Biological characterization of *Moraxella bovis* lipopolysaccharide

by

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INTRODUCTION

Moraxella bovis, a small, gram-negative, nonfermenting coccobacillus, is recognized as the primary etiologic agent of infectious bovine keratoconjunctivitis (IBK), or pinkeye. IBK may result in temporary or permanent blindness of affected eyes, as well as reduced feed conversion, and so is a disease of humanitarian consideration and economic importance (Marshall et al., 1985; Horsnell and Teale, 1987).

Although other factors, such as ultraviolet irradiation (Kopecky et al., 1980) and infection with Mycoplasma bovoculi (Rosenbusch, 1983) and infectious bovine rhinotracheitis virus (Pugh et al., 1970), have been shown to contribute to lesion production, M. bovis has two recognized virulence factors. These are the beta hemolysin and pili, both of which are outer membrane constituents and are required for virulence (Sandhu et al., 1977; Pedersen et al., 1972). Most attempts to develop a vaccine for IBK have met limited success in the field (Pugh et al., 1982). Many of these trials have involved whole cell bacterins (from hemolytic and nonhemolytic strains) or pili preparations; however, although there is some protection against homologous challenge, heterologous protection is variable at best (Pugh et al., 1976). Additionally, treatment for IBK may be time consuming and expensive, especially when considering reports that all animals in an infected herd are most likely exposed to M. bovis within several days (Ostle and Rosenbusch, 1985).

The outer membrane has been the principal site for investigation of M. bovis virulence factors because of the importance of the gram-negative

outer membrane interface with the host environment (Ostle and Rosenbusch, 1986). The outer membrane is the site for many virulence and resistance factors for gram-negative organisms; it is also accessible for recognition by the host immune system and thus, outer membrane antigens may be valuable for diagnosis. One major component of the outer membrane of gram-negative bacteria is the lipopolysaccharide (LPS), which is responsible for a large number of pathophysiologic effects. Lipopolysaccharide may also be protective to the organism, since it is important in serum resistance. Due to its structure and outer membrane location, LPS is highly immunogenic. To date, though, research on the LPS of Moraxella bovis and its potential role as a virulence or resistance factor in IBK has been limited. Few data on the LPS of Moraxella spp. are available (Adams et al., 1970; Veslemoy et al., 1980; Horisberger and Dentan, 1980), although the pilin gene has already be cloned (Marrs et al., 1985).

The purpose of this work was to lay the foundation for ascertaining the role of M. bovis LPS in the pathogenesis of IBK by characterization of its LPS, which included analysis of its chemical composition, polyacrylamide gel profile, and a number of biological assays.

LITERATURE REVIEW

Perspective

Bacterial products may often have a profound effect on their hosts. One such class of bacterial products are endotoxins, which are part of the cell wall of gram-negative bacteria. This designation serves to distinguish endotoxins from excreted toxic bacterial products which are termed "exotoxins". Endotoxins are composed of lipopolysaccharide (LPS) and associated protein. Endotoxin (and LPS) may cause a tremendous variety of reactions in the whole animal and on many different cell types, which include the induction of cytokines and causing massive changes in the blood coagulation system. LPS has also been shown to have a role in the pathogenicity of some gram-negative microorganisms.

LPS Structure and Reactivity

Before delving into the biological effects of LPS and endotoxin, it is perhaps worthwhile to discuss the structure of the molecule and relate it to the biological activity of the molecule. To begin such a review, it is important to distinguish clearly the difference between endotoxin and LPS. Lipopolysaccharide is comprised of three distinct regions, the "O-antigen", the core polysaccharide and lipid A. Endotoxin is LPS plus associated protein; this protein contributes to differences in biological activity seen between LPS and endotoxin (Morrison and Ulevitch, 1978; Bradley, 1979). The amount of protein extracted along with the LPS depends upon the method employed for extraction (Morrison and Ulevitch,

1978). So-called Boivin LPS is extracted using trichloroacetic acid and produces LPS contaminated with proteins (endotoxin) (Staub, 1965), while so-called Westphal LPS is extracted with phenol and yields relatively pure preparations of LPS (Westphal and Jann, 1965); LPS may also be extracted successfully using aqueous butanol (Morrison and Leive, 1975). In addition, endotoxin-associated protein (EAP) has been found to exhibit interleukin 1 (Il-1) properties, such as the direct stimulation of serum amyloid A production by resting human T cells; its activity is not blocked by polymyxin B (Johns et al., 1988), an antibiotic polypeptide which binds the lipid A moiety of LPS and abrogates its activity. Other studies have demonstrated that EAP has a granulopoietic colony-stimulating activity on human peripheral blood lymphocytes and bone marrow precursor cells (Bjornson et al., 1988).

The lipid A region of LPS is generally considered to be the most conserved part of the molecule and usually consists of a diglucosamine backbone with amide and ester-linked long chain fatty acids, as well as pyrophosphate groups (Wilkinson, 1977). The amide linked fatty acids appear to be uniformly beta-hydroxy substituted. There appeared to be no particular significance, in terms of reactivity, for chain length of ester-linked fatty acids and both odd and even numbered chains are found; however, it has been recently found that fatty acid substituents of synthetic lipid A are important in imparting immunoreactivity (Kumazawa et al., 1988). In these studies, different length acyl groups (C12-C16) and different stereoisomers for lipid A subunit analogs (4-O-phosphono-D-glucosamine derivatives) were used to study the mitogenicity,

pyrogenicity, lethal toxicity, local Shwartzman reaction, as well as cytokine induction (e.g., TNF-inducing activity); it was found that fatty acid substituents with the (RR) configuration had greater endotoxic activities than the corresponding (SS) stereoisomers and that acyl chain length also affected reactivity.

Some investigations have suggested that protein may be covalently bound to the lipid A region (Wilkinson, 1977). Lipid A is also the most internal moiety of LPS and is probably intercalated in the lipid bilayer of the outer leaflet of the cell (Osborn, 1979). Lipid A is linked through a unique sugar to the core region. This unusual sugar is 2-keto 3-deoxy-octulosonate (KDO). The core itself may be divided into inner and outer core regions. The inner core contains KDO and heptose with ethanolamine and phosphate as additional constituents. The outer core consists usually (for the Enterobacteriaceae) of glucose, galactose and ocasionally N-acetyl-glucosamine (Wikinson, 1977; Galanos and Luderitz, 1984). The core region may be quite similar for different strains or groups of bacteria. The most external fraction of LPS is the O-antigen, a long polysaccharide chain consisting of repeating oligosaccharide units. The repeating units may contain from one to as many as 7 sugars and many rare or novel sugars have been found in this region (Wilkinson, 1977). The basic oligosaccharide unit may be repeated 30-40 times and perhaps as many as 60-70 times for some organisms (Wilkinson, 1977). So-called rough mutants lack various amounts of the O-antigen and core regions. Because it extends beyond the cell wall, antibodies are most often directed against O-polysaccharide epitopes (Galanos and Luderitz, 1984). E. coli

O-antigens often contain mannose, rhamnose, glucose, N-acetyl glucosamine and N-acetyl galactosamine as constituent monosaccharides (Wilkinson, 1977). Lipopolysaccharide, then, is an amphipathic molecule responsible for many pathophysiological effects. Both regions of the molecule are responsible for some of these effects, such as complement activation, while many are attributed to the lipid A moiety, such as tumor necrosis factor induction.

LPS-Cellular Interactions

While the effects of LPS on different, isolated organs have not been clearly delineated (Morrison and Ulevitch, 1978), some of the effects of LPS on a plethora of cell types have been determined, including platelets, neutrophils, mast cells/basophils, macrophages/monocytes, endothelial cells and lymphocytes (Morrison and Ulevitch, 1978; McCartney and Wardlaw, 1985). The interactions of LPS with each of these cells types will be considered below.

Platelets

Platelets may react with LPS depending upon whether or not they possess immune adherence receptors, or receptors for the C3b component of complement on the cell membrane (McCartney and Wardlaw, 1985). Des Pres and co-workers were able to show aggregation of rabbit platelets, generation of platelet factor 3, and serotonin release upon interaction of platelets with LPS (Des Pres, 1967); additionally, it had been shown that the response depended on the presence of divalent cations (Des Pres and

Bryant, 1966) and that the terminal components of complement were required for lysis of platelets (McCartney and Wardlaw, 1985). Morrison and his co-workers proposed that the alternative pathway of complement activation was involved in this response, and this group further demonstrated that the lipid A moiety of the LPS was involved in the binding of LPS to the platelets (Morrison and Oades, 1979). Primate and human platelets, which lack immune adherence receptors, appear to respond in a complement-independent manner to lipid A by secretion of granule constituents (Nagayama et al., 1971; Hawinger et al., 1975, 1977).

Polymorphonuclear leucocytes

Polymorphonuclear leucocytes (PMN) have been shown by Wilson et al. (1987) to bind LPS in vitro, but without demonstrable consequential effect. Morrison and Ulevitch, though, were able to provide evidence that the presence of LPS could enhance the phagocytic activity of PMN, in addition to increasing glycolysis and the ability to reduce nitroblue tetrazolium, in vitro (Morrison and Ulevitch, 1978). In vivo, LPS induces a leucopenia followed by a leucocytosis (Athens et al., 1961). It was also demonstrated that the leucopenia was due to sequestration of PMNs in capillary beds, while release of granulocytes from bone marrow reserves was responsible for the leucocytosis (Athens et al., 1961).

Neutrophils may also be affected by a neutrophil chemotactic factor, which is different from Il-1 and synthesized by human mononuclear leukocytes upon stimulation with LPS (Yoshimura et al., 1987). Thus, neutrophils may be recruited to sites of gram-negative sepsis or LPS-

induced biological reactions, such as the local Shwartzman reaction, through this 10 kilodalton chemotactic factor.

Recently, it was found that binding of a cationic antimicrobial protein, CAP57 from human neutrophils, to S. typhimurium was dependent upon the length of the O-polysaccharide chain of the LPS. In these studies, the longer the O-antigen and core oligosaccharide of the LPS, the less sensitive the Salmonella strain was to the effects of cationic granule proteins (Farley et al., 1988). It was also found that in polymyxin B-resistant mutants with substituents that block anionic groups on lipid A (such as 4-aminoarabinose on the 4' phosphate of lipid A) show increased resistance to CAP57 through the loss of hydrophobic binding sites which stablize the interaction of CAP57 with the bacterial surface (Farley et al., 1988). These studies give insight into the mechanisms by which some gram-negative microorganisms are able to resist host defenses as well as why organisms with a smooth LPS tend to be pathogenic and rough organisms nonpathogenic.

Macrophages and monocytes

LPS has a profound effect on macrophages and a variety of mediators are released from such cells upon incubation with LPS. Among the products secreted are tumor necrosis factor (TNF), IL-1, colony stimulating factor (CSF), collagenase, endogenous pyrogen, prostaglandins, lymphocyte activating factor and plasminogen activator. Endotoxin has also been found to have an inhibitory effect on the expression of certain cell-surface proteins (termed immune response associated antigens, or Ia

antigens) on murine macrophages by Steeg et al. (Steeg et al., 1982). These investigators believe that LPS inhibits gamma-interferon (INF-gamma) stimulation of macrophage Ia antigen expression through the induction of prostaglandin E2 (PGE2) synthesis and the resulting increase in intracellular cAMP (Steeg et al., 1982). Indomethancin abrogated these inhibitory effects of LPS, thus indicating a role for a cyclooxygenase pathway product; additionally, PGE2 was able to induce the same inhibitory effects as LPS (Steeg et al., 1982). Complementing this work are recent studies by Koerner and co-workers which suggest that the LPS controls the regulation of Ia expression at the level of accumulation of mRNA (Koerner et al., 1987). In these studies, INF-gamma was shown to induce, in a dose- and time-dependent manner, the accumulation of mRNA for Ia in murine peritoneal macrophages; LPS suppressed this induction in a dose-dependent manner, from 40-80 %, and the suppression correlated with decreased amounts of surface Ia protein (Koerner et al., 1987).

A new role for macrophages in the catabolic pathway of LPS and as a host defense mechanism is suggested by the recent work of Peterson and Munford who were able to demonstrate dephosphorylation of lipid A from the rough E. coli D21f2 mutant; however, there was no evident difference in the ability of LPS-responsive C3H/HeN or LPS-hyporesponsive C3H/HeJ murine macrophages to dephosphorylate lipid A (Peterson and Munford, 1987). Lipid A analogs lacking phosphate at either the 1 or 4' position have been shown to have reduced toxic effects and pyrogenicity, thus dephosphorylation may be a mechanism by which macrophages are able to modulate LPS immunoreactivity (Peterson and Munford, 1987).

One of the early responses to LPS by macrophages may be the elicitation of macrophages which are specialized for antigen degradation and associated functions, and consequently have a negative regulatory effect on acquired immunity to LPS (Cluff and Ziegler, 1987). In these studies, the catabolism of macrophages elicited three days after LPS injection was three times greater than that of resident macrophages; in addition, these macrophages exhibited low Ia antigen levels, were able to rapidly destroy the antigenicity of Listeria monocytogenes antigens studied, and were slow to process and present antigen in vitro. Cluff and Ziegler also believe that this catabolic response is under the control of the lps gene locus (Watson, 1979), as elicited macrophages from LPS-hypopresponsive C3H/HeJ mice did not show an enhanced rate of catabolism. The authors speculate that the impact of LPS treatment is a decrease in T cell responses due to reduced antigen presentation, a manifestation of the down regulation of immune responses through elimination of antigen.

