized with galactosamine (22) were used to determine the relative toxicity of the treponemal LPS and endotoxin and the 50% lethal dose (LD_{50}) was determined by the method of Reed and Muench (43). Briefly, the mice were provided with drinking water containing 2 mg/ml galactosamine *ad libitum* for 48 hours. Mice were then given an intraperitoneal injection (i.p.) of 16 μg galactosamine followed by an intravenous injection (i.v.) injection of the indicated dose of LPS or endotoxin. The assay was set up with 5

mice per group using 5 different concentrations of LPS or endotoxin. Mice were observed for 2 days and deaths recorded.

Limulus amebocyte lysate assay (LAL) A chromogenic LAL assay (QCL-1000 Whittaker M.A. Bioproducts, Walkersville, MD) was performed as described by the manufacturer.

Adjuvanticity Both in vivo (59) and in vitro (26) assays were used to determine adjuvant activity of the stimulants. In vivo, BALB/cByJ mice were given an interperitoneal injection of a suboptimal dose of sheep red blood cells (SRBC, 0.5%). Mice were simultaneously treated i.p. with either LPS or endotoxin at the indicated doses. After 4 days, mice were sacrificed by cervical dislocation, spleens were removed, minced, washed in Hank's balanced salt solution, and resuspended. The anti-SRBC plaque forming cell (PFC) response was determined using the Cunningham slide method (26).

The in vitro immune response to SRBC was examined using spleens from BALB/cByJ and C3H/HeN mice, previously primed with 0.05% SRBC i.p. 3 days earlier. Mice were sacrificed and the spleens were aseptically removed, minced, the cells were washed and resuspended to 1×10^7 cells/ml in minimal essential medium (MEM) supplemented with 37 μg/ml $NaHCO_3$, 67 μg/ml gentamycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 5×10^{-2} mM 2-mercaptoethanol, and 10% fetal calf serum (FCS, JR Scientific, Irvine, CA). One half ml of cell suspension was added to each well of a 24 well cluster dish (Costar #3424, Cambridge, MA). Thirty μl of a 1% SRBC suspension (5 ml of 10% washed SRBC in glucose (0.5

mg/ml)-PBS treated with neuraminadase (0.01 unit/ml) for 1 hour at 37°C) (30) in HBSS were added to all wells except controls. LPS or endotoxin was added at the indicated concentrations. Cultures were incubated for 5 days at 37°C in modular inculator chambers (Billups-Rothenberg, Del Mar, CA) flushed with a 7% O_2, 10% CO_2, 83% N_2 gas mixture. Cultures were fed daily with 1 drop of a Mishell-Dutton feeding cocktail (18.0 ml Hanks Balanced salt solution (10x), 20 ml of a 0.1 g dextrose/ml HBSS, 8 ml MEM amino acid solution (50x), 4 ml nonessential amino acid (100x), 4 ml 200 mM L-glutamine, 15 ml 7.5% $NaHCO_3$, 0.6 ml gentamycin (50 mg/ml), supplemented with fetal calf serum (33% final concentration)). Cultures were harvested on day 5 and assayed for the anti-SRBC PFC response performed as described above.

Pyrogenicity New Zealand White rabbits (3 - 4 kg) were injected with LPS or endotoxin i.v. in the marginal ear vein at the indicated dose in 0.5 ml pyrogen-free saline. Rectal temperatures were taken every 15 - 20 minutes with a digital thermometer (Norelco, Stamford, CT).

Dermal Shwartzman reaction New Zealand White rabbits weighing 3 - 4 kg had their backs shaved 24 hours prior to receiving preparative intradermal injections (i.d.) of LPS or endotoxin in 0.1 ml. Twenty-four hours after the preparative administration, 50 μg *E. coli* LPS in 0.2 ml pyrogen-free saline was given i.v. in the marginal ear vein (provocative dose). The dermal injection sites were observed at 24, 30, and 48 hours for induration, erythema and necrosis. Animals were euthanized at 48 hours and tissue was taken for routine histologic examination (hematoxyalin and eosin staining) (19).

Reagents Unless stated otherwise, all reagents and chemicals were obtained from Sigma Chemical Company, St. Louis, MO.

Statistics The results are expressed as the mean ± S.E.M. where applicable, and significance was determined using the student t test.

RESULTS

Chemical analysis of LPS and endotoxin The chemical analyses shown in Table 1 indicate that the treponemal LPS preparations containing negligible protein and a large percentage of carbohydrate (almost 80% for *T. hyodysenteriae* and 47% for *T. innocens*). The endotoxins had a larger amount of protein than the LPS preparations. The carbohydrate content was similar in 3 of the 4 preparations examined (Table 1) while the *T. hyodysenteriae* LPS contained almost twice the amount of carbohydrate in comparison to other preparations. The KDO content was determined to be approximately 0.4 - 0.45 % except in the *T. hyodysenteriae* LPS where the amount was approximately 0.12 %.

TABLE 1. Chemical analysis of treponemal LPS and endotoxin

	T. hyodysenteriae		*T. innocens*	
Component	LPS	Endotoxin	LPS	Endotoxin
Protein (%)	<0.1[a]	8	<0.1	26
Hexose (%)	76	34	47	38
KDO (%)	0.12	0.45	0.45	0.4

[a]Values are expressed as percent weight (w/w).

SDS-PAGE Silver stained polyacrylamide gels (Fig. 1A) showed a distinct profile with 5 bands for the *T. hyodysenteriae* LPS and an

unresolved profile for the *T. innocens* LPS. As can be seen in Figure 1A,lane E, the *T. innocens* LPS failed to resolve into distinct bands or a "tiger tail" pattern as is associated with the enteric LPS (lane B). However, *T. innocens* endotoxin (lane F) did resolve into distinct bands but failed to migrate into the gel as far as either the *T. hyodysenteriae* or *E. coli* preparations. Coomaisse blue stained gels (Fig. 1B) showed a major protein band with an apparent molecular weight of 35 - 40 kDa in both *T. hyodysenteriae* and *T. innocens* endotoxin preparations. *T. innocens* endotoxin also showed 3 minor protein bands (Fig. 1B, lane F).

Mitogenic response Treponemal endotoxins were observed as very potent mitogens of BALB/cByJ spleen cells (Fig. 2). The treponemal LPS preparations failed to stimulate a measurable mitogenic response. In contrast to the lack of a response induced by treponemal LPS, the treponemal endotoxin preparations stimulated a mitogenic response in LPS-hyporesponsive C3H/HeJ mice (data not shown).

Endotoxin activity The relative toxicity of LPS and endotoxin preparations has been examined by mouse lethality. The LD_{50} of *T. hyodysenteriae* LPS and endotoxin was determined to be 350 μg/mouse and 80 μg/mouse, respectively (data not shown). This is in comparison to *E. coli* strain K235 LPS which was previously determined to have an LD_{50} of 0.6 μg/mouse (59).

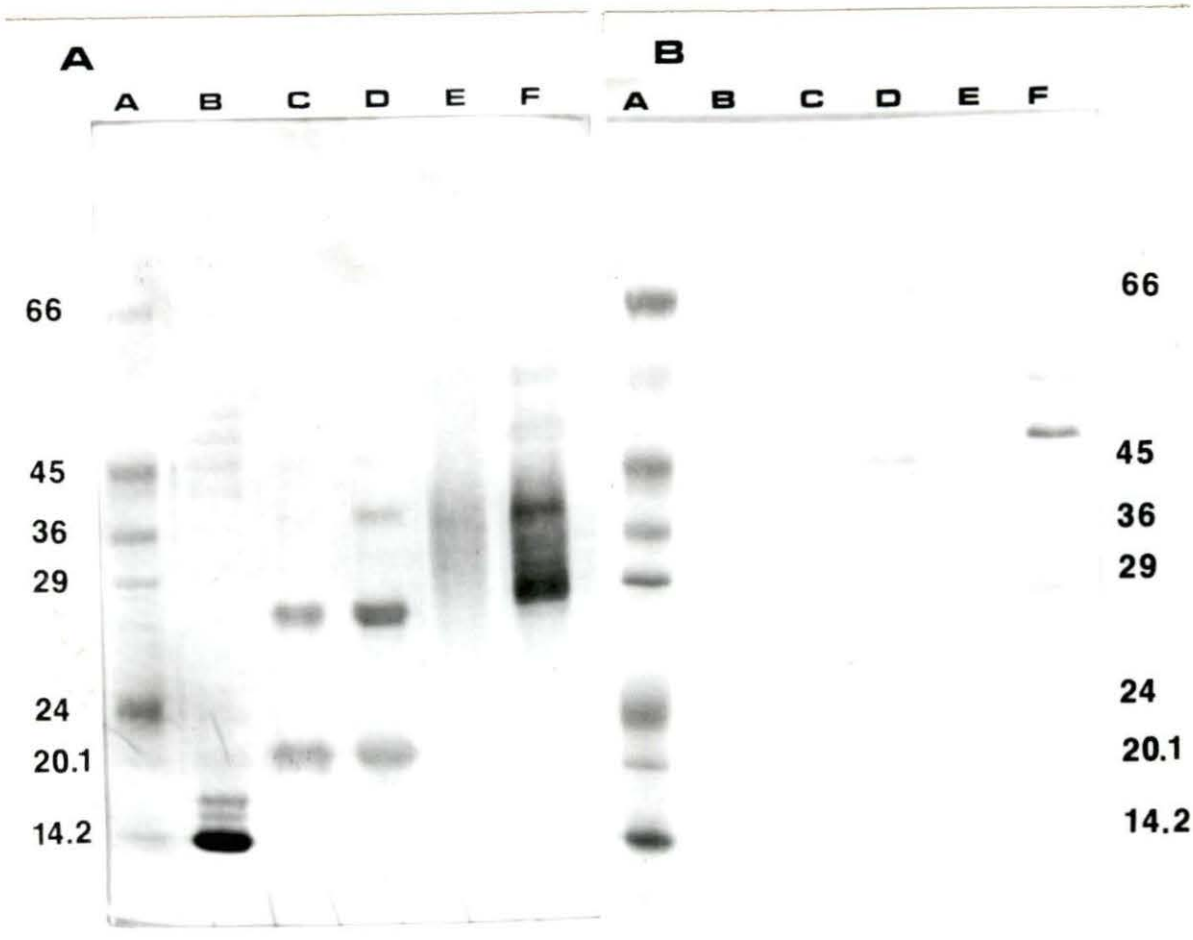


FIG. 1. Electrophoretic separation of LPS and endotoxin from *T. hyodysenteriae* strain B204 and *T. innocens* strain B1555a were performed on a 7.5 - 15% separating gel. The LPS and endotoxin were visualized by silver staining (A) and by Coomaisse blue staining (B). Lane A., molecular weight standard; B., *E. coli* LPS, 20 μg; C., B204 LPS, 80 μg; D., B204 endotoxin, 40 μg; E., B1555a LPS, 40 μg; F., B1555a endotoxin, 40 μg. Molecular weight determinations (KDa) are indicated on left side of panel A and the right side of panel B.

Limulus amebocyte lysate assay The LAL assay indicated that the treponemal preparations had endotoxin units/ng comparable to standard *E. coli* LPS. It was determined there were 1.2 and 1.4 units endotoxin activity per ng *T. hyodysenteriae* LPS and endotoxin, respectively. *T. innocens* LPS and endotoxin had 0.2 and 2.4 units of endotoxin activity per ng. This was compared to the 1.2 units of endotoxin activity per ng of *E. coli* K235 LPS.

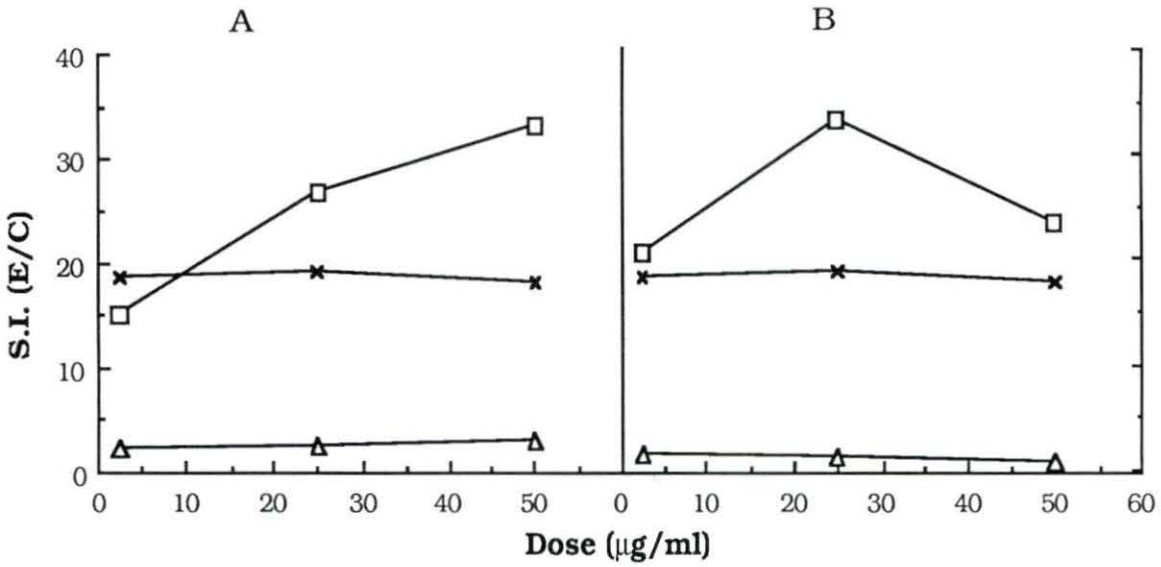


FIG. 2. Mitogenic responses of BALB/cByJ splenocytes stimulated by treponemal LPS or endotoxin. The assay was measured by ^{3}H-thymidine incorporation. A.) *T. hyodysenteriae* B204 LPS (triangle); B204 endotoxin (square); *E. coli* LPS (x); B.) *T. innocens* B1555a LPS (triangle); B1555a endotoxin (square); and *E. coli* LPS (x). Values from triplicate cultures are expressed as a stimulation index (S.I.) defined as the experimental CPM/ control CPM (E/C).

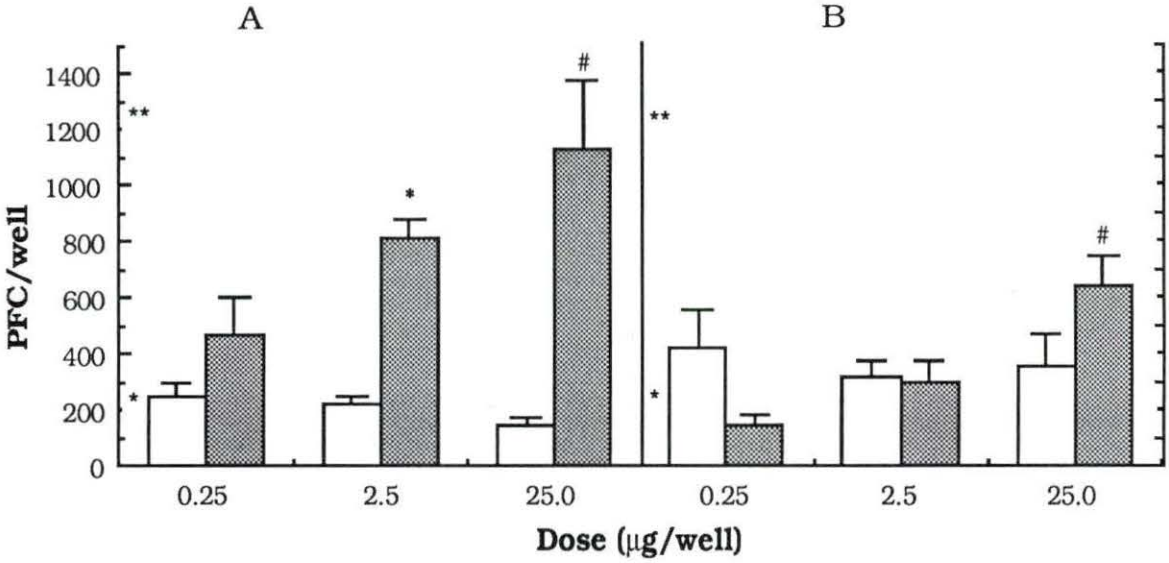
Adjuvanticity The adjuvant activity of LPS and endotoxin from both *Treponema* species was determined in vitro (Fig. 3) and in vivo (Fig. 4) using SRBCs as the test antigen. The results depicted in Figure 3 demonstrate that the treponemal LPS was a weak adjuvant in comparison to *E. coli* LPS or the treponemal endotoxins. In the in vivo test (Fig. 4), the *T. hyodysenteriae* preparations at 100 μg/mouse did not enhance PFC response as well as *E. coli* LPS at 10 μg/mouse.

Pyrogenicity Rabbits were injected with 50, 100, and 250 μg of the treponemal LPS or endotoxin. The results presented in Figure 5 demonstrated that 250 μg of the treponemal preparartions were less pyrogenic than 10 μg of *E. coli* LPS. A mild febrile response was noted in rabbits treated with the *T. hyodysenteriae* endotoxin (Fig. 5A). Niether the LPS or endotoxin preparartion of *T. innocens* were pyrogenic (Fig. 5B). There was no pyrogenic response observed in rabbits receiving 50 or 100 μg of the treponemal preparations (data not shown).

Local Shwartzman reaction Gross lesions induced by LPS and endotoxin of the *Treponema* species were either nonexistent or consisted of very mild edema and erythema, while the *E. coli* LPS induced a necrotic lesion (Fig. 6). Hematoxylin-eosin staining of sections treated with *T. hyodysenteriae* endotoxin (500 μg) as the preparatory treatment and *E. coli* LPS as the provocative dose showed a mild cellular response with some edema. At 500 μg, the *T. innocens* endotoxin induced a response with multifocal areas of inflammatory

cells. No lesions were observed on sites inoculated with the treponemal LPS preparations.

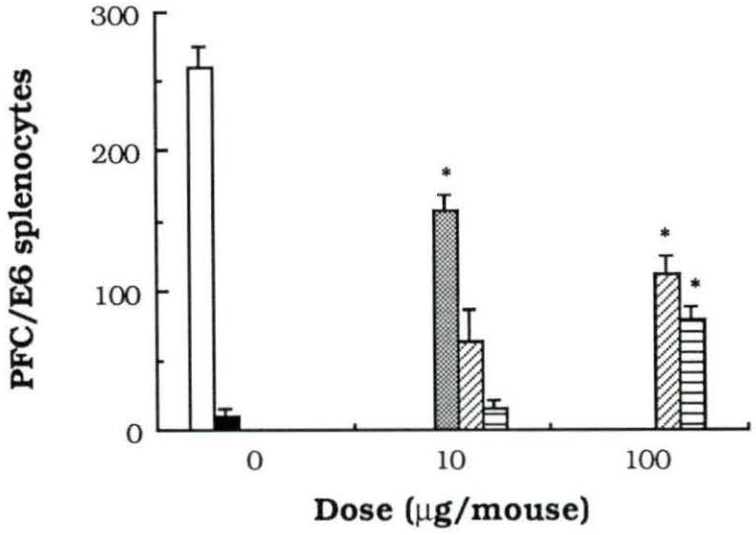
Experiments were performed using either *E. coli* LPS or *T. hyodysenteriae* LPS as the provocative dose for the Shwartzman reaction with no difference in the resulting responses (data not shown).