Obviously, the unleashing of this array of enzymes and cytokines from macrophages exposed to LPS can have far reaching effects on other immune mediators or cells. For instance, TNF released from human macrophages stimulated by LPS has been demonstrated to induce TNF receptors on human T cells and augment T cell responses, such as increasing the expression of high affinity Il-2 receptors and HLA-DR antigens (Scheurich et al., 1987), or cause the down regulation of T cell responses as mentioned above.

Lymphocytes

Lipopolysaccharide effects on lymphocytes and the resultant impact on the immune response have recieved substantial attention over the last twenty years. Lipopolysaccharide can initiate both antibody secretion and polyclonal proliferation from B cells. Gery et al. (1972) demonstrated that LPS is a B cell mitogen, but only for murine B cells. Lipopolysaccharide has also been shown to augment human B cell differentiation by acting directly upon B cells which are pokeweed mitogen (PWM)-responsive, while Il-1 did not exhibit a similar effect (Anderson and Lawton, 1987). Other researchers have shown that B cell stimulatory factor-1 (BSF-1) or interleukin 4 preincubated with murine B cells was able to enhance the secretion of IgG1 when the B cells were subsequently stimulated with LPS (Snapper and Paul, 1987). This team believes that BSF-1 may act upon the IgG1 constant heavy gene or its switch region thus enhancing the likelyhood of a selective recombination after a second stimulus, i.e., LPS.

T cells do not appear to respond to LPS by proliferation, and their interaction with LPS is not well defined. However, new roles for T cell subsets, such as natural killer cells, are being discovered in interactions with LPS. Natural killer cells have been found recently to be activated by surface LPS and show enhanced cytotoxicity (Lindemann, 1988). Suppressor T lymphocytes have been shown by Baker and co-workers (1988) to be inactivated by monophosphoryl lipid A from the Re mutant of S. typhimurium, while the activity of other T cell subsets (e.g., T-helper cells) was not inhibited. In addition, it appears that LPS may act

indirectly on T cells via the immunomodulators it induces, such as TNF.

Epithelial cells

Epithelial cells are also affected by endotoxin, and may also be induced to elaborate various immunomodulators, similar to some of those secreted by macrophages. One such cytokine is called corneal epitheial cell-derived thymocyte activating factor (CETAF) and is synthesized by the established corneal cell line, SIRC, as well as primary rabbit corneal epithelial cells incubated with LPS (Grabner et al., 1983). This cytokine is similar to a murine epidermal cell-derived thymocyte activating factor (ETAF; Sauder et al., 1984). Interleukin 1, CETAF and ETAF (human and murine) all stimulate thymocyte proliferation. Epidermal cell-derived thymocyte activating factor and CETAF both appear to by chemotactic for PMNs as well (Grabner et al., 1982). Grabner and co-workers believe as well that CETAF may be pyrogenic, which has been shown by Sauder et al. to be true for ETAF. In contrast to macrophages, the epithelia of ciliary body and iris are induced by LPS to express Ia antigens (Kim et al., 1986). Kim and co-workers beleive that this enhancement of Ia antigen expression may lead to better antigen presentation by ciliary body epithelial cells to lymphocytes, thus enhancing the immune response (Kim et al., 1986).

Endothelial cells

Endothelial cells may also take up endotoxin after intravenous injection, as well as being damaged in a manner which is not dependent

upon complement activation by-products (Morrison and Ulevitch, 1978). After detachment of endothelial cells, exposure of a vascular basement membrane may provide a suitable site for activation of the Hageman factor, leading to localized coagulation and elaboration of vasoactive substances (e.g., bradykinin), thus exacerbating the initial endothelial cell injury (Morrison and Cochrane, 1974).

Basophils

The effects of LPS on mast cells or basophils are unclear. Some investigators have been unable to demonstrate any deleterious effect of LPS on mast cells, while some researchers have noted degranulation and a decrease in the number of mast cells recovered from the peritoneum after intraperitoneal injection of LPS (Morrison and Ulevitch, 1978). Lipid A associated protein, however, has been found by Morrison and Betz to be cytotoxic at high concentrations to mast cells, as well as causing the induction of noncytotoxic vasoamine secretion (Morrison and Betz, 1977).

LPS cellular activation

Little is still known about how LPS binds to cellular membranes and the existence of an LPS receptor per se is perhaps unresolved (Kabir et al., 1978; Morrison and Rudbach, 1980). However, uptake of LPS may depend upon the particular cell type and its location. While examining the uptake of endotoxin by Kupffer cells and peritoneal macrophages, Fox and co-workers were able to confirm earlier reports of an LPS receptor on rabbit peritoneal macrophages; in contrast, however, their data supported

an absorbtive pinocytic process for endotoxin uptake in Kupffer cells (Fox et al., 1987).

One hypothesis for the cellular action of LPS has been proposed by Bradley. In this model, LPS binds to the cell membrane, is taken up by endocytosis and is transferred in a vacuole to a primary lysosome which then transfers the LPS to a specific receptor on the mitochondrial membrane. LPS causes the mitochondrial proton gradient to be destroyed, causing accumulation of ADP and NADH, which results in increased glycolysis. Lysosomal enzymes are induced, causing autophagy and the release of lysosomal hydrolases. The release of these enzymes would be a pivotal event leading to the characteristic pathophysiologic effects of LPS (Bradley, 1979).

LPS and Biological Reactions

As well as acting upon many different cell types, LPS is recognized as causing a variety of pathophysiologic effects, such as pulmonary hypotension and disseminated intravascular coagulation (DIC). LPS also induces the release of a variety of cytokines and enzymes, as indicated above.

Limulus amoebocyte gelation

Endotoxin and LPS can cause the gelation of the horseshoe crab, Limulus polyphemus, amoebocytes. The earliest report of intravenous coagulation in L. polyphemus was in 1902 by Loeb (Mills, 1978), while Bang reported in 1956 on a disease in L. polyphemus that resulted in complete

intravascular coagulation and death (Bang, 1956). Levin and Bang, in the 1960s, were able to show that endotoxin was responsible for this vascular coagulation, and that gelation of the Limulus amboecyte lysate resulted from an enzymatic reaction involving clottable proteins from these cells (Levin and Bang, 1968). The mechanism of this reaction was demonstrated to entail the activation of a pro-clotting enzyme by LPS in the presence of Ca ++ (Young et al., 1972; Tai and Lui, 1977). The activated enzyme is a serine protease (Niwa et al., 1975; Tai and Lui, 1977) which causes the proteolysis of a clottable protein, "coagulogen". The coagulogen is cleaved into peptides A and B, which are polymerized and incorporated into the clot; a third peptide, C, is produced and is insoluble (Tai and Lui, 1977). The active moiety of LPS in this scheme is lipid A (Levin et al., 1970; Niwa et al., 1975). The Limulus amoebocyte lysate (LAL) assay has been accepted by the US Food and Drug administration for testing for the presence of endotoxin in various biological products, drugs and medical devices (McCartney and Wardlaw, 1985). However, some substances inactivate the reaction, and include complement (Johnson and Ward, 1972), esterases (Skarnes, 1970), lipoprotein (Freundenberg et al., 1980) and antibody (Levin et al., 1970; Young, 1975); thus some researchers find the use of LAL for clinical blood specimens dubious. Also, some researchers question the specificity of LAL for detection of endotoxin or LPS, as peptidoglycan from gram-positive bacteria has been shown to produce gelation, albeit at activities 1000-40,000 times less than that of LPS (Elin and Wolff, 1973; Wildfleuer, 1974; Brunson and Watson, 1976). Still, LAL is able to detect minute quantities (0.1 ng/ml), and recent

modifications, such as the use of a chromogenic substrate (Iwanaga et al., 1978; Scully et al., 1980) have increased the sensitivity of the assay as well as giving a more objective or quantitative endpoint (McCartney and Wardlaw, 1985).

Disseminated intravascular coagulation

Disseminated intravascular coagulation (DIC), is an important pathophysiologic effect of LPS. LPS can activate both the intrinsic and extrinsic coagulation pathways (Morrison and Ulevitch, 1978), most likely by causing the activation of the Hageman factor of the intrinsic pathway, and by acting upon mediator cells, such as monocytes, by the extrinsic pathway (Morrison and Ulevitch, 1978). Again, lipid A is important in the activation of monocytes (Niemetz and Morrison, 1977; Rickles and Rick, 1977), and LPS appears to provide the appropriate, negatively charged surface to initiate the activation of the Hageman factor (Morrison and Cochrane, 1974). LPS activation of the Hageman factor also results in fibrinolysis, through the activation of plasma prekallikrein to its proteolytic form, kallikrein; kallikrein causes the proteolysis of plasminogen to plasmin, and plasmin is active in fibrinolysis (Revak et al., 1977). DIC can be induced with a single injection of sufficient LPS dose in LPS-sensitive species.

Shwartzman reaction

Coagulation also plays a role in the pathological lesion produced by the Shwartzman reaction, which can take a generalized or local form.

These require two injections of LPS given 24 hr apart; however, the generalized Shwartzman requires two intravenous injections of sublethal doses of LPS (usually administered to a rabbit), while in the local Shwartzman reaction, the first (priming) dose is administered intradermally while the second (provocative) dose is given intravenously.

Generalized Shwartzman The generalized Shwartzman reaction results in the development of bilateral renal cortical necrosis in the rabbit. The role of intravascular coagulation in this reaction is demonstrated by anticoagulative therapy, which prevents the formation of lesions (Good and Thomas, 1953). However, the roles of cell types such as platelets and granulocytes are somewhat controversial. Margaretten and McKay used thrombocytopenic and normal rabbits given heparin one hour prior to administration of anti-platelet antibody and several hours prior to the provocative dose of LPS. They found that none of the thrombocytopenic rabbits developed glomerular capillary thrombi, while 6 of 10 control rabbits given antiserum previously absorbed with platelets developed typical lesions. These authors concluded that platelets were necessary for the induction of the generalized Shwartzman reaction (Margaretten and McKay, 1969). Other workers, however, have concluded that platelets are not essential for the development of this reaction; these include two teams that also used anti-rabbit platelet antibody to deplete circulating platelets (Levin and Cluff, 1965; Muller-Berghaus and Kramer, 1976). Levin and Cluff found that the induced thrombocytopenia did not inhibit the localized Shwartzman reaction (see below), while Muller-Berghaus and Kramer found that platelets did not contribute to

precipitation of soluble fibrin. Other evidence for a nonessential role of platelets was provided by a set of experiments utilizing rabbits that were both thrombocytopenic and neutropenic. Disseminated intravascular coagulation was not induced in these rabbits upon the appropriately spaced injections of endotoxin; however, transfusion to replace granulocytes restored ability of the rabbits to generate microthrombi, which was not the case when platelets were replenished by transfusion (Bohn and Muller-Berghaus, 1976; Muller-Berghaus et al., 1976). Currently, neutrophils and particularly monocytes appear to have a major role in the development of the generalized Shwartzman reaction (Morrison and Ulevitch, 1978). This role is supported by recent work using TNF to induce many of the pathobiologic effects induced by LPS (Beulter and Cerami, 1986).

Local Shwartzman In the local or dermal Shwartzman reaction, the priming injection can produce erythematous lesions at the injection site consisting of platelet and leucocytic aggregates (McCartney and Wardlaw, 1985). The provocative dose induces edema, necrosis, some fibrin deposition and hemorrhage at the site of the priming injection. The localized Shwartzman reaction may also be prevented by administration of anticoagulants and a prominent role for neutrophils and monocytes is suggested (McCartney and Wardlaw, 1985). Recently, Beck et al. have proposed that Il-1 serves as the common mediator of the inflammatory response and that Il-1 may also have the same role in priming and provocation of the local Shwartzman reaction. These workers showed that Il-1 can produce characteristic lesions when used as the priming agent or to provoke the reaction 24 hr after intradermal injection of either E.

coli 0111:B4 LPS or Il-1 (Beck et al., 1986). Furthermore, these authors demonstrated the accumulation of neutrophils in less than one hour at the site of intradermal Il-1 injection, with the migratory activity of the neutrophils peaking at 4 hr. Based upon their results and the findings of others (Taichman, 1971; Fleming and Dunn, 1985), Beck and co-workers postulate a mechanism for the local Shwartzman reaction. The priming injection induces a transient hypersensitivity to the provocative agent. Because of the diversity of agents that can provoke the reaction (Taichman, 1971), Beck et al. believe that there is a common endogenous mediator of these changes, namely Il-1, that is produced locally. Increased expression for Il-1 receptors, then, on target cells such as the endothelial cells lining small venules or leukocytes migrating in response to Il-1, may account for the transient hypersensitivity to the provocative agent(s). Il-1 from the provoking dose may induce PMN to discharge their granules, initiating skin necrosis, or lesion development may occur because of increased adhesiveness of endothelial cells for PMN in response to Il-1 (Fleming and Dunn, 1985). Other work has demonstrated an essential role of complement in the development of the local (and generalized) Shwartzman reaction by use of cobra venom factor (Fong and Good, 1971), while recent work by Ito (Ito, 1985) has refuted the notion that the local Shwartzman reaction was a special case of hypersensitivity (Stetson and Good, 1964). Ito, using germ free rabbits, demonstrated that pre-existing natural antibody to endotoxin was not required for the development of the lesions. In his work, 11 of 11 germfree rabbits developed lesions, while 8 of 11 conventional rabbits developed lesions in response to E. coli 055

LPS (Boivin preparation).