* Significant at the 0.05 level.

\# Significant at the 0.1 level.

FIG. 3. In vitro anti-SRBC PFC response. To measure adjuvant activity, C3H/HeN murine splenocytes were treated with 30 μl of 10% SRBCs with the indicated dose of treponemal LPS or endotoxin. On day 5, the anti-SRBC PFC response was measured. A.) *T. hyodysenteriae* strain B204; B.) *T. innocens* strain B1555a; LPS (open bars); endotoxin (shaded bars); SRBC alone, (single star); and SRBC plus 10 μg *E. coli* LPS (two stars). Values are expressed as the mean ± S.E.M. anti-SRBC PFCs per million splenocytes from triplicate cultures.



*Significant at the 0.05 level.

FIG. 4. The in vivo adjuvant activity of *T. hyodysenteriae* preparations were determined by injecting BALB/cByJ mice i.p. with 0.2 ml of a 0.5% SRBC suspension containing the indicated dose of LPS or endotoxin. On day 4, anti-SRBC PFC responses were determined. 10% SRBC (open bar); 0.5% SRBC (closed bar); *E. coli* LPS plus 0.5% SRBC (shaded bar); *T. hyodysenteriae* strain B204 LPS plus 0.5% SRBC (hatched bar); B204 endotoxin plus 0.5% SRBC (horizontal hatched bar). Values are expressed as the mean ± S.E.M. anti-SRBC PFC response per million splenocytes using 5 mice per group.

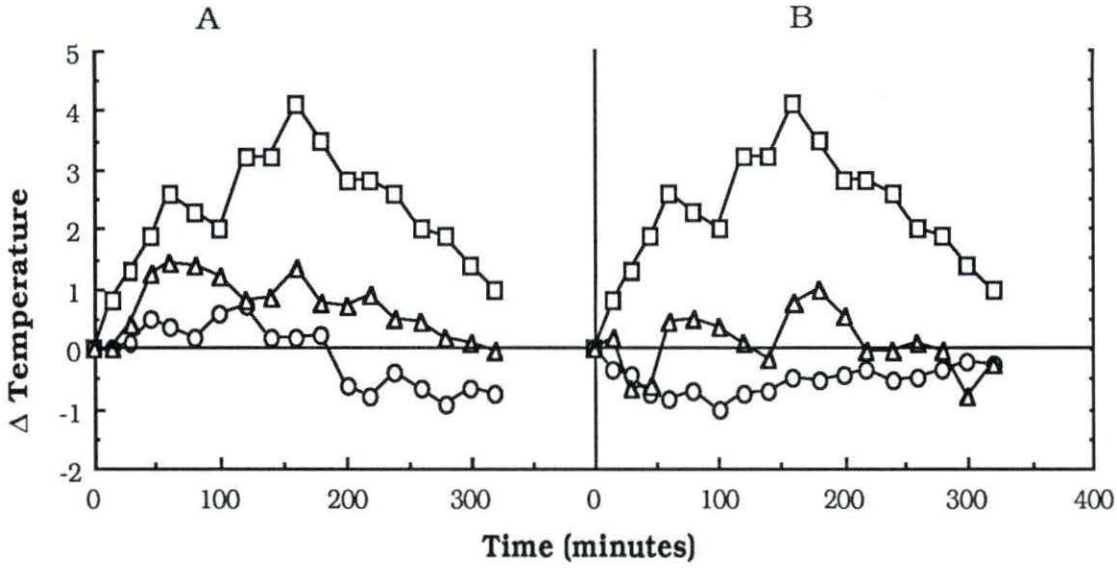


FIG. 5. Rectal temperatures of rabbits stimulated with LPS or endotoxin of *Treponema* species were measured. The results are expressed as the average change in temperature of 2 rabbits following treatment with either 250 μg of a treponemal preparation or 10 μg of *E. coli* LPS (square). A.) *T. hyodysenteriae* B204 LPS (circle); B204 endotoxin (triangle); B.) *T. innocens* B1555a LPS (circle); B1555a endotoxin (triangle).

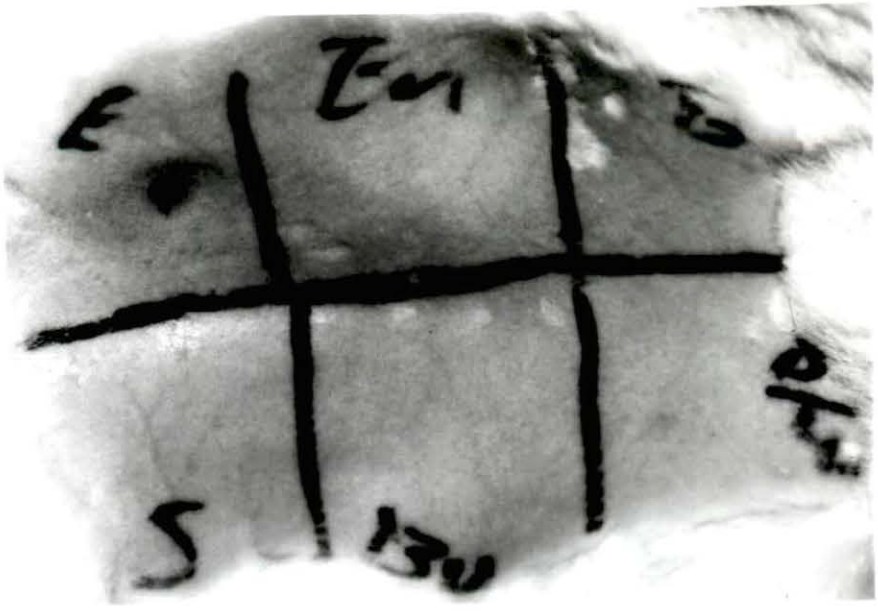


FIG. 6. Dermal Shwartzman reaction. Rabbits received an i.d. injection of LPS or endotoxin from each treponemal species. Two groups (two rabbits/group) were given an i.v. injection in the marginal ear vein of either *T. hyodysenteriae* endotoxin or *E. coli* LPS. The rabbit depicted received 50 μg of *E. coli* LPS i.v. 20 hours after i.d. injections. The injection pattern from left to right on the top row was *E. coli* LPS (100 μg), *T. hyodysenteriae* LPS (100 μg), *Bacteroides fragilis* LPS (100 μg); bottom row was saline, *T. hyodysenteriae* endotoxin (250 μg), *T. hyodysenteriae* LPS (250 μg).

DISCUSSION

Lipopolysaccharide of *T. hyodysenteriae* has been previously isolated and characterized as containing 85 - 90% carbohydrate and 5 - 10% protein (3). The use of treponemal LPS and rabbit anti-serum established seven serotypes (3, 25). A study of the biologic activity of *T. hyodysenteriae* strain B204 LPS reported the preparation to be mitogenic, toxic, and enhanced phagocytosis (37). Nuessen and Joens (38) demonstrated that the LPS was responsible for the serotype-specific opsonization of *T. hyodysenteriae* using convalescent pig serum. Additionally, Nuessen et al. (39) reported that the LPS of *T. hyodysenteriae* was lethal to C3HeB/FeJ mice but not for LPS-hyporesponsive C3H/HeJ mice, and was chemotactic for murine macrophage. These papers suggested that the LPS of *T. hyodysenteriae* had biologic activity and played a role in the pathogenesis of swine dysentery.

Other spirochetes, besides *T. hyodysenteriae*, have been shown to contain LPS (4, 8, 15, 29, 56). Mergenhagen et al. (29) demonstrated the presence of endotoxin in *B. buccalis* and *B. vincentii*. The presence of LPS has also been reported in *B. burgdorferi*, the Lyme disease spirochete (4). *B. burgdorferi* LPS was pyrogenic, mitogenic, and clotted the LAL (4). However, Takayama et al. (54) have challenged the existence of LPS in *B. burgdorferi* since they were unable to demonstrate the presence of LPS in either a phenol/water extract or a phenol/chloroform/petroleum ether extract of the organism. This conclusion was based on the inability to detect KDO, glucosamine, and

hydroxy fatty acids in the phenol/water extracts or the ability of the preparations to induce either prostaglandin E_2 production or gelation of the LAL (54).