Pyrogenicity

Pyrogenicity has long been recognized as a property of endotoxin and LPS. After intravenous administration of LPS, a biphasic rise in temperature is usually observed in LPS-sensitive rabbits. Humans and rabbits are the most sensitive animals to the pyrogenic effects of LPS and because humans respond similarly to the same dose of LPS, the rabbit pyrogenicity test is currently used for detection of endotoxin in drugs, etc. by the US and European Pharmacopeias (McCartney and Wardlaw, 1985). However, because of its cost and imprecision (Bangham, 1971), the LAL assay (see above) is an attractive alternative. The minimum dose of LPS pyrogenic for man is 1-5 ng/kg and a monophasic febrile response is observed within 90-120 minutes (Smith, 1981). Repeated daily injection of LPS in the rabbit will result in the loss of the second febrile peak; in man this induced tolerance leads to a decrease in febrile response (Smith, 1981). This state of tolerance can be overcome by increasing the dose of LPS; in rabbits, human INF-beta has been shown to restore the febrile response (Kawasaki et al., 1987b). The mechanism of the pyrogenic response may be due to LPS acting directly on the hypothalamus and also through the induction of various "endogenous pyrogens" by LPS effects on cells such as leukocytes or monocytes. LPS has been shown to induce PGE2 synthesis from monocytes and PGE2 has been implicated by Skarnes and co-workers as the mediator of the first phase of LPS fever. Also, prostaglandin inhibitors, such as aspirin and indomethacin suppress the

febrile response (Skarnes, 1970). Recently, Kawasaki et al. were able to demonstrate the augmentation of endotoxin-induced fever by recombinant human beta interferon (INF-B) in rabbits. In this work, nonpyrogenic doses of endotoxin (0.1-1ng/kg) produced a fever of about 1 C when injected with a nonpyrogenic dose of recombinant human beta interferon (Kawasaki et al., 1987a). These authors also demonstrated significantly higher PGE2 levels in plasma from rabbits injected with both LPS and IFN-B. Interleukin 1 and TNF have also been implicated in the mediation of the fever response to LPS. The febrile response to LPS, then, is a good example of the direct and indirect effects of LPS that cause a physiologic response.

Lethality

Lethality is perhaps the end result or sum of the effects of LPS on an animal, although lymphoid cell involvement in the terminal stages of lethality appear to be important, as genetically resistant C3H/HeJ mice can be sensitized to the lethal effects of LPS by transfer of spleen cells from the histocompatible, endotoxin sensitive C3H/HeN strain (Watson, 1979). Susceptibility to the lethal effects of endotoxin in mice can be increased a thousand-fold or more with the administration of inhibitors of protein synthesis, such a D-galactosamine (Galanos et al., 1979). However, this state of hypersensitivity is short-lived, and Galanos et al. have shown that injection of LPS one hour prior or 4 hr after the administration of D-galactosamine was not lethal. The same group demonstrated that D-galactosamine increases sensitivity of rats and

rabbits to the lethal effects of LPS, as well as for mice. In addition, Lehmann et al. have shown that LPS and TNF were identical in their lethal effects on D-galactosamine-treated mice, and that TNF was lethal for D-galactosamine-sensitized, LPS-resistant C3H/HeJ mice. In this work, in the absence of D-galactosamine, C3H/HeJ mice were equally as susceptible to TNF as endotoxin-sensitive C3H/TifF mice, and these results support to the hypothesis that TNF is an important mediator of endotoxin lethality (Lehmann et al., 1987).

LPS and Cytokine Induction

Interleukin-1 (IL-1)

It is obvious that one of the consequences of LPS is the release of a wide variety of cytokines and other mediators which impact or exacerbate the effects of LPS on cells directly. One such cytokine already mentioned is Il-1, whose release from monocytes is stimulated by LPS. Bakouche et al. have recently examined Il-1 release from human monocytes treated with LPS encapsulated in liposomes. They observed Il-1 release by macrophages which had phagocytosed LPS associated with lyophilized liposomes, while LPS in classical liposomes or free IL-1 did not inhibit nor increase the amount of IL-1 released (Bakouche et al., 1987). Additionally, this group hypothesizes that LPS interaction with the monocyte membrane may be required for activation leading to IL-1 release; the results of their work indicate that LPS density at the liposome surface may also play a role in monocyte responsiveness: low LPS density may lead to the accumulation of intracellular IL-1, while high LPS density leads to the release of IL-1.

There were other intriguing findings in this study, namely that liposomes containing media only or LPS did not stimulate the release of IL-1, which would indicate that phagocytosis alone is not sufficient for stimulation of IL-1 secretion and that encapsulated LPS lost its ability to stimulate IL-1 release. Interestingly, the polysaccharide moiety of Haemophilus actinomycetemcomitans LPS was found to block the release of Il-1 from murine macrophages; however, the production of intracellular Il-1 and membrane-bound Il-1 was not suppressed (Nishihara et al., 1988).

Fuhlbrigge and his co-workers have shown that while resting peptone-elicited peritoneal exudate cells (PEC) and resident PEC do not express biologically active Il-1, stimulation of such PEC by adherence or incubation with LPS induce similarly increased levels of mRNA for Il-1-alpha and Il-1-beta; these results imply that the regulation of Il-1 mRNA is important in the production of increased amounts of bioactive Il-1 (Fuhlbrigge et al., 1987). Other studies on the genetic regulation of LPS-induced Il-1 production have demonstrated that the Il-1 response appears not to be linked to the major histocompatibility complex, as high and low Il-1-responder mouse strains were used which share the same H-2 haplotype (Brandwein et al., 1987). Additionally, these studies showed that a major gene locus controls the Il-1 response to LPS and that one or more loci modify the magnitude of the response (Brandwein et al., 1987)

The release of IL-1 from macrophages by LPS stimulation is bound to affect other aspects, cells and mediators of the immune system. Indeed, IL-1 possesses several properties in common with LPS, including the induction of IL-2, PGE2, fever and inflammation (Dinarello et al., 1984;

Dinarello et al., 1986a) and this fact increases the complexity of dissecting LPS effects from those caused by LPS-induced Il-1.

Tumor necrosis factor (TNF)

Tumor necrosis factor, or cachectin, is another cytokine released by macrophages in response to LPS. LPS alone in sufficient concentrations can activate macrophages to release TNF, but INF-gamma has been shown by Gifford and Lohmann-Mathes to prime macrophages for activation by very small amounts of LPS (0.1 ng, insufficient for activation by LPS alone) (Gifford and Lohamann-Matthes, 1987). A central role for TNF in endotoxin shock is suggested by the demonstration that BALB/c mice passively immunized against TNF become resistant to LPS (Beutler et al., 1985). Further work by this group gives insight into the mechanism of endotoxin resistance. In studies utilizing macrophages from the LPS-sensitive BALB/c and C3H/HeN mouse strains and the LPS-resistant C3H/HeJ mouse strain, Beulter et al. were able to show that transcription levels of mRNA for TNF were increased threefold in the presence of endotoxin in responsive strains; C3H/HeJ macrophages did not show similar transcriptional activation of TNF mRNA at low concentrations of LPS (10 ng/ml) (Beutler et al., 1986). At high LPS concentrations (1 ug/ml), C3H/HeJ macrophages did express large amounts of TNF mRNA, although these cells failed to produce detectable quantities of TNF, suggesting that a post-translational defect occurs in these mice (Beutler et al., 1986). These researchers speculate that under normal cellular conditions, macrophages contain small pools of TNF mRNA which are not translated; endotoxin mobilizes this mRNA for translation as well

as inducing the synthesis of more TNF mRNA. Finally, Beutler and co-workers note the presence of a conserved octameric sequence in the 3' untranslated region in a number of genes for endotoxin-inducible mediators, such as lymphotoxin, TNF, Il-1, the interferons and others. This group speculates that the conserved sequence may be involved in translational regulation of TNF gene expression (Beutler et al., 1986; Beutler and Cerami, 1986).

TNF, like IL-1, also has several biological effects that are similar to those caused by LPS. These include a monophasic or biphasic febrile response in rabbits; this febrile response is caused by TNF acting directly on the hypothalamus and through the induction of IL-1 (Beutler and Cerami, 1986). TNF also increases the production of PGE2 and collagenase by human fibroblasts and synovial cells (Dayer et al., 1985; Beutler and Cerami, 1986; Dinarello et al., 1986b), stimulates the production of procoagulant activity by vascular endothelial cells (Nawroth and Stern, 1986), and activates neutrophil adherence, degranulation (Beulter and Cerami, 1986) and phagocytosis (Shalaby, 1985), in addition to causing the activation of eosinophils (Beutler and Cerami, 1986). It is apparent that these TNF effects may mediate or synergize with LPS effects in LPS-caused pathobiology, such as the generalized and local Shwartzman reaction. In fact, mounting evidence suggests that TNF is the principal mediator of lethal endotoxic shock. Tracey and co-workers were able to show that recombinant TNF, administered in quantities similar to those produced endogenously in response to LPS, produced pathophysiological changes akin to those induced by LPS, including

hypotension, metabolic acidosis, and diffuse pulmonary inflammation and hemorrhage (Tracey et al., 1986). Bauss and co-workers were also able to show mimicry of LPS effects by TNF. They demonstrated decreased plasma glucose levels, leucopenia, and increases in hematocrit and plasma lactate levels (Bauss, Droge and Mannel, 1988). However, this team did not detect Il-1 in plasma of mice injected with TNF in contrast to the results of Dinarello et al. Dinarello's group, using high doses (10 ug/kg) of recombinant TNF-alpha induced biphasic pyrogenic responses in rabbits. This group attributed the second febrile peak to Il-1 production induced by TNF, and supported their observation by using an anti-human Il-1 monoclonal antibody and heating to 70 C for 30 minutes, which destroys the pyrogenic activity of Il-1, but not of recombinant TNF-alpha; also, the recombinant TNF-alpha had been shown to induce Il-1 from human mononuclear cells in vitro (Dinarello et al., 1986b).

Current Advances

Much remains to be elucidated about the biosynthesis and gene regulation of LPS. Some steps in this direction are already being taken. For instance, Palermo and co-workers were recently able to clone what they speculate is a gene for a core biosynthetic enzyme(s) of Neisseria gonorrhoeae and obtain its expression in E. coli K-12-- that is, the cloned gene was able to modify E. coli core LPS moieties such that they contained gonococcus-like epitopes recognized by antigonococcal serum on western blots (Palermo et al., 1987). Stein and co-workers, also working with N. gonorrhoeae, have been able to use transformation to

alter the LPS of several N. gonorrhoeae strains, producing LPS with donor phenotype, donor and recipient phenotype, as well as new LPS molecules different from either the donor or recipient, exhibiting novel SDS-PAGE profiles (Stein et al., 1988).

The lipid A moiety of LPS is responsible for many of the biological reactions of the molecule, and lipid A antiserum has been previously shown to be protective against fever and skin necrosis (Shwartzman reaction) (Reitschel and Galanos, 1977). The use of synthetic LPS or LPS precusors for treatment or prophylatic purposes of gram-negative sepis is an area of LPS research holding some promise for the future. Studies by Golenbock and his colleagues show that lipid X may offer protection from the lethal efects of endotoxin (Golenbock et al., 1987, 1988). Lipid X is a monosaccharide precursor of lipid A and has some of the properties of lipid A and LPS, but no toxicity. Lipid X can cause the gelation of Limulus amoebocyte lysates, murine B cell mitogenesis as well as activate macrophages, albeit to a much lesser extent than LPS or lipid A (Golenback et al., 1987). Pretreatment with lipid X has also been shown to protect sheep from the deleterious effects of endotoxin (Golenbock et al., 1987), in addition to protecting neutropenic ICR mice, alone or in combination with the antibiotic ticarcillin, from lethal infection with E. coli (Golenbock et al., 1988).

Discerning which of its effects are truly direct and which are due to immunomodulators, such as Il 1, TNF and PGE2, already has shown protective benefits, since mice given anti-TNF antiserum are protected from the lethal toxicity of LPS (Beutler et al., 1985). Further studies examining

the genetic regulation of such cytokines and how LPS is able to induce their increased production at the mRNA level may yield new intervention strategies in preventing the pathobiological effects of LPS.

Regardless of how LPS binds to and initiates cellular changes, it is still evident that much work remains to be done to resolve which pathophysiologic effects are attributable to LPS alone, and which result from the complex interplay of mediators, enzymes and factors induced by the direct effects of LPS. Developing the full pharmacologic benefits of its derivatives also presents contemporary researchers with a practical challenge.

Lipopolysaccharide has been extensively studied for many years. It has been found to cause some sort of effect in nearly every system studied. LPS acts on an amazing variety of cells types and induces an array of cytokines, enzymes, and immunomodulatory responses which result in pathophysiologic phenomena such as the Shwartzman reaction and lethality. LPS also possesses adjuvanticity, can increase nonspecific resistance to infection, and can induce nonspecific LPS-tolerance; despite the cornucopia of literature on this bacterial product, much remains to be uncovered about its biosynthesis, potential of synthetic derivatives as therapeutic agents, as well as sifting through its direct and indirect effects to elucidate the mechanisms involved in LPS pathobiology.

MATERIALS AND METHODS

Organisms

Four Moraxella strains were used in this work: M. bovis 62L, a field isolate, and three others obtained from the American Type Culture Collection (ATCC): M. bovis ATCC 10900, M. ovis ATCC 33078, and M. phenylpyruvica ATCC 23333. LPS from Escherichia coli K235 and J5 and Salmonella typhimurium prepared by phenol-water extraction were commercially obtained (Sigma Chemical Company, St. Louis, Mo.) S. minnesota Rd LPS was also commercially obtained (List Biological Laboratories, Cambell, Ca.), and Bacteroides fragilis LPS was prepared by phenol-water extraction in the laboratory of Dr. M. J. Wannemuehler, Veterinary Medicine Research Institute, Iowa State University, Ames, Ia.

Animals

Female New Zealand White rabbits weighing 3.0-3.5 kg were obtained from Small Stock Industries, Pearidge, Ar., and housed at the Laboratory Animal Resource facility in the College of Veterinary Medicine, Iowa State University, Ames, Ia. C3H/HeJ and BALB/c/ByJ mice, 8-16 weeks of age when used, were obtained from the Jackson Laboratories, Bar Harbor, Me. C3H/HeN mice, 8-16 weeks of age when used, were obtained from Harlan Sprague Dawley, Madison, Wi.

Hot Phenol-water Extraction of LPS

LPS was extracted as described by Hanson and Phillips (Hanson and Phillips, 1981). Cultures were grown in PNB broth, composed of 20 g/l proteose peptone #3 (Difco, Detroit, Mi.), 5 g/l sodium chloride, and 0.5 % bovine serum albumin (BSA; United States Biochemical Corporation, Cleveland, Oh.) at 37°C in a shaking water bath for 18 hr. Cultures were harvested by centrifugation, washed once in sterile phosphate buffered saline (PBS), pH 7.4 and stored at -70°C until used. Thirty ml of 90 % phenol was added to 10 g of cells (wet weight), suspended in 60 ml of distilled water heated to 68°C with vigorous stirring. This mixture was kept at 68°C for 15 min, then cooled to 10°C in an ice bath. To separate the LPS-containing aqueous layer, the cooled mixture was centrifuged at 10,000 x g for 30 min. The aqueous layer was removed and retained, while the bottom protein pellet and the middle phenol layer were extracted once more, as just described, by adding another volume of hot water. The aqueous layers were pooled and dialyzed for three days against distilled water containing 0.2% sodium azide to inhibit microbial contamination, with several changes of dialysate per day to remove any remaining phenol. The water extract was then digested in 2 ug/ml DNase with 3 ug/ml $MgCl_2$ by incubation at 37°C for 2.5 hr, followed by digestion with 2.5 ug/ml RNase and 1 ug/ml proteinase K for 1 hr at 37°C. The LPS was recovered by centrifugation at 100,000 x g for 4 hr and resuspended in pyrogen free saline. Relative purity of the preparation was assessed by scanning spectrometer readings from 240 nm to 300 nm, and repeating the centrifugation until the absorbance readings were close to 0. Finally, the

LPS was resuspended in pyrogen-free distilled water, quick frozen in a 90% ethanol-dry ice bath and lyophilized. Protein content of the lyophilized preparation was determined by using a 0.1 ml aliquot of a 1 mg/ml solution and subjecting it to a commercial assay. LPS was stored lypophilized at 4 C with desiccant. Confirmation that LPS had been extracted was performed by silver staining, mild acid hydrolysis and the the detection of the presence of 2-keto-3-deoxy-octonate (KDO).

Compositional Analysis of LPS

The compositional analysis of LPS was performed using several different preparations for each strain, while the biological assays described below were performed using only one preparation from each strain.

The absence of DNA or RNA in LPS preparations was confirmed by reading the absorbance of the LPS from 240nm to 300nm and determining if any peaks at 260nm and 280nm existed, indicating the presence of nucleic acids, and possibly protein.

Protein determinations were performed according to the Bio-Rad method, using commercial reagents (Bio-Rad, Richmond, Ca.) and bovine serum albumin as the protein standard.

Hexose content was determined colorimetrically by the method of Ashwell (Ashwell, 1966) by adding 0.05 ml 80% phenol to 2 ml of LPS solution (containing 0.1 mg LPS in 2 ml pyrogen free water), incubating at room temperature for 30 minutes, then adding 5 ml concentrated H SO to the sample and mixing. Absorbance of each sample was read at 485 nm and

the percent carbohydrate determined using the plot of a standard curve of D-galactose (Sigma Chemical Company, St. Louis, Mo.).

The KDO (Sigma Chemical Company, St. Louis, Mo.) content was determined by the method of Karkhanis et al. (Karkhanis et al., 1978), by adding 1 ml of 0.2 N H_2SO_4 to a tube containing 0.1 mg LPS. This mixture was heated at 100°C for 30 min, then centrifuged at maximum speed in a clinical centrifuge for 5 min. After centrifugation, the clear, upper 0.5 ml of each sample was pipetted into another tube, leaving the hydrolyzed LPS behind. The samples were treated with 0.25 ml of 0.04M HIO_4 in 0.125 N H_2SO_4 was added, the mixture vortexed and allowed to incubate at room temperature for twenty minutes before the addition of 0.25 ml of 2.6% $NaAsO_2$ in 0.5 N HCl. After vortexing and allowing the brown color to disappear, 0.5 ml of 0.6% 2-thiobarbituric acid (TBA) was added, and the mixtures vortexed and heated at 100°C for 15 min. While the samples were still hot, 1 ml of dimethyl sulfoxide (DMSO) was added, and the samples were cooled to room temperature before reading absorbance at 548 nm. A standard curve was simultaneously prepared using appropriate concentrations of KDO taken in 0.5 ml of 0.2 N H_2SO_4. Percent KDO was calculated as twice the amount interpolated from the graph divided by the amount of starting material, and multiplied by 100.

Determination of endotoxin units for each Moraxella LPS was performed using a commercial Limulus amoebocyte lysate (LAL) assay kit (Mallinkrodt Inc., St. Louis, Mo.). The assay was performed according to the manufacturer's instructions. One standard endotoxin unit (E. U.) was equal to 0.2 ng of E. coli endotoxin.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis of the LPS was performed using 12.5 % polyacrylamide (Bio-Rad, Richmond, Ca.) gels with 4M urea. Ten ul of 1 mg/ml solutions of LPS were boiled for 1.5 min in a treatment buffer containing 2-mercaptoethanol prior to loading. Gels were run at 40 mA for 2.5 hr, or until the dye front was 1 cm from the bottom of the slab. Gels were silver stained, or transferred to nitrocellulose for western blotting, as described below.

Silver Staining of LPS

Gels were stained using the Hitchcock modification of the Tsai and Frasch method (Hitchcock and Brown, 1983). Gels were fixed overnight with 25% isopropanol in 7% acetic acid, then oxidized for five minutes in 150 ml distilled water with 1.05 g periodic acid plus 4 ml of fixative solution. The gels were then washed eight times in distilled water with gentle shaking for thirty minutes each time. The washes were followed by 10 minutes of staining in 0.1 N NaOH, 1.2 ml of concentrated ammonium hydroxide, and 5 ml 20% silver nitrate in 150 ml distilled water, followed by 4- ten minute washes in distilled water. Gels were allowed to develop for 5-15 minutes in developer containing 25 mg citric acid and 0.25 ml of 37% formaldehyde in 500 ml distilled water, then development was stopped by shaking in 200 ml water with 10 ml 7% acetic acid for one hour.

Western Blot Analysis

Lipopolysaccharides were transferred overnight in a 20% methanol-25 mM sodium phosphate buffer, pH 6.8, onto nitrocellulose (Schleicher & Schuell, Inc., Keene, N. H.) at 20 mV. The nitrocellulose was then washed 3 times in tris saline buffer (1.2 g/l THAM, 0.9 g/l NaCl, and 0.02 g/l sodium azide, pH 8.6) with 0.05% tween 20, then blocked with 1% gelatin in the tris saline buffer. The LPS samples were then allowed to react with a 1.5% solution of bovine globulins from a gnotobiotic calf that had been infected with M. bovis 62L. Outer membrane preparations from M. bovis 62L were also reacted with the bovine globulin solution. Epitopes recognized by the globulins were visualized by reaction with 1.5 uCi of ^{125}I-labeled protein G (Amersham, Arlington Heights, Il.) and subsequent autoradiography. Western blot analysis was also performed using a 4.3 % solution of a monoclonal antibody developed against M. bovis 62L. The monoclonal antibody was tested against all LPS preparations and against an outer membrane preparation of M. bovis 62L (Ostle and Rosenbusch, 1986). Epitopes recognized by the monoclonal antibody were visualized by reaction with 1.5 uCi of rabbit anti-mouse ^{125}I-labeled IgG (Amersham, Arlington Heights, Il.) and subsequent autoradiography.

Pyrogenicity Assay

Female New Zealand White rabbits weighing 3.0 to 3.5 kg were used for pyrogenicity assays. Rabbits were preconditioned to rectal probing by handling several days prior to the start of the experiment. Baseline rectal temperatures for each rabbit were established before administration

of LPS. Two or three rabbits were randomly assigned to receive each Moraxella LPS dilution, given intravenously in the marginal ear vein in 0.5 ml pyrogen free saline. As controls, two rabbits received either 0.5 ml pyrogen free saline or 0.01 mg E. coli K235 LPS. Temperatures were monitored rectally with electronic probes (Norelco, Stamford, Ct.) every fifteen minutes after the injection of LPS for the first hour, and every 20 minutes thereafter until biphasic increases in temperature were observed and temperatures returned to near baseline (about 5.5 hours). Means and SEM for temperature changes were determined for each time point.

Local Shwartzman Reaction

Female New Zealand White rabbits weighing 3.0 to 3.5 kg were used for the induction of a local Shwartzman reaction, using a protocol similar to that described by Beck et al. (1986). Rabbit backs were shaved 24 hr prior to the start of the experiment, at which time the rabbits received priming intradermal injections of the Moraxella LPS dilutions (100, 50, 25 and 10 ug in 0.5 ml pyrogen free saline), plus control sites of saline only or E. coli K235 LPS (10 ug). After 24 hr (day 2), the rabbits were then given a provocative intravenous dose of homologous LPS (50 ug) and after 48 hr (day 3), the external morphology of the lesions was described (e.g., diameter of swelling, degree of redness or necrosis). The rabbits were then euthanized and the lesions were excised, fixed in 10% buffered formalin and subjected to hematoxylin and eosin staining, then examined microscopically to assess the degree of inflammation as evidenced by such

hallmarks as polymorphonuclear leukocyte infiltration, coagulation, hemorrhage and edema. Lesions were scored based on their external morphology, as well as on the extent of neutrophil infiltration, edema, hemorrhage, and fibrin deposition. Sites with no apparent redness or swelling were scored as -; lesions with a slight swelling (diameter of 5 mm or less) and some redness were scored as +; lesions with moderate swelling (diameter of 6-12 mm) and redness were scored as ++; lesions with substantial swelling (diameter of 15-19 mm) and erythema were scored as +++; lesions with maximum swelling (diameter of 20 mm or greater), erythema and necrosis were scored as ++++.

Stimulation of Murine Peritoneal Exudate Cells

BALB/c/ByJ mice 8-16 weeks old were injected intraperitoneally with 2.0 ml of sterile thioglycollate 3 days prior to culturing of the peritoneal exudate cells (PEC). Mice were sacrificed by cervical dislocation, and the peritoneal cavity subjected to lavage using 8-10 ml PBS with 1% fetal bovine serum (FBS; KC Biological, Inc., Lenexa, Ks.) and 5 units heparin (Lypho-Med, Inc., Chicago, Il.) with a 20 gauge needle. The body cavity was vigorously massaged to loosen macrophages, then the wash containing the PEC was withdrawn and the lavage repeated. PEC were washed twice in RPMI 1640 (Gibco, Grand Island, N. Y.) supplemented with 100 units l-glutamine, 100 units/ml penicillin, 100 ug/ml streptomycin and 10 mM HEPES. PEC were then counted using a model ZF Coulter counter (Coulter Electronics, Hialeah, Fl.) and plated at 10^6 cells/ml, 1 ml per well, in a 12- well Costar plate (Costar, Cambridge, Ma.). Harvested PEC

were allowed to adhere for 2 hr, then nonadherent cells were removed by vigorous washing with RPMI 1640 three times. To the adherent cells remaining (macrophages), LPS dilutions were added in 1 ml RPMI, and the plates were incubated at 37°C in 5% CO_2 for 24 to 72 hr. Supernatants were harvested, cells were removed by centrifugation and the cell-free supernatants were stored in 0.2 to 0.5 ml aliquots at -70°C until used in TNF and IL-1 assays (see below).

Peritoneal Exudate Cell Toxicity

The potential toxicity of Moraxella LPS on murine PEC was assessed using BALB/c/ByJ PEC elicited as described above, as well as resident PEC which were similarly obtained, but without any prior stimulation. Cells were resuspended to one million cells/ml in RPMI 1640 with the same supplements as above. Different doses (0.5, 5, and 50 ug in 50 ul of RPMI 1640) of each Moraxella LPS, or E. coli K235 LPS were added to 1 ml of cells in polypropylene tubes (Fisher Scientific, Pittsburgh, Pa.) and incubated overnight with rotation (to prevent adherence) at 37°C. Each sample was stained with 10 ul of a sterile 0.5 mg/ml solution of propidium iodide (indicates dead cells) and 10 ul of 0.5 % fluoroscein diacetate in acetone, diluted 1:1000 in PBS (indicates viable cells). The percentages of live and dead cells were determined using an Epic 7, model 752 Coulter fluorescence activated cell sorter (FACS; Coulter Electronics, Hialeah, Fl.); five thousand cells per sample were counted.