Isogai et al. (15) and Vinh et al. (56) have isolated LPS from *Leptospira interrogans.* The *L. interrogans* LPS was shown to be toxic for mice and produced a febrile response in rabbits but neither response was comparable to the responses obtained with *E. coli* LPS (15). The leptospiral LPS also induced adjuvant activity, induced clot formation in the LAL, and caused bone marrow and liver necrosis in treated mice (15). Vinh et al. (56) determined that the fatty acid composition of LPS from *L. interrogans* consisted of hydroxylauric ($C_{12:0}$), palmitic ($C_{16:0}$), and oleic acid ($C_{18:1}$) without any C_{14} fatty acids (i.e., myristic acid ($C_{14:0}$) and 3-hydroxymyristic acid (3-OH-$C_{14:0}$)) (56). They also demonstrated that the *L. interrogans* LPS contained an unusual form of KDO (56). In contrast to Takayama et al. (54), the biochemical and biological information indicate the presence of an LPS-like molecule in *L. interrogans.*

In the present study, the treponemal LPS preparations (Table 1) were shown to have a negligible protein component, while the *T. innocens* and *T. hyodysenteriae* endotoxin preparations contained 26% and 8% protein, respectively. The presence of protein in the endotoxin preparations was expected due to the nature of the butanol/water extraction procedure (33, 51, 57, 58). However, the three-fold difference in protein content between the endotoxin preparations was not understood. The carbohydrate content of the *T.*

hyodysenteriae LPS was similiar to that reported previously by Baum and Joens (76% and 89%, respectively) (3). As expected, treponemal endotoxins had a smaller proportion of carbohydrate than the LPS preparations due to the increase in the protein content in the sample. The KDO content (0.4 - 0.45%) was similiar in 3 of the 4 treponemal preparations. However, the KDO content (0.12%) of *T. hyodysenteriae* strain B204 LPS was almost four times less than the other preparations. These differences may be due to the physicochemical nature of the molecule extracted by phenol/water vs. butanol/water techniques and/or the differences between species of *Treponema*. Using gas-liquid chromatography, the LPS of *T. hyodysenteriae* was shown to contain 14 and 16 carbon fatty acids, glucosamine, KDO, heptose, rhaminose, mannose, galactose and glucose (personal communication, Dr. Hyoik Ryu, Veterinary Microbiology and Preventive Medicine, Iowa State University). In addition, mild acid hydrolysis of treponemal LPS preparations yielded a water insoluble precipitate similar to that obtained from *E. coli* LPS (11). Collectively, these results indicate that the phenol/water preparations from *T. hyodysenteriae* contain LPS-like molecules.

The LAL assay has been used to measure endotoxin activity, but not quantity, in biological materials (28). This assay demonstrated that the treponemal preparations had endotoxic activity comparable to *E. coli* LPS.

Treponemal preparations were analyzed by SDS-PAGE and two different profiles were obtained for the *T. hyodysenteriae* and *T.*

innocens preparations (Fig. 1). In comparison to the *T. hyodysenteriae* LPS (Fig. 1A, lane C), the *T. innocens* LPS (Fig. 1A, lane E) did not migrate as far into the gel and did not resolve into distinct bands. The endotoxin preparation of *T. innocens* was also less mobile in the gel but did resolve into numerous bands (Fig. 1A, lane F). These observations may be related to the differences in lipid content previously reported by Matthews et al. (27). However, these apparent physicochemical differences did not result in varying biologic activities between the *T. hyodysenteriae* and *T. innocens* preparations.

Even though the treponemal LPS preparations were not mitogenic, the endotoxin preparations from both *T. hyodysenteriae* and *T. innocens* induced substantial mitogenic responses in murine splenocytes (Fig. 2). These results are in contrast to a previous report in which *T. hyodysenteriae* LPS was shown to be mitogenic (37). However, the protein content of the LPS preparation was reported as 8% (37) and this would be consistent with the endotoxin preparations used in the present study. The protein component(s) of endotoxin preparations have been shown to be mitogenic (52). This would suggest that the protein and not the lipid A component of the endotoxin complex from the *Treponema* species was the major mitogenic moiety.

In comparison to *E. coli* LPS (LD_{50} = 0.6 μg) (59), the *T. hyodysenteriae* B204 LPS (LD_{50} = 350 μg) and endotoxin (LD_{50} = 80 μg) were at least 100 times less toxic for galactosamine treated mice. In addition to being mitogenic, the protein component of endotoxin

appeared to potentiate the toxicity of the LPS molecule. However, there is no reported evidence to suggest that the enterobacterial LAP or porin proteins are toxic (36).

Endotoxin and LPS has adjuvant activity which non-specifically activates the immune response (44). Adjuvant activity of the treponemal preparations were examined both in vitro (Fig. 3) and in vivo (Fig. 4). It was observed, in vitro, that *T. hyodysenteriae* LPS failed to induce an adjuvant response while the endotoxin did induce a significant adjuvant response at 2.5 μg per culture (Fig. 3A). The *T. innocens* LPS, like the *T. hyodysenteriae* LPS, was a poor adjuvant but the endotoxin preparation stimulated the anti-SRBC response at 25 μg per culture (Fig. 3B). In contrast to the in vitro data, LPS from *T. hyodysenteriae* as well as endotoxin (Fig. 4) induced in vivo adjuvant responses to SRBCs in vivo. However, the responses observed, even with 100μg of LPS or endotoxin, were not as strong as the response obtained with 10μg of *E. coli* LPS. As for the mitogenic activity, the enhanced adjuvant activity of the endotoxin preparations in vitro may be due to the presence of LAP or porins (52).

Endotoxin and LPS may contribute to the pathogenesis of a disease by eliciting a pyrogenic response in the host. The ability of the treponemal preparations to elicit a febrile response in New Zealand White rabbits was examined (Fig. 5). The endotoxins (250 μg/rabbit) of both species induced a slight but discernible biphasic febrile response in rabbits, while the LPS from both species failed to induce detectable febrile responses. Even though as much as 25 times the amount of *E*

coli LPS was given to the rabbits, the treponemal LPS preparations were not pyrogenic. The mild pyrogenic activity observed with the endotoxin preparations may have been associated with the protein component and/or their ability to stimulate endogenous pyrogens (i.e., interleukin 1 and tumor necrosis factor (Fig.1 and 3, pages 66 and 68, Section II)).

A local Shwartzman reaction is often used as a measure of the relative toxicity of an endotoxic preparation. This response is observed in rabbits which have received an i.d. injection of LPS (priming dose) followed 18 - 24 hr later with an i.v. injection of LPS (provocative dose). Regardless of the source of the provocative dose, dermal necrosis was not observed when treponemal preparations were used as the priming dose (Fig. 6). However, a necrotic lesion was obtained when an *E. coli* LPS priming dose was followed by a *T. hyodysenteriae* endotoxin provocative dose (personal observation). The ability of the treponemal endotoxin to serve as the provocative but not the priming dose appears somewhat contradictory. The mechanisms by which a local Shwartzman reaction is induced are not clearly understood, but the activation of the host's coagulative enzymes and the deposition of fibrin thrombi in tissue play a major role in the development of necrotic lesions (35). The actions of the two injections are assumed to be the same; however, it is likely that the priming injection renders the host hyper-responsive to the provocative dose. The inability of the treponemal preparations to prime the rabbit for a local Shwartzman reaction would indicate that the host is not rendered hyper-responsive

and this would be consistent with the reduced toxicity and pyrogenicity observed. In addition, the ability of the treponemal preparations to provoke a Shwartzman reaction would be consistent with the ability of the treponemal preparations to gel the LAL. Even though the treponemal preparations are not as active as *E. coli* LPS, they do possess some endotoxic properties and may contribute to the development of lesions during infection with *T. hyodysenteriae.*

In conclusion, the results indicated that the treponemal preparations were much less active than *E. coli* LPS in that the LPS of both treponemal species did not induce an inflammatory response, were not mitogenic, pyrogenic, or lethal even at high doses. In comparison to the LPS preparations, the endotoxins were more toxic, were able to provoke a Shwartzman reaction, and induced good mitogenic responses. This suggested that the presence of protein renders the endotoxin complex biologically active or that the activity is due to the protein itself. Even though the role of LPS in the development of dysenteric lesions is not fully understood, the virulence or avirulence of *T. hyodysenteriae* and *T. innocens* can not be attributed solely to the biologic activity of LPS or endotoxin.

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SECTION II. ABILITY OF TREPOMENAL LPS AND ENDOTOXIN TO INDUCE CYTOKINE RELEASE FROM LYMPHORETICULAR CELLS

ABSTRACT

The biologic activity of lipopolysaccharide (LPS, phenol/water) and endotoxin (butanol/water) preparations from virulent *Trepomena hyodysenteriae* and avirulent *T. innocens* was examined. The treponemal preparations showed toxic activity on murine peritoneal exudate cells (PEC) though not at concentrations as low as *E. coli* LPS. Both treponemal LPS preparations showed no stimulatory activity for induction of interleukin 1 or tumor necrosis factor from PECs; however, the endotoxins did stimulate induction of these products but at doses 50 fold higher than *E. coli* LPS. Natural killer cell activity was augmented by treponemal endotoxin-treated mice. The *T. innocens* endotoxin augmented NK activity to levels equivalent to *E. coli* LPS. Suppression of the immune response was observed by the *T. hyodysenteriae* preparations. The results suggest that no major differences exist in the biologic activities between *T. hyodysenteriae* and *T. innocens* LPS or endotoxin.

INTRODUCTION

Lipopolysaccharide (LPS) and endotoxin have been extensively studied and have been the subject of numerous review articles (i.e., 4, 23, 25, 26). The biological effects attributed to LPS and endotoxin have been established using preparations isolated from *Escherichia coli* and *Salmonella* species. These molecules have multiple effects on the immune system which affect B lymphocytes, T lymphocytes, macrophages, as well as other cells of the body. Of these cells, macrophages play a major role in the immune system as antigen presenting cells (39). Macrophages also interact with lymphocytes, are highly bactericidal and tumoricidal, and secrete a wide range of bioactive molecules (39).

The list of cytokines secreted by macrophage is quite extensive. Stimulation and/or activation of macrophage causes production and secretion of interleukin 1 (IL 1) (21), tumor necrosis factor (TNF) (3), interferon (14), as well as other products such as collagenase, prostaglandins (1), and complement components (26). Through secretion of these cytokines, B cells are affected (26), T cells are induced to produce interleukin 2 (39), and natural killer cell activity is augmented (7b). Because of the numerous activities induced by the LPS molecule, many host defense mechanisms against gram negative bacteria are stimulated and aid in recovery from disease. For example, LPS induces fever which speeds up metabolism to fight an infection and

it can initiate the coagulation system which would contain an infection (26, 25).

In addition to the *Enterobacteriaceae* there are numerous gram negative organisms which have LPS with varying biological and chemical characteristics. Some examples include *Pseudomonas aureginosa* (30), *Bacteroides fragilis* (17), and *Brucella abortus* (22, 35). *P. aureginosa* has small structural differences in its LPS when compared to the enteric LPS but this LPS has biologic activity in the LPS-hyporesponsive C3H/HeJ mouse (30, 32). The LPS of *B. fragilis* contains KDO (31), heptoses, has little O-specific polysaccharide, and has a fatty acid composition that is quite divergent when compared to *E. coli* LPS (42). *B. abortus* LPS is isolated from the phenol phase of a phenol/water extraction method (Westphal and Jann) (19). This LPS was different from classical LPS in that *Brucella* LPS stimulate spleen cell mitogenesis from LPS-hyporesponsive C3H/HeJ mice (22, 31, 32, 35, 41).

Spirochetes are also gram negative organisms. *Leptospira interrogans* and *Borrelia burgdorferi* possess a rough type of LPS and induce varying biologic effects on the host immune system in comparison to *E. coli* LPS (2, 6, 40). *L. interrogans* LPS was reported to cause fever without a biphasic response, acted as an adjuvant, and was less lethal than *E. coli* LPS (15). *B. burgdorferi* LPS was reported to be mitogenic, pyrogenic, clot the Limulus amebocyte lysate, and stimulate interleukin 1 production (2, 11).

Nuessen et al. (28) first implicated the LPS of *Treponema hyodysenteriae* as a virulence attribute in 1982. It was also reported that the LPS was toxic to murine macrophages and enhanced both complement (C3) and immunoglobulin G-Fc receptor-mediated phagocytosis (28). Additionally, the LPS was mitogenic for murine splenocytes and stimulated chemotaxis of porcine leukocytes (28). In 1983, Nuessen et al. (29) reported that *T. hyodysenteriae* LPS was lethal for LPS-responsive mice but not for LPS-hyporesponsive C3H/HeJ mice.

The present report compares the biologic activity of LPS and endotoxin extracted from *T. hyodysenteriae* and *T. innocens*. Results indicate that the treponemal LPS was toxic for murine macrophages, a poor inducer of monokines, and failed to induce natural killer cell activity. However, the endotoxin preparations were often potent stimulators of lymphoreticular cells, though no as potent as *E. coli* LPS.

MATERIALS AND METHODS

Bacteria and cell lines *Treponema hyodysenteriae* strain B204 and *T. innocens* strain B1555a were obtained from at Iowa State University (ISU), Ames, IA. *Escherichia coli* strain K235 was obtained from Dr. Suzanne Michalek, Department of Microbiology, University of Alabama in Birmingham. The *Treponema* species were grown in trypticase soy broth containing dextrose (BBL #11768, Cockeysville, MD) and supplemented with 5 g/l yeast extract (BBL), 20 ml of each VPI A and B salt solutions (stock A - 0.4 g $CaCl_2$, 0.4 g $MgSO_4$, 1 liter H_2O; stock B - 2.0 g K_2HPO_4, 2.0 g KH_2PO_4, 20.0 g $NaHCO_3$, 1 liter H_2O), and 0.5 g/l L-cysteine. Medium was adjusted to pH 7.3. After autoclave sterilization, 50 ml/l horse serum (Hyclone Laboratories, Inc., Logan, UT) was added and cultures grown under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2) for 18 - 24 hours. The cells were harvested by centrifugation at 10,000 x g for 20 minutes, washed twice in phosphate buffered saline (PBS - 8 g NaCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, to 1 liter H_2O, pH 7.2) and once in distilled water. Whole cells were frozen at -20°C until the LPS or endotoxin extraction was performed.

The cell lines used were purchased from the American Type Culture Collection and grown as prescribed. The IL 1 assay was run using the IL 1 dependent T helper cell clone D10.G4.1 (TIB 224). The TNF assay used a TNF sensitive, murine fibrosarcoma cell line L929 (CCL 1) (5), and to act as a target cell for the natural killer assay, YAC-1 cells (TIB 160) were used.

Animals Original breeder pairs of C3H/HeJ and BALB/cByJ mice were obtained from Jackson Laboratory (Bar Harbor, ME), and C3H/HeN mice were obtained from Harlan Sprague Dawley (Madison, WI). Mice were housed at the Laboratory Animal Resources (LAR) facility at the College of Veterinary Medicine, ISU. The mice were given autoclave-sterilized water and feed (Purina Lab Chow #5010, Purina Mills, Inc., St. Louis, MO) *ad libitum*.

LPS and endotoxin extraction Lipopolysaccharide was extracted by a modified hot phenol/water extraction procedure (41). Briefly, 200 mg of lyophilized whole cells were suspended in 10 ml water and mixed with an equal volume of liquified phenol at 68°C. The mixture was stirred for 15 minutes at 68°C. After cooling, the mixture was centrifuged at 200 x g to separate the two phases. The aqueous phase was collected and saved and the phenol phase was extracted twice with half the original volume of water. The aqueous phases were pooled and dialyzed exhaustively against distilled water. The LPS containing material was concentrated and then centrifuged at 100,000 x g for four hours. The pellet was resuspended in pyrogen-free water and the centrifugation was repeated two times. After the last centrifugation, the pellet was resuspended in pyrogen-free water and lyophylized. The LPS was stored at -20°C until used. *E. coli* strain K235 LPS was prepared by the Westphal and Jann method (41).

Endotoxin was prepared by the butanol/water extraction procedure (29). Briefly, 0.25 g wet weight of whole cell paste was suspended in 1 ml water with an equal volume of butanol added. The endotoxin

partitioned to the aqueous phase below the butanol. The butanol phase was extracted with half the original volume of water three times. The pooled aqueous phase was digested with Pronase (20 μg/ml final concentration) in 0.2 M Na_3PO_4 buffer (pH 7.0) overnight at 37°C. The digested endotoxin preparation was centrifuged at 10,000 x g for 40 minutes and the precipitated interphase removed. The endotoxin solution was exhaustively dialyzed, concentrated, lyophylized, and stored at -20°C until assays were performed.

The LPS and endotoxin preparations were dissolved in pyrogen-free saline and sterilized by heating to 100°C for 10 minutes. These solutions were stored at 4°C until use. The prepartations were heated for 1 - 2 minutes at 100°C before use in assays.

Macrophage viability Peritoneal exudate cells (PECs) from BALB/cByJ mice were harvested by peritoneal lavage 3 - 4 days after an interperitoneal injection of 2 mls fluid thioglycollate (Difco, Detroit, MI). PECs were lavaged with phosphate buffered saline (PBS - 8 g NaCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, to 1 liter H_2O, pH 7.2) supplemented with 10 units/ml heparin and 1% fetal calf serum (FCS, JR Scientific, Irvine, CA). PECs were washed in RPMI supplemented with 37 mg/ml $NaHCO_3$, 25 units/ml penicillin, 25 μg/ml streptomycin (P/S), 2 mM L-glutamine (L-glu), and 1% FCS, then resuspended in the same medium at 5 x 10^6 cells/ml and aliquoted into sterile 13 x 100 mm polypropylene snap cap tubes. Dilutions of LPS or endotoxin were added to the tubes. The tubes were rotated end over end at 37°C for 24 hours and then stained with 10 μl of PBS containing 0.5 mg/ml

propidium iodide (33) and/or 100 µl of PBS containing a 1:1000 dilution of a 0.5% (w/v) fluorescein diacetate-acetone stock solution. Using fluorescent microscopy, this procedure allows the differentiation of live (green) and dead (orange to red) cells. Cells were incubated for 15 - 30 minutes at room temperature and were counted using a fluorscent microscope (Leitz Orthoplan, Wetzlar, Germany) or a fluorescence activated cell sorter (Coulter, Luton, England).

Interleukin 1 assay Thioglycollate elicited PECs from BALB/cByJ mice were harvested (see above), washed, resuspended, and adjusted to 1 x 10^6 cells/ml in RPMI 1640 (P/S, L-glu, $NaHCO_3$). Cultures (1 ml) were stimulated for varying lengths of time with various doses of LPS or endotoxin and incubated at 37°C in 5% CO_2. At the indicated time, supernatant fluid was harvested by centrifugation and stored at -70°C until use. D10.G4.1 cells were centrifuged through a density gradient (Ficoll-paque 1.077) to concentrate live cells. After washing three times to remove excess gradient material, cells were resuspended to 5 x 10^5 cells/ml in RPMI 1640 (P/S, L-glu, 10 mM HEPES, 5% FCS) and 0.1 ml was added to each well of a 96 well microtiter plate (Costar #3799, St. Louis, MO). Cultures were incubated 72 hours and 0.5 µC of ^{3}H-thymidine (Arlington Heights, IL) was added during the last 8 hours. Samples were harvested on to filter paper discs with an automatic harvester (Bellco, Vineland, NJ), dried, and counted using liquid scintillation.