Interleukin 1 Assay

To ascertain the ability of Moraxella LPS to stimulate Il-1 secretion from murine PEC, proliferation of the Il-1 dependent T cell line D10.G4.1 (Kaye et al., 1984; American Type Culture Collection, Washington, D. C.) was measured by uptake of tritiated thymidine. Briefly, D10.G4.1 cells depleted of Il-1 were centrifuged through a density gradient (Ficoll-Paque 1077, Sigma Chemical Company, St. Louis, Mo.) at 1000 rpm in a clinical centrifuge for 30 min, then washed twice in RPMI 1640 supplemented with 10% FCS, 5 ug/ml concanalvin A, 100 u/ml penicillin, 100 ug/ml streptomycin, 100 u/ml L-glutamine and 10 mM HEPES. The D10.G4.1 cells were then resuspended to a density of 10^6 cells/ml in the same medium, and 0.1 ml per well of this suspension was plated in a flat bottom 96-well microplate. Supernatants from stimulated PEC were added at various dilutions (e.g., 1:10, 1:20, 1:40) in triplicate and the plates incubated for 48 hr at 37°C in 5% CO_2, then pulsed by adding 0.5 uCi of tritiated-thymidine in RPMI 1640, and incubated an additional 16 hr. Samples were harvested onto filter paper using a multi-well cell harvester. The filter paper was allowed to dry, and then the sample discs placed in scintillation vials with 2.5 ml scintillation fluid, and counted using standard liquid scintillation techniques.

Tumor Necrosis Factor Assay

To determine if Moraxella LPS was capable of stimulating the production of tumor necrosis factor (TNF) from murine PEC, the TNF-sensitive fibroblast cell line, L929 (American Type Culture Collection,

Washington, D. C.), was used. Briefly, the L929 cells were harvested by scraping with a cell scraper into RPMI 1640 supplemented with 10% horse serum (HS; Hyclone, Logan, Utah), L-glutamine, 10 mM HEPES and 100 units/ml penicillin and 100 ug/ml streptomycin and counted using a model ZF Coulter counter, then plated at a density of 10^5 cells per well of a 96-well microplate in the same RPMI (0.1 ml). The L929 cells were then incubated overnight at 37 °C in 5% CO_2 before serial dilutions (1:10 to 1:31250, final dilution) of LPS-stimulated cell-free PEC supernatants were added. All dilutions were performed in quadruplicate, and a 0.1 ml volume supplement of RPMI 1640 medium containing 5 ug/ml actinomycin D was added before incubating the cells for an additional 18 hr at 37 °C in 5% CO_2. After incubation, supernatants were discarded by shaking the inverted plates, and the remaining cells were washed twice with 0.1 ml PBS with 2% normal buffered formalin (NBF), then stained for 20 minutes using 0.13 % crystal violet in 5% ethanol with 2 % NBF, according to a published procedure (Gentry and Dalrymple, 1980). The plates were then washed 5 times in tap water and allowed to air dry. The stain was resolubilized with 100 ul of 50% ethanol before the samples were read on an automated ELISA reader (Microplate model EL310, Bio-Tek, Winooski, Vt.) at 595 nm. The percent cytotoxicity was determined by dividing the mean absorbance obtained with stimulated supernatants for each dilution by the absorbance obtained by control wells incubated with RPMI 1640 only. To confirm that the cytotoxicity of the supernatants was due to TNF, some cultures were incubated with rabbit anti-mouse TNF antisera (Genezyme Corporation, Boston, Ma.).

Splenic Blastogenesis Assay

To assess the ability of Moraxella LPS to induce B cells to undergo blastogenesis, responder C3H/HeN and BALB/c/ByJ and hyporesponsive C3H/HeJ mice were used as described by Wannemuehler et al. (1984). Spleens from these mice were aseptically removed and minced, using a 100 mesh screen, as a source of B cells. Cells were allowed to settle, and were then washed in RPMI 1640 by centrifugation at 1000 rpm in a clinical centrifuge. Washed cells were resuspended in RPMI 1640, 2 ml per spleen, and counted using a model ZF Coulter counter. Cells were plated at 10^5 cells per well in a 96-well flat bottom microplate. Dilutions of Moraxella LPS or control mitogens (e.g., phytohemagglutinin (PHA) and concanalvin A (Con A) at 1.0, 0.5, 0.1, and 0.01 ug/ml) were added to the wells in triplicate, along with additional controls of RPMI 1640 only and various concentrations of E. coli K235 and J5 LPS, Salmonella minnesota Rd and Bacteroides fragilis LPS. Cells were incubated at 37°C for 40 hr with 5% CO_2, then pulsed with 0.5 uCi of tritiated-methyl-thymidine and incubated another 6-8 hr. Samples were harvested onto filter paper using a multiwell cell harvester; the paper was allowed to dry, and the discs placed into scintillation vials with 2.5 ml cocktail and counted using standard scintillation techniques. The mean CPM +/- SEM was determined for each sample. The stimulation index was determined by dividing mean sample CPM by the CPM of the control containing RPMI 1640 only.

Lethality in Galactosamine-sensitized Mice

The lethal dose required to kill 50% of the sensitized mice (LD_{50}) for each Moraxella LPS was determined using BALB/c/ByJ mice sensitized to the lethal effects of LPS by treatment with 2 mg/ml D-galactosamine in their drinking water ad libitum for 48 hr prior to LPS injection (Galanos et al., 1979). After sensitization, LPS doses (from 0.1 ug to 40 ug) were given to groups of 6 mice i.v. via the tail vein in 0.2 ml pyrogen free saline followed by an i.p. injection of 16 mg of D-galactosamine in pyrogen-free saline. As controls, one group of 6 mice received saline only i.v. with the i.p. D-galactosamine, while another group received 0.1 ug of E. coli K235 LPS. The mice were observed for 3 days following LPS administration and deaths were recorded.

RESULTS

Moraxella LPS Compositional Analysis

Confirmation that LPS had been extracted was evidenced by successful silver staining (see below) and lack of Coomasie blue staining of the preparations, as well as the presence of KDO (see below) and the production of a white precipitate upon mild acid hydrolysis.

The protein content of each *Moraxella* LPS preparation was undetectable by the protocol employed; these results are given in Table 1. The KDO content of each *Moraxella* LPS preparation is presented in Table 1, and represents the mean % KDO +/- SD for at least three independent determinations. The hexose content of the preparations indicated in Table 1 represent the mean % hexose for at least five separate determinations. As also indicated in Table 1, all *Moraxella* LPS preparations were found to have 2 E.U. per ng LPS (Table 1).

SDS-PAGE Analysis

The silver stain SDS-PAGE profile of *Moraxella* LPS is presented in Figure 1. *M. bovis* 62L exhibits a smooth type LPS (Figure 1, Lane E), with a "ladder" such as those produced by *E. coli* K235 and *S. typhimurium* (Figure 1, Lanes B and D, respectively) while *M. bovis* ATCC 10900 has a rough profile (Figure 1, Lane F). Both *M. ovis* ATCC 33078 and *M. phenylpyruvica* ATCC 23333 exhibit rough profiles (Figure 1, Lanes G and H, respectively), similar to that produced by the rough *E. coli* J5 (Figure 1, Lane C). Secondary staining of a silver stained gel with Coomassie blue

failed to stain any of the LPS preparations, although it did stain an outer membrane preparation of M. bovis 62L (not shown).

Western Blot Analysis

The results of western blot analysis using a 1.5 % solution of globulins from convalescent serum from a gnotobiotic calf infected with M. bovis 62L are presented in Figure 2. The globulins reacted with both the smooth and rough M. bovis 62L and M. bovis ATCC 10900 LPS (Figure 2, Lanes D and E, respectively), as well as with the rough M. ovis LPS (Figure 2, Lane F). The globulins failed to react with M. phenylpyruvica LPS (Figure 2, Lane G). These globulins recognized many determinants on an outer membrane preparation from M. bovis (Figure 2, Lane H). Also, E. coli K235, J5, and S. typhimurium LPS were recognized (Figure 2, Lanes A, B, and C, respectively). The epitopes recognized were located on molecules with various relative mobilities (Mr).

The results of western blot analysis using a 4.3 % solution of a monoclonal antibody reactive with a M. bovis 62L LPS moiety or epitope are shown in Figure 3. The monoclonal antibody reacted only with M. bovis 62L and ATCC 10900 LPS (Figure 3, Lanes E and D, respectively) and an outer membrane preparation from M. bovis 62L (Figure 3, Lane A). The monoclonal antibody failed to react with M. ovis or M. phenylpyruvica LPS (Figure 3, Lane C and B, respectively). LPS from E. coli K235 and J5 and S. typhimurium also failed to react with this monoclonal antibody (Figure 3, Lanes H, G, and F, respectively). The epitope recognized was located on bands of the same Mr for M. bovis 62L and ATCC 10900 LPS and

Table 1. Compositional Analysis of *Moraxella* LPS

Strain	%KDO[a]	%Hexose[b]	Protein	E.U.[c]
M. bovis 62L	10.2 +/- 2.3	20.8 +/- 5.8	n.d.[d]	2
M. bovis ATCC 10900	5.3 +/- 1.9	9.7 +/- 4.2	n.d.	2
M. ovis	5.6 +/- 0.4	14.0 +/- 3.6	n.d.	2
M. phenylpyruvica	10.8 +/- 3.9	17.2 +/- 5.2	n.d.	2

[a] KDO = 2-keto-3-deoxy-octonate.

[b] Standard was D-galactose.

[c] E.U. = endotoxin unit per ng LPS.

[d] n.d. = not detected.

Figure 1. SDS-PAGE analysis of Moraxella LPS using a 12.5% polyacrylamide gel with 4M urea
Lane A, LMW; Lane B, E. coli K234; Lane C, E. coli J5; Lane D, S. typhimurium; Lane E, M. bovis 62L; Lane F, M. bovis ATCC 10900; Lane G, M. ovis ATCC 33078; Lane H, M. phenylpyruvica ATCC 23333. 10 ug of each LPS was subjected to electrophoresis.


A
B
C
D
E
F
G
H

for the outer membrane preparation of M. bovis 62L.

Pyrogenicity

M. bovis strains

The results of pyrogenicity studies performed in rabbits with M. bovis 62L and ATCC 10900 LPS are represented by Figure 4. Typical biphasic peaks are observed with both LPSs, as well as with the E. coli K235 control. The first peak occurred within 45-60 min of LPS injection and the second peak occurred about 180 min after LPS injection. No pyrogenic effect was observed in pyrogen free saline controls. The maximum mean temperature increase for M. bovis 62L at 100 ug was 3.9 °F, while the maximum temperature increase for M. bovis ATCC 10900 at 100 ug was 1.6 °F. E. coli K235 LPS at 10 ug produced the greatest mean maximum pyrogenic response, 4.6 °F.

Non-M. bovis strains

The results of pyrogenicity studies performed in rabbits using M. ovis and M. phenylpyruvica LPS, and run simultaneously with the M. bovis strains are shown in Figure 5. Biphasic peaks in temperature increase were observed with both LPSs, occurring 45-60 min and 180 min after LPS injection. M. ovis LPS at 25 ug gave a maximum temperature increase of 3.8 °F, while M. phenylpyruvica LPS at 25 ug gave a maximum temperature increase of 3.0 °F. Again, the pyrogenic response to E. coli K235 LPS at 10 ug was a maximum 4.6 °F.

Figure 2. Autoradiograph of western blot analysis using globulins from convalescent gnotobiotic calf serum
A 1.5% solution of immunoglobulins from a gnotobiotic calf was reacted with LPS transferred to nitrocellulose after SDS-PAGE electrophoresis using a 12.5 % gel with 4 M urea. Lane A, E. coli K235; Lane B, E. coli J5; Lane C, S. typhimurium; Lane D, M. bovis 62L; Lane E, M. bovis ATCC 10900; Lane F, M. ovis ATCC 33078; Lane G, M. phenylpyruvica ATCC 23333; Lane H, M. bovis 62L outer membrane preparation (10 ug protein). Epitopes recognized by the globulins were visualized using 1.5 uCi of ^{125}I-labeled protein G and autoradiography.

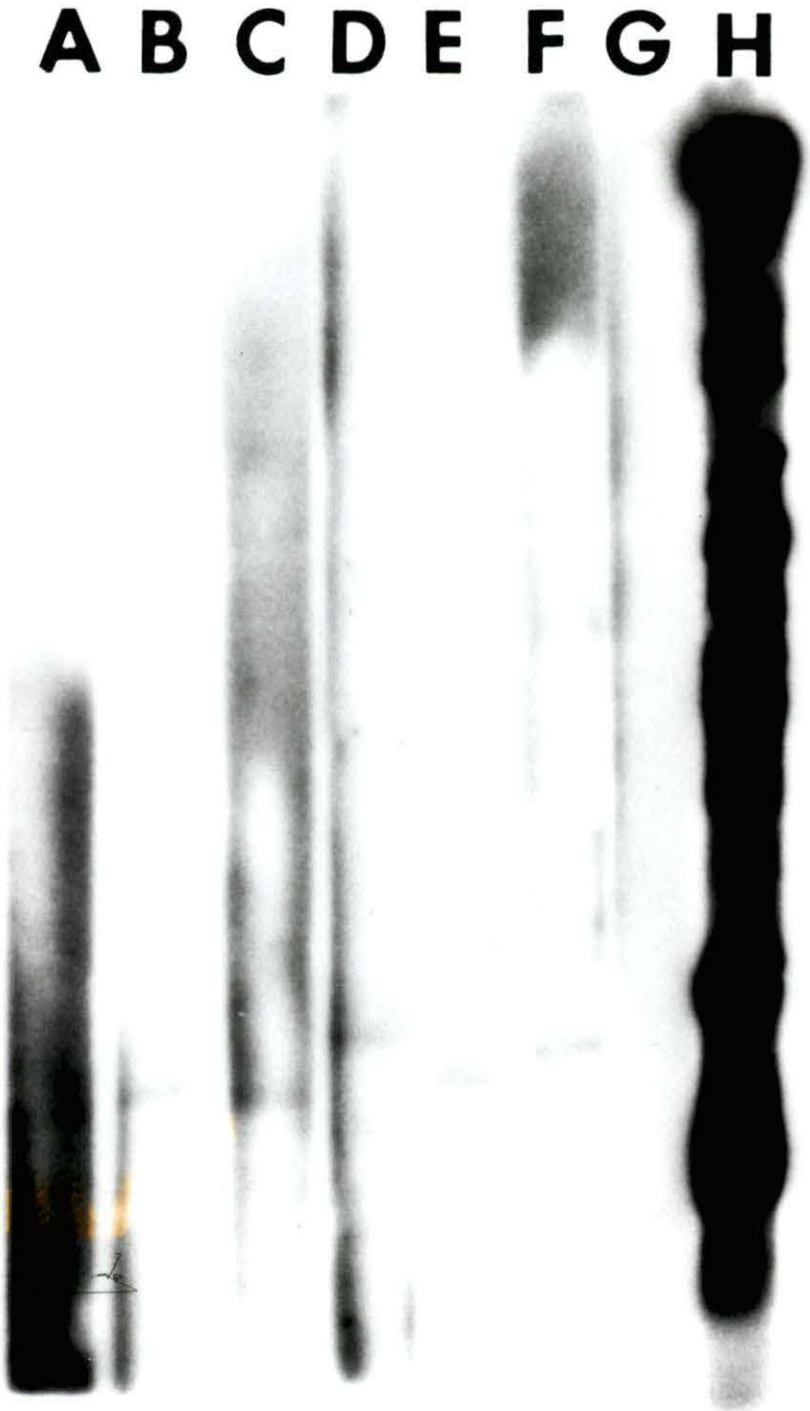
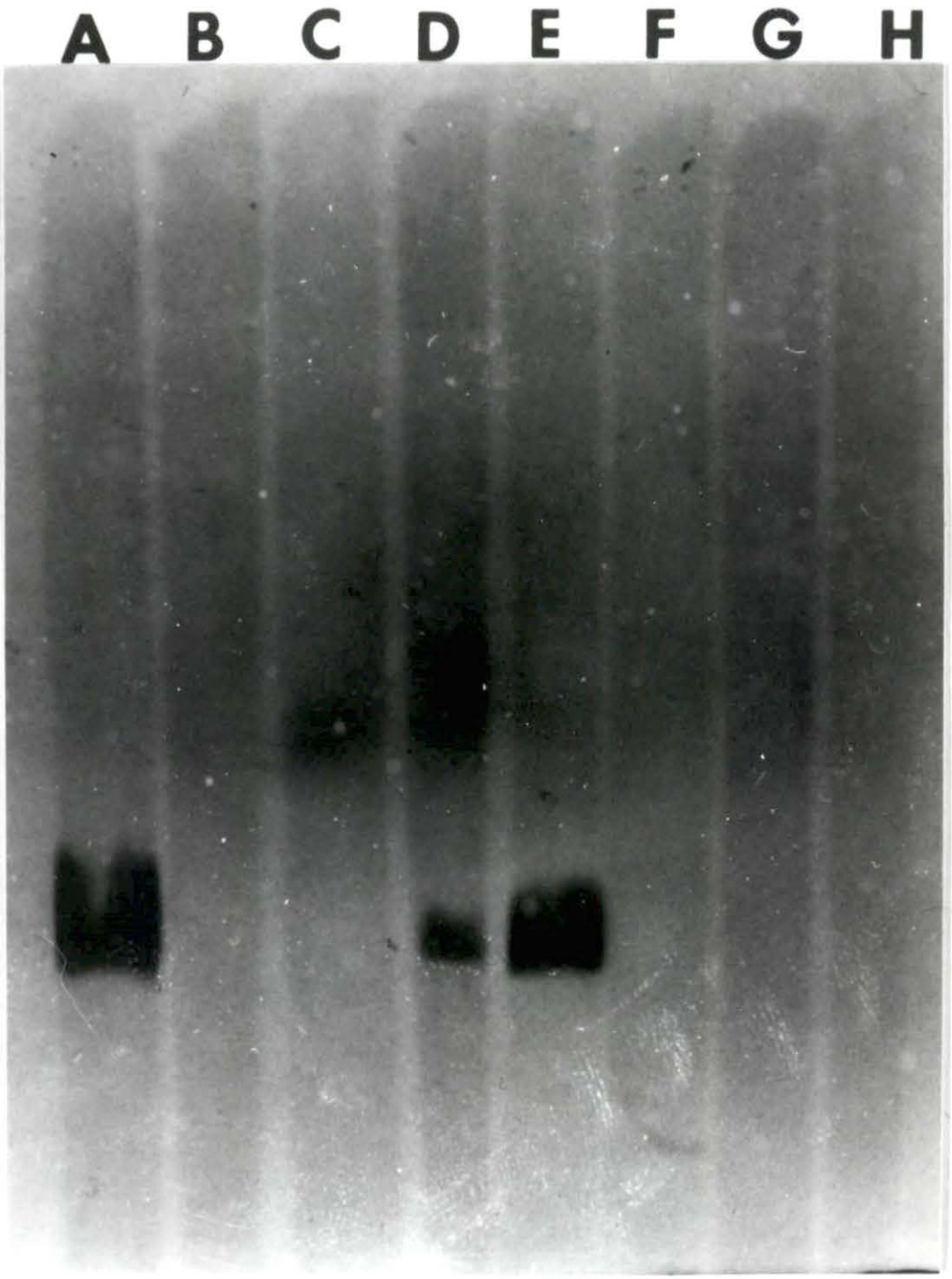

A B C D E F G H

Figure 3. Autoradiograph of western blot analysis using monoclonal antibody 29.6
A 4.3% solution of monoclonal antibody 29.6, reactive with an M. bovis 62L LPS moiety, was reacted with LPS transferred to nitrocellulose after SDS-PAGE electrophoresis using a 12.5 % gel with 4 M urea. Lane A, M. bovis 62L outer membrane preparation; Lane B, M. phenylpyruvica ATCC 23333; Lane C, M. ovis ATCC 33078; Lane D, M. bovis ATCC 10900; Lane E, M. bovis 62L; Lane F, S. typhimurium; Lane G, E. coli J5; Lane H, E. coli K235. Epitopes recognized by the monoclonal antibody were visualized using rabbit anit-mouse ^{125}I-labeled IgG and autoradiography.


A
B
C
D
E
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G
H

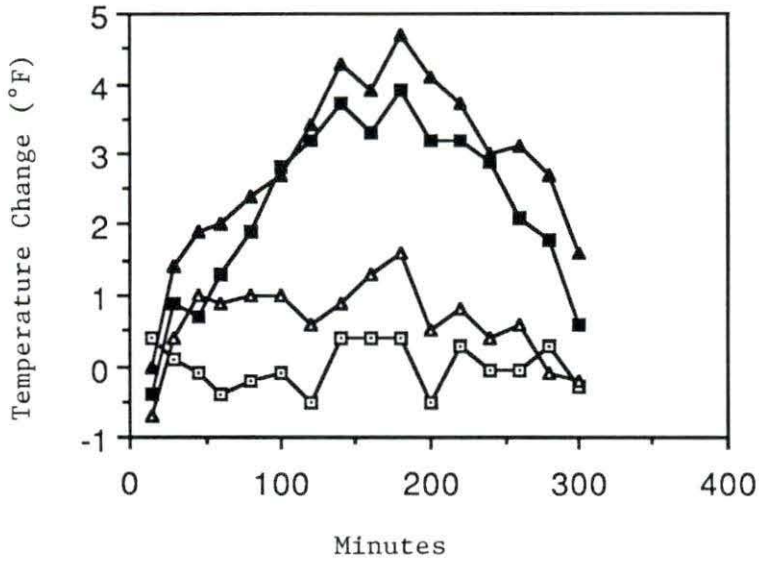
Local Shwartzman Reaction

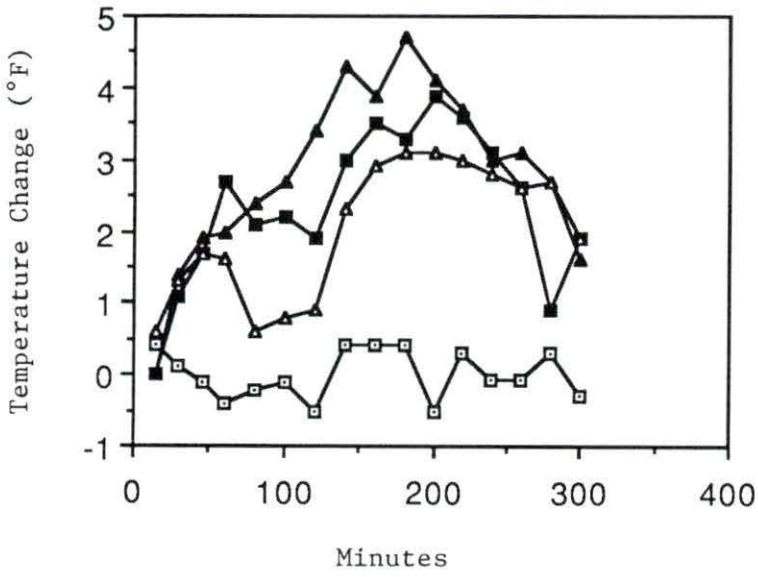
External morphology

The external morphology of the lesions provoked during the local Shwartzman reaction is presented in Figure 6. Although no lesions, swelling or redness were apparent 18 hr after the priming, i.d. injections of LPS, 24 hr after the provocative dose, lesions were produced with necrotic centers at the highest i.d. doses (100 ug). Saline control sites never developed lesions. E. coli K235 LPS (10 ug) i.d. sites sometimes had no external evidence of reaction (e.g., M. bovis ATCC 10900/ M. bovis ATCC 10900 rabbit and M. phenylpyruvica/ E. coli rabbit; Table 2), or exhibited lesions with swelling only (M. bovis 62L/ M. bovis 62L rabbit and all LPS/ saline rabbit; Table 2) and sometimes gave lesions fifteen mm in diameter with some redness (M. ovis/ M. ovis rabbit; Table 2). The sites primed with 10 ug Moraxella LPS showed little or slight swelling (up to 6 mm diameter swelling with redness; Table 2). Similarly, the 25 ug priming sites of all Moraxella LPS showed little or slight swelling (up to 5 mm in diameter with slight redness; Table 2). However, nearly all the priming i.d. sites of higher doses showed reaction. At 50 ug, all but one Moraxella LPS (that of M. bovis ATCC 10900; Table 2) showed substantial swelling (up to 15 mm in diameter) with redness; on the M. phenylpyruvica/ E. coli rabbit the lesion at this dose had a necrotic center. At 100 ug, all Moraxella LPSs produced lesions with substantial swelling (from 15 to 22 mm in diameter) and erythema. Again, at this dose, the M. phenylpyruvica/ E. coli rabbit had a lesion with a necrotic center. The rabbit primed with all Moraxella LPSs and "provoked" with saline had

Figure 4. Pyrogenicity with M. bovis strains
3.0-3.5 kg female New Zealand White rabbits were used to assess Moraxella LPS pyrogenicity. M. bovis 62L (●) and M. bovis ATCC 10900 (▲) LPS was given i.v. at 100 ug in 0.5 ml saline. Control rabbits received 10 ug E. coli K235 (■) LPS in 0.5 ml saline or the same volume of saline (□) alone. Mean change in temperature was recorded over the indicated time course.

Figure 5. Pyrogenicity with non-M. bovis strains
3.0-3.5 kg female New Zealand White rabbits were used to assess Moraxella LPS pyrogenicity. M. ovis ATCC 33078 (●) and M. phenylpyruvica ATCC 23333 (▲) LPS was given i.v. at 25 ug in 0.5 ml saline. Control rabbits received 10 ug E. coli K235 (■) LPS in 0.5 ml saline or the same volume of saline (□) alone. Mean change in temperature was recorded over the indicated time course.


Temperature Change (°F)
5
4
3
2
1
0
-1
0
100
200
300
400
Minutes


Temperature Change (°F)
5
4
3
2
1
0
-1
0
100
200
300
400
Minutes

little or no reaction with saline, E. coli K235 LPS or with M. ovis LPS at 100 ug. However, some swelling and redness was observed at the i.d. sites of the other Moraxella LPSs at 100 ug (from 12 mm in diameter for the M. bovis 62L site to 28 mm in diameter for the M. phenylpyruvica site). The results of the external morphology of the local Shwartzman reaction are summarized in Table 2.

Microscopic morphology

None of the saline control sites showed PMN infiltration, congestion, hemorrhage or any other inflammatory effects (Figure 7; Table 2). The E. coli K235 priming sites usually showed PMN infiltration and occasionally hemorrhage (Figure 8; Table 2). The i.d. injection sites of the higher doses showed multiple foci of PMN infiltration (Figures 7-10; Table 2), fibrin deposition (Figures 9 and 10), hemorrhage and congestion (Figures 8 and 9; Table 2), as well as edema (Figure 10) and microthrombi. The microscopic morphology of the stained sections was indistinguishable from rabbit to rabbit. These results are summarized in Table 2.