Tumor necrosis factor assay L929 cells were scrapped and resuspended to 5 x 10^5/ml in MEM (P/S, L-glu, $NaHCO_3$)

supplemented with 10% horse serum (Gibco, Irvine, CA). One tenth ml was added to each well of a 96 well microtiter plate (Costar #3799) and incubated overnight at 37°C in 5% CO_2. Supernatant fluid from LPS or endotoxin stimulated PECs was added at increasing dilutions along with MEM medium containing actinomycin D (final concentration of actinomycin D in a well was 2.5 μg/ml). Anti-TNF rabbit serum (Genzyme, Orvala, FL) was added to additional wells to inhibit TNF activity present in the supernatant. This assay was terminated after 18 hours by washing plates with PBS to remove nonadherent cells. Residual monolayers were stained with a 0.2% crystal violet - 2% formalin solution for 20 minutes (10). Plates were washed in water 3 times, with the third wash standing 10 minutes. The stain was then solublized in 50% ethanol and quantitated by measuring the adsorbance at 595 nm on an ELISA reader (Biotek Instruments model EL 301, Winooski, VT) (34). Quadruplicate wells were set up with results expressed as the percent cytotoxicity ± S.E.M.

Natural killer cell assay C3H/HeN mice were injected i.p. 24 hours prior to the assay with LPS or endotoxin. Mice were sacrificed by cervical dislocation and individual spleens were removed and minced into single cell suspensions. Red blood cells were removed by hypotonic shock induced by resuspending each spleen in 2 ml of RPMI 1640 (1% FCS, P/S, L-glu, HEPES) to which 6 ml of sterile distilled water was added. After 30 seconds, 2 mls of 3.5% saline was added. Cells were then washed three times in the same medium to remove red blood cell debris. Cells were then resuspended to 5 $x10^6$ cells/ml in

NK medium (RPMI 1640 supplemented with P/S, L-glu, HEPES, 10% FCS, 5 x 10^{-4} M 2-mercaptoethanol) and 0.1 ml added to each well of a 96 well U-bottom microtiter plate (Falcon #3910, Oxnard, CA). YAC-1 cells (1.2 x 10^7) were labeled with 200 μCi of ^{51}Cr in NK medium supplemented with 0.25% sucrose for 90 minutes. Cells were washed in NK medium three times.

Spleen cells were resuspended to 2 x 10^5/ml and 0.1 ml was added to each well of a 96 well U-bottom microtiter plate. ^{51}Cr labeled YAC-1 cells were then added to each well at ratios of effector cell (spleen) to target cell (YAC-1) of 100:1, 50:1, or 25:1. Plates were incubated four hours at 37°C in 5% CO_2. Harvesting of the supernatant was carried out using harvesting frames (Skatron #7072, Sterling, VA) with a plastic tube (USA Scientific Plastics #USA8845, Ocala, FL). Samples were immediately counted in a gamma counter (Beckman Biogamma II, Beckman Instruments, Inc., Fullerton, CA) The results were expressed as

$$\% \text{ cytotoxicity} = \frac{\text{experimental CPM - spontaneous CPM}}{\text{total CPM - spontaneous CPM}} \times 100$$

Suppression assay BALB/cByJ mice were injected with endotoxin or LPS i.p. 24 hours prior to an injection of 10% sheep red blood cell suspension (SRBC). Four days after the injection of the SRBCs, mice were sacrificed by cervical dislocation, spleens were removed, minced, washed in Hank's balanced salt solution, and resuspended in the same solution. The anti-SRBC plaque forming cell (PFC) response was determined using the Cunningham slide method (20).

RESULTS

Macrophage viability The effect of LPS and endotoxin on macrophage viability was assayed by fluorescent microscopy using propidium iodide and fluorescein diacetate. The results depicted in Table 1 show that the treponemal LPS preparations were toxic to elicited PECs at 100 μg/million cells while the endotoxin preparations were not toxic. In comparison to *E. coli* LPS, the treponemal LPS preparations were less toxic for murine PECs.

IL-1 production Both time and dose dependent assays were performed to determine the optimum conditions for IL 1 production. The data in Figure 1 shows that treponemal endotoxins were able to induce the secretion of IL 1 while the LPS preparations failed to stimulate IL 1 production. The optimal responses were observed at doses greater than 0.5 μg/ml (Fig. 2). The treponemal endotoxins induced responses much better than their corresponsing LPS preparations (Fig. 1 and Fig. 2). The endotoxins (5 μg/ml) elicited responses similiar to those obtained with *E. coli* LPS (0.1 μg/ml).

Production of TNF There was no difference in the levels of TNF activity in culture supernatants from PEC stimulated with treponemal endotoxin preparations (Fig. 4). At the lower dilutions of PEC supernatants (1:5 and 1:25), the *T. innocens* LPS induced better TNF activity (≥ 65% cytotoxicity) than *T. hyodysenteriae* LPS (≤ 45% cytotoxicity). The levels of TNF induced by 5 μg/ml of the treponemal endotoxins were similiar to those induced by 0.1 μg/ml of *E. coli* LPS.

The presence of TNF in the supernatants from PECs stimulated with *E. coli* LPS and *T. hyodysenteriae* endotoxin was confirmed by the ability to inhibit the cytotoxic activity with rabbit anti-TNF antisera (Fig. 5).

Table1. Effect of treponemal LPS or endotoxin on BALB/cByJ peritoneal exudate cell viability

Preparation	amount added (μg)	Viability (%) of PEC[a]	
		Elicited[b]	Resident[c]
None		67.7	70.9
E. coli K235			
LPS	25	24.5	56.4
	100	15.2	53.8
T. hyodysenteriae B204			
LPS	25	63.1	78.0
	100	38.5	74.3
endotoxin	25	73.2	78.0
	100	66.4	80.3
T. innocens B1555a			
LPS	25	60.1	72.1
	100	45.4	56.5
endotoxin	25	76.1	83.3
	100	78.6	88.1

[a]Proteose peptone elicited or resident BALB/cByJ PECs, 1 x 10^6 cells/ml, were incubated for 24 hours in the presence or absence of LPS or endotoxin.

[b]Viability of elicited PECs was determined using a fluorescent activated cell sorter. For each sample, 10,000 cells were counted.

[c]Viability of resident PECs was determined by ultraviolet microscopy and 300 cells or 10 microscopic fields (250x) were enumerated per sample.

The TNF activity of all PEC supernatants could be inhibited with the addition of the anti-TNF rabbit serum (data not shown).

Natural killer cell assay Augmentation of natural killer (NK) cell activity can be achieved by treating mice i.p. with LPS and endotoxin. This augmentation is the result of the elaboration of interferon. Therefore, as a crude measure of in vivo interferon production, the ability of treponemal LPS or endotoxin to enhance NK cell activity was determined (Fig. 6). The NK activity in mice treated with *T. innocens* or *T. hyodysenteriae* endotoxin was significantly higher than control response ($p \leq 0.05$). The *T. hyodysenteriae* LPS preparation augmented NK activity above the saline control ($p \leq 0.1$) but the *T. innocens* LPS preparation did not augment the NK response above control levels.

Suppression assay In addition to the ability of LPS to enhance an immune response, it has been shown to inhibit the antibody response to antigens administered subsequent to LPS injection. The suppression of an anti-SRBC PFC response by LPS or endotoxin was assayed using C3H/HeN mice. Mice were given an i.p. injection of LPS or endotoxin 24 hours before immunization with SRBC. At doses of 10 and 100 μg/mouse, the *T. hyodysenteriae* endotoxin inhibited the subsequent anti-SRBC PFC response by 54% (Fig. 7). At 100 μg/mouse, the *T. hyodysenteriae* LPS suppressed the PFC response by only 38% while 10 μg/mouse of *E. coli* LPS suppressed the response by 65%.

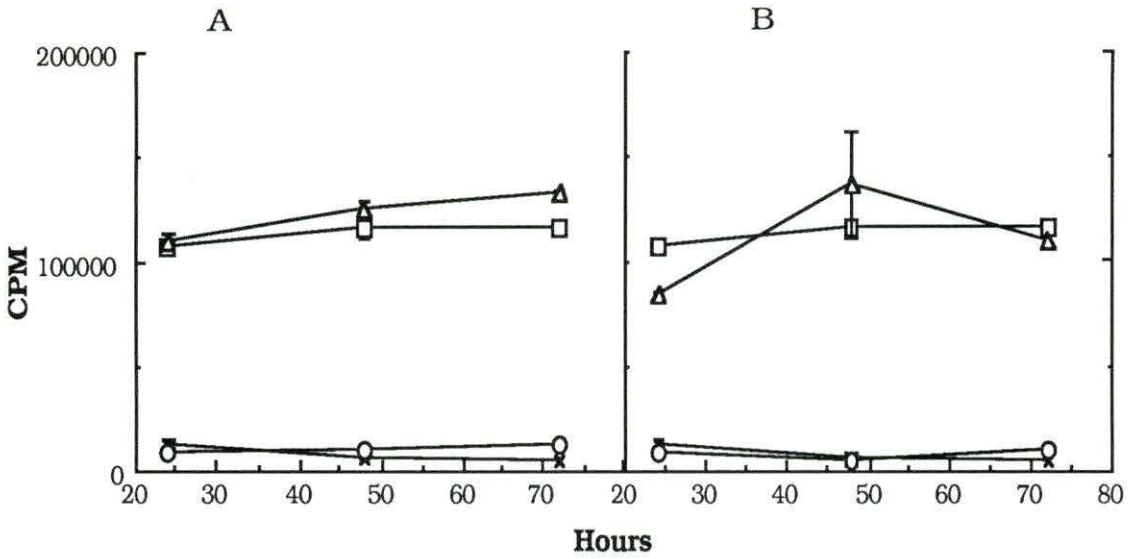


FIG. 1. A time dependent IL 1 response was determined for PEC cultures treated with treponemal LPS or endotoxin. The IL1-dependent D10.G.4 T cell clone was used to measure IL 1 activity. The supernatants (1:20 dilution) were prepared using 0.1 µg/ml *E. coli* LPS (square), 5 µg/ml LPS (circle), and 5 µg/ml endotoxin (triangle), and RPMI control (X). A.) *T. hyodysenteriae* B204; B.) *T. innocens* B1555a. Values represent the mean CPM ± S.E.M. of triplicate cultures.

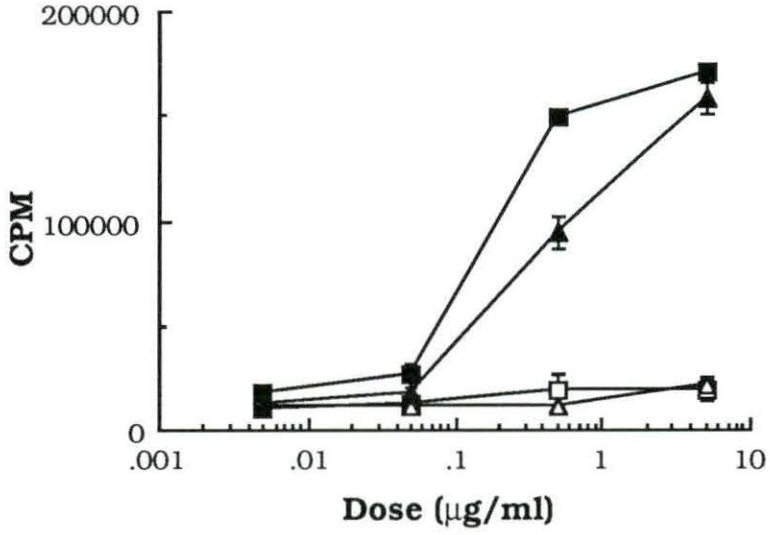


FIG. 2. A dose dependent response for the production of IL1 was determined for PECs stimulated with treponemal LPS or endotoxin. Supernatants were diluted 1:10. *T. hyodysenteriae* B204 LPS (open square); B204 endotoxin (shaded square); *T. innocens* B1555a LPS (open triangle); and B1555a endotoxin (shaded triangle). Supernatants from PECs stimulated with 0.1 μg/ml *E. coli* LPS induced 110,000 CPM. Values are expressed as the mean ± S.E.M. of triplicate cultures.

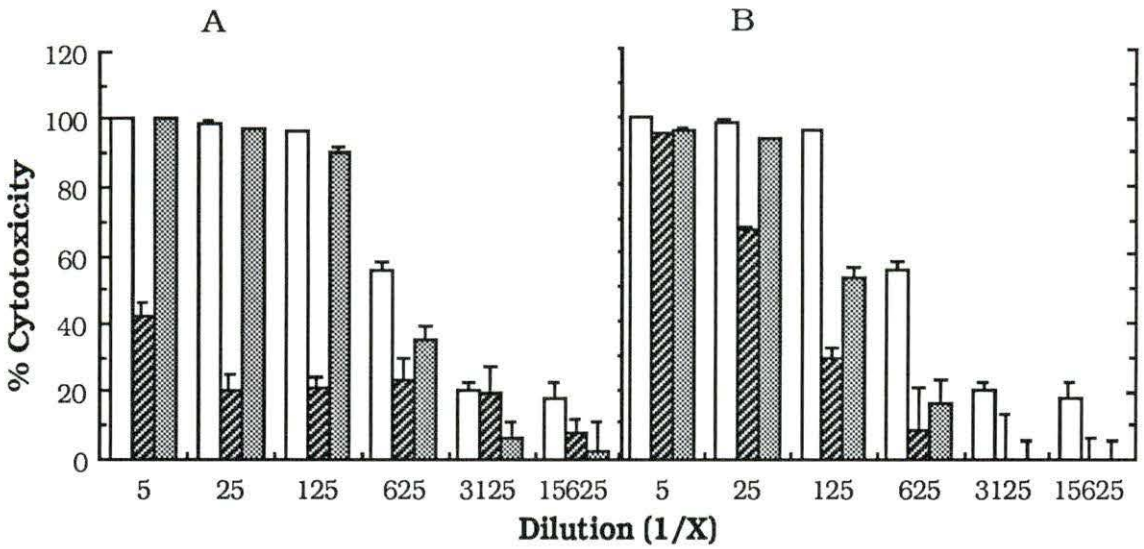


FIG. 3. Tumor necrosis factor activity was assayed by adding diluted culture supernatant from murine peritoneal exudate cells, stimulated with LPS or endotoxin, to L929 cells. After 20 - 24 hours, plates were washed and then stained with crystal violet to visualize adherent cells. Absorbance of the samples were read at 595 nm. Each value represents the mean ± S.E.M. of quadruplicate cultures and divided by the average of control cultures and multiplied by 100. A.) *E. coli* LPS, O.1 μg/ml (open bar); *T. hyodysenteriae* B204 LPS, 5 μg/ml (slashed bar); B204 endotoxin, 5 μg/ml (shaded bar); B.) *E. coli* LPS, 0.1 μg/ml (open bar); *T. innocens* B1555a LPS, 5 μg/ml (slashed bar); and B1555a endotoxin, 5 μg/ml (shaded bar).

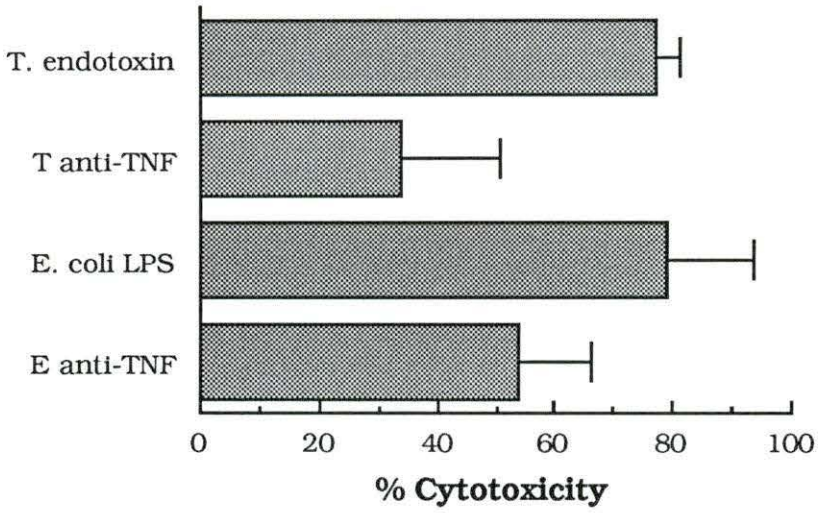
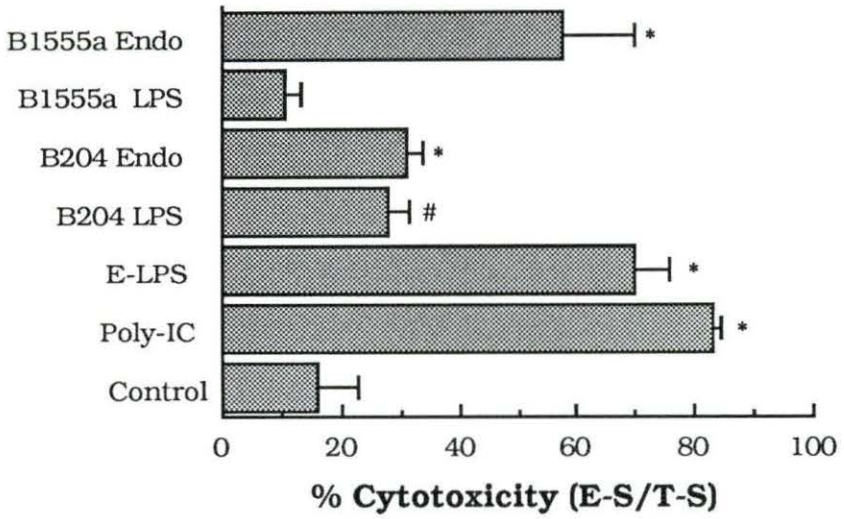


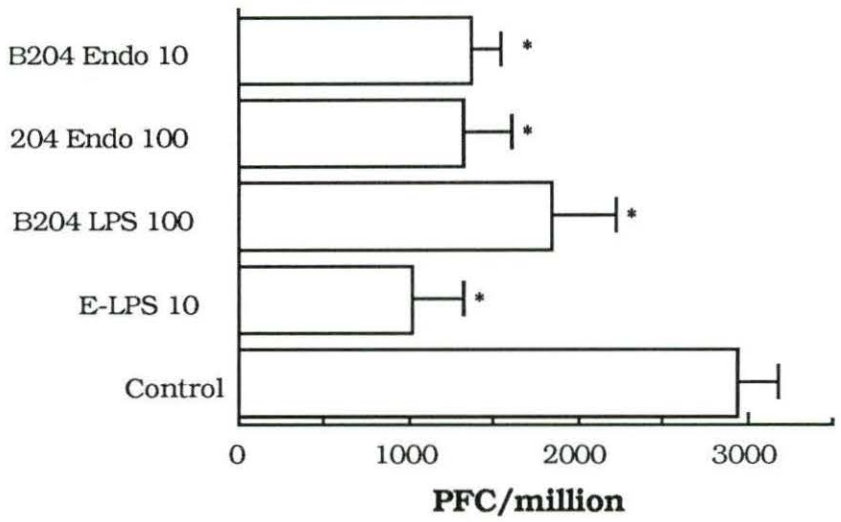
FIG. 4. Tumor necrosis factor activity was suppressed by the addition of rabbit anti-TNF serum (2 units/well). Thioglycollate elicited BALB/cByJ PECs were stimulated with *T. hyodysenteriae* B204 endotoxin (0.5 μg/ml) and *E. coli* LPS (0.1 μg/ml) and supernatants were collected at 48 hours. The supernatants were diluted 1:75 and treated with 2 units/well anti-TNF and compared to untreated supernatant. Results are expressed as mean ± S.E.M. of duplicate cultures.