Splenic Blastogenesis

C3H/HeN mice

All the Moraxella LPS preparations induced C3H/HeN splenocytes to undergo blastogenesis (Table 3). Some proliferative activity was seen with concentrations of 0.01 ug and 0.1 ug, although the effect was much greater at amounts of 1, 5, 10, 20 and 50 ug LPS. For M. bovis 62L, maximum activity occurred at 5 ug, while for M. bovis ATCC 10900 and M.

Table 2. Results of the local Shwartzman reaction

Agent/Dose[b]	Provoking Agent[c]	Lesion Morphology[a] External	Lesion Morphology[a] Microscopic
E. coli K235 10	*M. bovis* 62L LPS	+	PMN[d],H[e]
Saline	same	-	-
M. bovis 62L 10	same	-	-
M. bovis 62L 25	same	+	PMN
M. bovis 62L 50	same	++	PMN,H[f]
M. bovis 62L 100	same	+++	PMN,C[f],H, MT[g]
E. coli K235 10	*M. bovis* ATCC 10900 LPS	-	-
Saline	same	-	-
M. bovis 10	same	+	PMN
ATCC 10900 25	same	-	-
M. bovis 50	same	-	-
ATCC 10900 100	same	++++	PMN,C,H,MT
E. coli K235 10	*M. ovis* ATCC 33078	+++	PMN,C,H
Saline	same	-	-
M. ovis 10	same	-	-
M. ovis 25	same	+	PMN,C,H
M. ovis 50	same	++	PMN,C,H,MT
M. ovis 100	same	+++	PMN,C,H,MT, E[h]

[a]Lesions scored on size and appearance of necrotic centers.

[b]ug LPS given in 0.5 ml pyrogen-free saline as the priming dose.

[c]50 ug of indicated LPS given in 0.5 ml pyrogen-free saline.

[d]PMN = Polymorphonuclear leukocyte infiltration; indicates inflammation.

[e]H = hemorrhage; lesion characteristic.

[f]C = congestion; lesion characteristic.

[g]MT = microthrombi; lesion characteristic.

[h]E = edema; indicates inflammation.

Agent/Dose		Provoking Agent	Lesion Morphology External	Lesion Morphology Microscopic
E. coli K235	10	*E. coli* K235 LPS	-	-
Saline		same	-	-
M. phenyl-	10	same	++	PMN,H
pyruvica	25	same	+	PMN,C
ATCC	50	same	+++	PMN,C,H,MT
23333	100	same	++++	PMN,C,H,MT, E
E. coli K235	10	Saline	-	-
Saline		same	-	-
M. bovis 62L	100	same	++	PMN,C,H
M. bovis	100	same	+++	PMN,C,H,MT
ATCC 10900				
M. ovis	100	same	+	PMN,C
M. phenyl-	100	same	++++	PMN,C,H,MT, E
pyruvica				

Figure 6. Local Shwartzman reaction: external morphology
2.5-3.0 kg female New Zealand White rabbits were used to assess the ability of Moraxella LPS to provoke the local dermal Shwartzman reaction. M. bovis ATCC 10900 LPS was injected i.d. into the shaved rabbit back in 0.5 ml saline, along with E. coli K235 LPS and saline only controls. The reaction was provoked 18 hr after i.d. injection by i.v. injection of 50 ug M. bovis LPS in 0.5 ml saline. Upper left, E. coli K235 LPS (10 ug); upper right, saline only control; middle left, 10 ug M. bovis ATCC 10900 LPS; middle right, 25 ug M. bovis LPS; lower left, 50 ug M. bovis LPS; lower right, 100 ug M. bovis LPS.

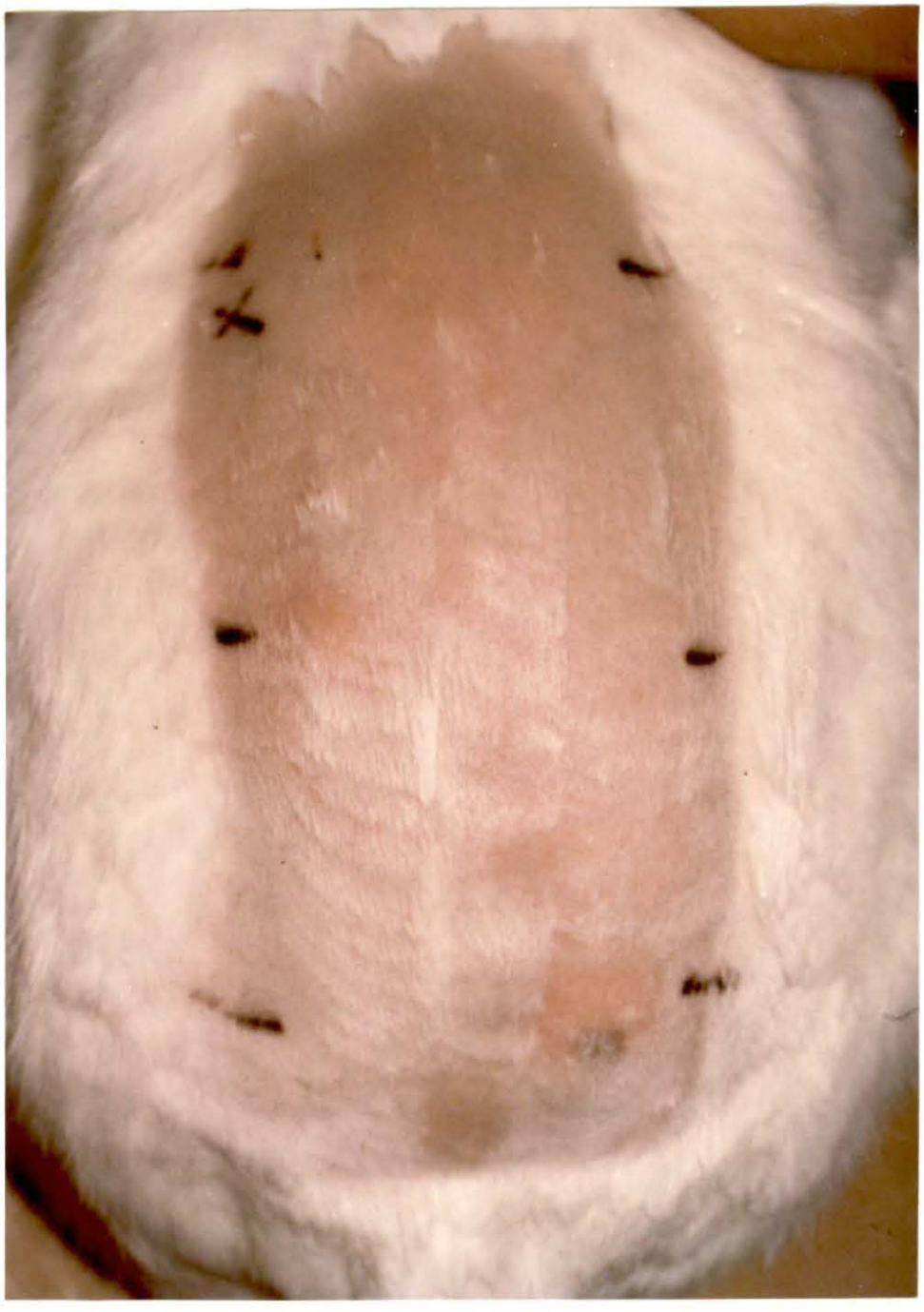
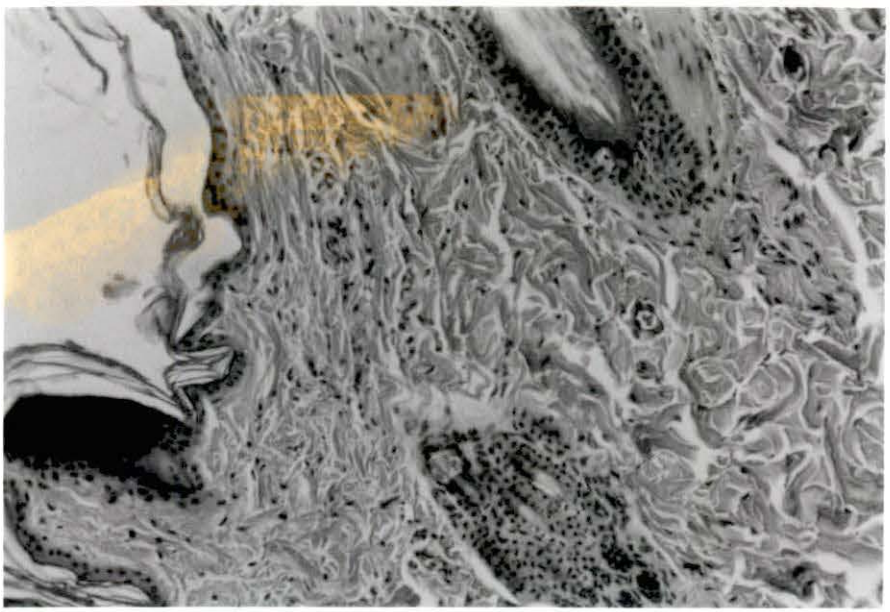


Figure 7. Local Shwartzman reaction: histological examination Example of saline control site with no evident reaction. Rabbit was injected with 0.5 ml saline 18 hr prior to i.v. injection of 50 ug M. bovis 62L LPS. Section was stained with hematoxylin and eosin. Magnification 160X.

Figure 8. Local Shwartzman reaction: histological examination Example of vascular coagulation. Rabbit was injected i.d. with 10 ug E. coli K235 LPS 18 hr prior to i.v. injection with 50 ug M. bovis 62L LPS. Section was stained with hematoxylin and eosin. Magnification 160X.



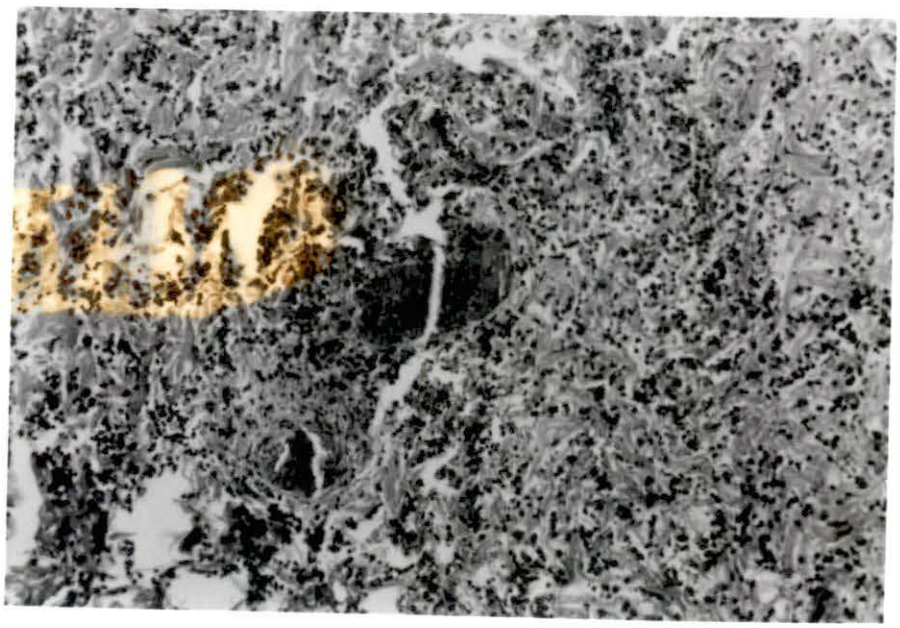
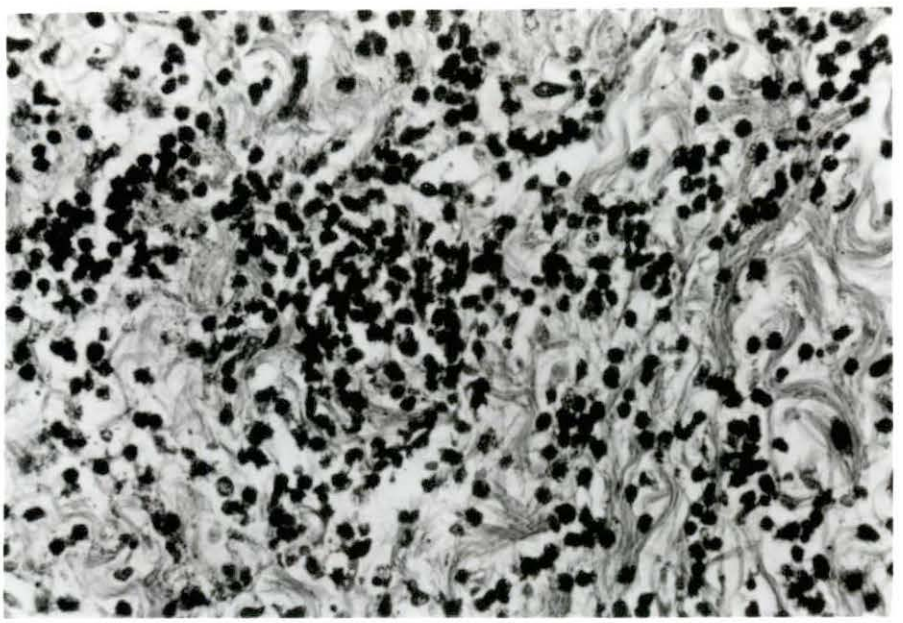
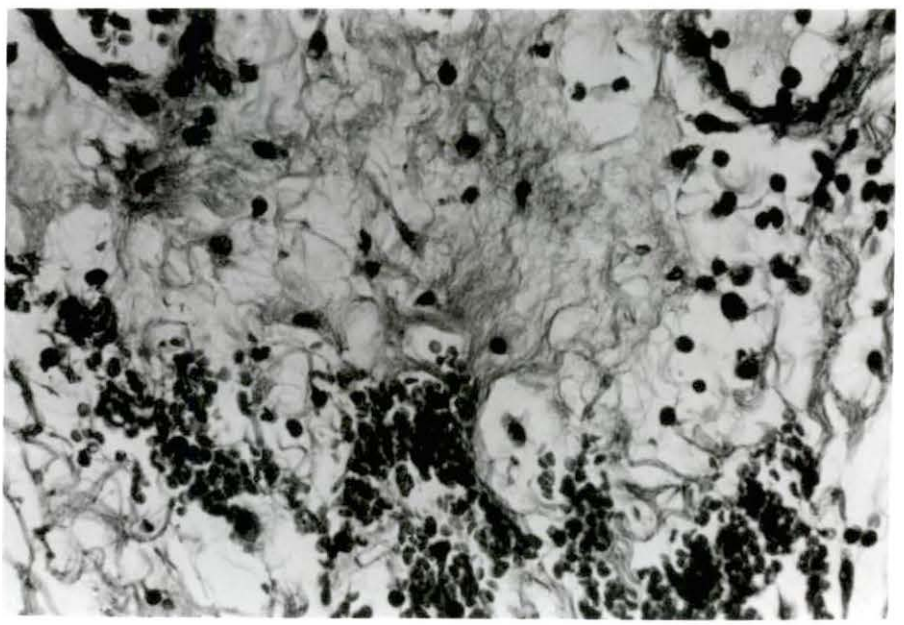


Figure 9. Local Shwartzman reaction: histological examination Example of PMN focus. Rabbit was injected i.d. with 100 ug M. phenylpyruvica ATCC 23333 LPS 18 hr prior to i.v. injection of 50 ug E. coli K235 LPS. Section was stained with hematoxylin and eosin. Magnification 400X.