*Significant at the 0.05 level.

#Significant at the 0.1 level.

FIG. 5. Natural killer cell activity was assayed by a ^{51}Cr release assay using YAC-1 cells as the target and spleen cells from C3H/HeN mice as effector cells. The ratio of effector cells to target cells was 50:1. Mice were given i.p. injections of LPS or endotoxin 24 hours prior to assay. Treponemal preparations were given at 100 μg/mouse, poly IC at 100 μg/mouse, and *E. coli* LPS at 20μg/mouse. This assay was carried out in triplicate and values are expressed as the mean ± S.E.M.



*Significant at the 0.05 level.

FIG. 6. Studies on the suppressive effect of LPS and endotoxin on the anti-SRBC PFC response. LPS was given to BALB/cByJ mice 24 hours prior to the SRBC immunization. On day 4, anti-SRBC PFCs were determined. Each group contained five animals and values are expressed as the mean ± S.E.M. per million splenocytes.

DISCUSSION

Swine dysentery is an inflammatory disease of the porcine cecum and large intestine (12). Symptoms of the disease include fever, anorexia, and yellow to gray soft feces. Diarrheic stools often contain fibrin, mucus, and blood flecks as the disease progresses. Gross lesions are characterized as necrotic, catarrhal, and hemorrhagic (12).

The specific component(s) of *T. hyodysenteriae* that induce the inflammatory condition have not been defined. The initial event in many infections is the development of an inflammatory response, and bacterial LPS or endotoxin has been shown to contribute to these inflammatory conditions (27). Habicht et al. (11) and Beck et al. (2) reported that macrophages produced IL 1 following stimulation with whole cells of *Borrelia burgdorferi*. In addition, it was shown that these IL 1 preparations could provide both the preparatory or provocative stimuli for a local Shwartzman reaction. As discussed in Section I, the treponemal preparations failed to induce a dermal Shwartzman reaction in rabbits, but were capable of providing the provocative stimulus following an intradermal injection of *E. coli* LPS. The inability of treponemal LPS or endotoxin to induce necrotic lesions indicated that these preparations were not inflammatory agents. This would be inconsistent with the results obtained with other LPS preparations (27) and may be explained by the inability of the treponemal preparations to induce endogenous pyrogens (i.e., IL 1 or TNF).

Macrophages have been shown to secrete both IL 1 and TNF following stimulation with LPS or endotoxin (7a, 8, 27). Interleukin 1 is a 15,000 dalton protein with hormone action (7a, 9). The effects of IL 1 on the immune system include enhanced expression of cell surface markers, enhanced antibody production, and stimulated T cell proliferation (7a, 9). Tumor necrosis factor is also a protein and has a molecular weight of 17,000 daltons (3). When macrophages are stimulated with LPS, TNF can account for as much as 1-2% of the total protein produced (3). Tumor necrosis factor has been shown to be the mediator of toxic shock following administration of endotoxin (37). Like IL 1, TNF induced the biosynthesis of various inflammatory agents including prostaglandins, leukotrienes, platelet activating factor, and collagenase (8). Depending on the magnitude of the response to an infectious agent, these monokines can have beneficial or deleterious effects on the host. The contribution of these monokines to the pathogenesis of swine dysentery is not known. However, the inflammation and edema observed in dysenteric lesions would be consistent with the synthesis and secretion of IL 1 and TNF.

Murine PECs stimulated with the treponemal endotoxins produced a dose-dependent response (Fig. 2). Though comparable IL 1 responses were elicited by *E. coli* LPS and the treponemal endotoxins, the dose of endotoxin was 50 fold higher than that of *E. coli* LPS. The treponemal LPS preparations did not stimulate IL 1 release (Fig. 1 and 2). Beck et. al. (2) demonstrated that IL 1 can provide the preparatory and provocative stimuli for a Schwartzman reaction. The lack of IL 1

secretion from PECs treated with treponemal LPS may explain the inability of these preparations to induce a local Shwartzman reaction (Section I).

The TNF activity, like IL 1 production, was stimulated by treponemal endotoxins, but not the LPS preparations (Fig. 4). The presence of TNF in the culture supernatants was demonstrated by the addition of rabbit anti-TNF serum (Fig. 4). As observed for IL 1 production, the level of TNF in PEC culture supernatant following stimulation with endotoxin was similar to that obtained with*E. coli* LPS, but the dose required was 50 fold higher.

The endotoxin protein of *P. aeruginosa* has been shown to induce interferon both in vitro and in vivo at greater levels than LPS (18). The ability of the treponemal preparations to induce both IL 1 and TNF seem to coincide with the presence of protein in the endotoxin preparations. Therefore, the NK cell tumoricidal assay was used to indirectly measure in vivo interferon production following stimulation with treponemal LPS or endotoxin (7b). The NK activity was augmented by both endotoxin preparations ($p \leq 0.05$), but little activity was seen from the treponemal LPS preparations (Fig. 5).

The treponemal endotoxins were observed to have very little effect on murine PEC viability (Table 1). For resident PECs, *T. innocens* LPS was toxic at 100 μg/ml, but not at 25 μg/ml. At 100 μg/ml, *T. hyodysenteriae* and *T. innocens* LPS were toxic for elicited PECs. It has been previously shown that the lipid component of LPS is responsible for the associated toxicity (26). On a weight basis, the increased

toxicity of the treponemal LPS in comparison to the endotoxin preparations may be related to the increased amount of the lipid component. Results of this study differ with the previous report by Nuessen et al. (28) in that the treponemal LPS was shown to be toxic at 15 μg/ml and higher rather than 100 μg/ml. This difference may be explained by the use of different preparations and/or the method used to determine macrophage viability. For example, the determination of macrophage viability based on the number of adherent cells remaining after treatment with LPS may have been incorrectly interpreted. It was recently shown that LPS will cause macrophages to release from glass surfaces without affecting viability (16). This may have led the previous authors (28) to report toxic effects at lower doses of LPS than observed in the present study. In addition, the toxicity of the treponemal LPS relative to *E. coli* LPS cannot be compared since the previous authors (28) did make that comparison.

Previous reports have established that endotoxin (butanol/water extraction) preparations contain LAP (23) as well as outer membrane porins (36). The addition of protein components change the LPS molecule, thus changing what is seen by the host system (13). The biologic differences between endotoxin and LPS could be accounted for by the addition of these protein moieties. These proteins may work alone to give the responses seen in this study.

In addition to the effects on macrophages, LPS has been shown to suppress the immune response to a T cell-dependent antigen (38). This suppression was observed when the LPS was given 24 to 48 hours

prior to antigen presentation. Treatment of mice with the treponemal preparations resulted in significant reduction ($p \leq 0.05$) of the anti-SRBC PFC response (Fig. 6).

We have shown that the treponemal LPS is biologically less active on the immune response than treponemal endotoxin or *E. coli* LPS. The results of the present study coincide with the results of the previous study (Section I). The contribution of treponemal LPS or endotoxin to the development of dysenteric lesions is unknown. These lesions may be the result of the host's own inflammatory response or the direct action of the spirochete. It has been shown that the treponemal endotoxin stimulated inflammatory agents (i.e., IL 1 and TNF) although at lesser amounts than that induced by classical LPS. This apparent difference in the ability to induce inflammatory agents may have little bearing on the development of lesions since the absolute amount of IL 1 or TNF required is not known.

The LPS preparations of *T. hyodysenteriae* and *T. innocens* were shown to be less toxic for murine PECs than *E. coli* LPS and did not induce monokine production. The endotoxin preparations stimulated PECs to produce IL 1 and TNF. Although activity was comparable to *E. coli* LPS, the doses of treponemal endotoxin required for these responses were 50 fold higher. In addition, the biologic activity of the treponemal preparations was dependent on the presence of the protein component. Finally, the results suggest that the differences in virulence between *T. hyodysenteriae* and *T. innocens* is not based on the biologic activity of their LPS or endotoxin components.

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GENERAL CONCLUSION

Lipopolysaccharide (LPS) and endotoxin from *Treponema hyodysenteriae* and *T. innocens* were studied to determine the biologic activity, primarily using the murine model system.

Chemical composition varied slightly between the different preparations. *T. hyodysenteriae* LPS had similar hexose content but relatively little protein content compared to the treponemal LPS in previous reports. The classic LPS biologic responses (pyrogenicity, mitogenicity, adjuvanticity, lethality, Limulus assay, and dermal Shwartzman reaction) of treponemal preparations were relatively weak compared to *Escherichia coli* LPS, with the exception of the Limulus assay giving comparable activities to both.

Stimulation of lymphoreticular cells by *Treponema* preparations was weak. It was observed that treponemal preparations were, when compared to *E. coli* LPS, less toxic to macrophage, weakly stimulatory for interleukin 1 and tumor necrosis factor production, weakly augmented natural killer cell activity, and showed mild suppressive effects on the murine immune system.

The results of this study indicate that there is little difference in the biologic activities of *T. hyodysenteriae* and *T. innocens* LPS and endotoxin. Virulence of the two organisms is not dependent on biologic activity of the LPS and the biologic activity of the endotoxin preparations coincided with the presence of protein.

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