Figure 10. Local Shwartzman reaction: histological examination Example of fibrin deposition and edema. Rabbit was injected i.d. with 50 ug M. phenylpyruvica ATCC 23333 LPS 18 hr prior to i.v. injection with 50 ug E. coli K235 LPS. Section was stained with hematoxylin and eosin. Magnification 400X.





ovis, maximum proliferative effects were seen at 10 ug and 20 ug, respectively. For M. phenylpyruvica LPS, the maximum effect seen on the C3H/HeN cells was at 50 ug. B. fragilis, and S. minnesota control (rough) LPSs induced greatest proliferation at 5 ug; the greatest proliferation for the rough E. coli J5 LPS was at 20 ug. The smooth E. coli K235 LPS optimum dose was 1.0 ug. These results are summarized in Table 3 for all doses and illustrated in Figure 11 for the 5 ug dose.

C3H/HeJ mice

The smooth LPS from M. bovis 62L did not induce a proliferative response in C3H/HeJ splenocytes, although the rough LPS from M. bovis ATCC 10900, M. ovis and M. phenylpyruvica at high doses (50 ug) induced moderate proliferation in these hyporesponsive splenocytes (Figure 11, Table 3). Generally, the proliferative response of C3H/HeJ cells to these rough Moraxella LPSs was greater than that to the rough control LPS (E. coli J5, B. fragilis and S. minnesota) at all doses (Figure 11, Table 3). The smooth E. coli K235 LPS failed to induce a proliferative response in these cells. These results are summarized in Table 3 for all doses and illustrated in Figure 11 for the 5 ug dose.

BALB/c/ByJ mice

All the Moraxella LPS were able to induce a proliferative response in BALB/c/ByJ mice comparable to that of E. coli K245 LPS at similar doses.

Table 3. Splenic blastogenesis

Dose, ug	C3H/HeJ CPM[a]	C3H/HeJ S.I.[b]	C3H/HeN CPM	C3H/HeN S.I.
RPMI only	698 +/- 84	1.0	922 +/- 40	1.0
ConA 0.5	53603 +/- 1791	76.8	48487 +/- 877	52.6
ConA 0.1	1813 +/- 75	2.6	1848 +/- 126	2.0
		M. bovis 62L		
50.0	1145 +/- 55	1.6	43607 +/- 1673	47.3
20.0	970 +/- 21	1.4	75017 +/- 1662	81.4
10.0	690 +/- 21	1.0	71909 +/- 1132	78.0
5.0	541 +/- 57	0.8	73224 +/- 1884	79.4
1.0	584 +/- 15	0.8	54586 +/- 3458	59.2
0.1	734 +/- 64	1.1	13831 +/- 536	15.0
0.01	1311 +/- 409	2.9	5290 +/- 606	5.7
		M. bovis ATCC 10900		
50.0	24622 +/- 1791	35.3	33253 +/- 1541	36.1
20.0	3563 +/- 282	5.1	55824 +/- 12928	60.5
10.0	1957 +/- 65	2.8	66429 +/- 1335	72.0
5.0	1309 +/- 65	1.9	55848 +/- 3900	60.6
1.0	868 +/- 61	1.2	56892 +/- 4363	61.7
0.1	619 +/- 23	0.9	20241 +/- 3323	21.9
0.01	817 +/- 172	1.2	5785 +/- 159	6.3
		M. ovis ATCC 33078		
50.0	8276 +/- 1235	11.9	33430 +/- 1324	36.3
20.0	2715 +/- 79	3.9	38601 +/- 2175	41.9
10.0	1660 +/- 95	2.4	36033 +/- 1691	39.1
5.0	1380 +/- 222	2.0	32095 +/- 2113	34.8
1.0	1007 +/- 105	1.4	16289 +/- 1420	17.7
0.1	632 +/- 11	0.9	5505 +/- 247	6.0
0.01	529 +/- 23	0.8	4338 +/- 153	4.7

[a]Mean CPM +/- SD for triplicate cultures.

[b]S.I. = Stimulation index; calculated as mean CPM of sample/ mean CPM RPMI only control.

Dose, ug	C3H/HeJ CPM	C3H/HeJ S.I.	C3H/HeN CPM	C3H/HeN S.I.
		M. phenylpyruvica ATCC 23333		
50.0	17937 +/- 164	25.7	70405 +/- 2097	76.4
20.0	9014 +/- 819	12.9	22287 +/- 892	22.2
10.0	4993 +/- 373	7.2	14292 +/- 1348	15.5
5.0	2875 +/- 103	4.1	8892 +/- 856	9.6
1.0	1392 +/- 64	2.0	5059 +/- 478	5.5
0.1	736 +/- 83	1.1	3863 +/- 141	4.2
0.01	647 +/- 54	0.9	2707 +/- 585	2.9
		E. coli K235		
10.0	1093 +/- 28	1.6	13689 +/- 152	14.8
1.0	590 +/- 11	0.8	14165 +/- 759	15.4
0.01	764 +/- 41	1.1	9499 +/- 340	10.3
		E. coli J5		
50.0	3705 +/- 475	5.3	22311 +/- 1372	24.4
20.0	1721 +/- 18	2.5	54909 +/- 559	59.6
10.0	1235 +/- 88	1.8	49661 +/- 2316	53.9
5.0	880 +/- 56	1.3	51390 +/- 2846	55.9
1.0	832 +/- 45	1.2	39837 +/- 653	43.2
0.1	608 +/- 58	0.9	11042 +/- 621	12.0
0.01	638 +/- 48	0.9	5266 +/- 523	5.7
		S. minnesota Rd		
20.0	5399 +/- 564	7.7	19284 +/- 709	20.9
5.0	493 +/- 37	0.7	29641 +/- 966	32.1
1.0	2142 +/- 320	3.1	18436 +/- 1042	19.9
0.1	1325 +/- 158	1.9	9869 +/- 442	10.7
		B. fragilis		
25.0	2274 +/- 58	3.3	18797 +/- 868	20.4
5.0	1560 +/- 121	2.2	22754 +/- 648	24.7

Tumor Necrosis Factor Assay

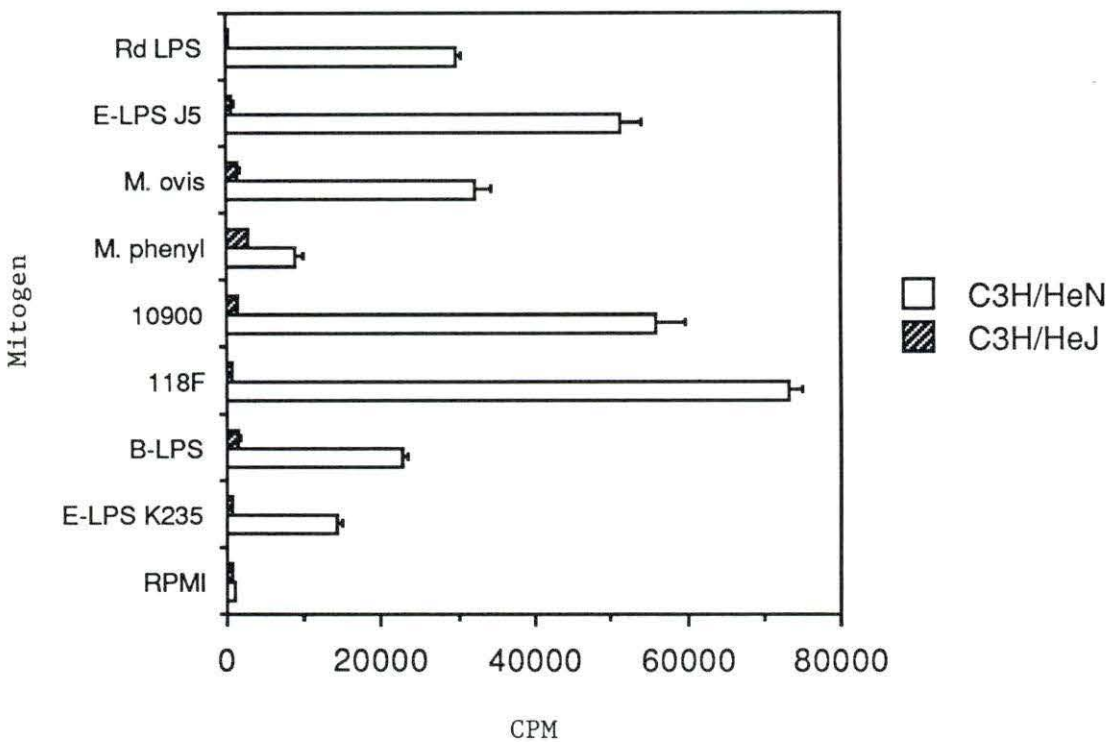
Controls

Cell-free 24, 48, and 72 hr supernatants from LPS-stimulated BALB/c/ByJ PEC were used measure the TNF activity induced by Moraxella LPS by determining their cytotoxicity on the fibroblast cell line, L929. Supernatants from PEC stimulated only with RPMI contained little TNF activity, which was quickly diluted out (i.e., 1:50 dilution). In addition, the greatest activity from these supernatants was observed after 24 hr incubation, and declined with the length of incubation. Thus, at 24 hr, maximum RPMI supernatant activity was seen with the 1:10 dilution and was equal to 53.1% cytotoxicity; by 48 hr the activity seen with the 1:10 dilution was only 19.5% cytotoxicity. Supernatants from PEC incubated with 0.1 ug E. coli K235 LPS showed the least TNF activity at 24 hr, while maximum TNF activity from the E. coli K235 supernatant was observed at 48 hr. The 72 hr supernatants were nearly equal to the the 48 hr supernatants in activity at low dilutions, but this activity was more quickly diluted out.

Moraxella strains

Moraxella LPS TNF activity was measured using PEC incubated with 0.5 and 5.0 ug of each Moraxella LPS for 24, 48 and 72 hr. Similar to the supernatnats from E. coli K235 LPS-treated PEC, all the supernatants from Moraxella LPS-treated PEC showed maximum TNF activity at 48 hr. However, the optimum dose of Moraxella LPS was 5 ug, while 0.1 ug was the dose used for E. coli K235 LPS. The activity from 24 hr supernatants was less than that of the 48 and 72 hr supernatants. The TNF activity from the 72 hr

Figure 11. Splenic blastogenesis assay
LPS responsive C3H/HeN (open) or hyporesponsive C3H/HeJ (striped) splenocytes (5×10^5) were incubated with 5 ug of LPS for 48 hr, then pulsed with tritiated thymidine (0.5 uCi) to assess B cell blastogenesis induced by Moraxella LPS. Mean CPM +/-SD for triplicate cultures are shown. Rd LPS = S. minnesota Rd LPS; E-LPS J5 = E. coli J5 LPS; M. ovis = M. ovis ATCC 33078 LPS; M. phenyl = M. phenylpyruvica ATCC 23333 LPS; 10900 = M. bovis ATCC 10900 LPS; 62L = M. bovis 62L LPS; E-LPS K235 = E. coli K235 LPS (1.0 ug); RPMI = RPMI only control. Mean CPM for Concanalvin A controls (0.5 ug) were 53600 +/- 1791 and 48500 +/- 877, for C3H/HeJ and C3H/HeN cells, respectively.


Rd LPS
E-LPS J5
M. ovis
M. phenyl
10900
118F
B-LPS
E-LPS K235
RPMI
Mitogen
0
20000
40000
60000
80000
CPM
C3H/HeN
C3H/HeJ

supernatants was diluted out more quickly than that from the 48 hr supernatants. Figure 12 shows the TNF activity of supernatants from PEC treated with 5 ug of M. bovis 62L and ATCC 10900 LPS for 48 hr, while Figure 13 presents the results for supernatants from PEC treated for 48 hr with 5 ug of M. ovis and M. phenylpyruvica LPS.

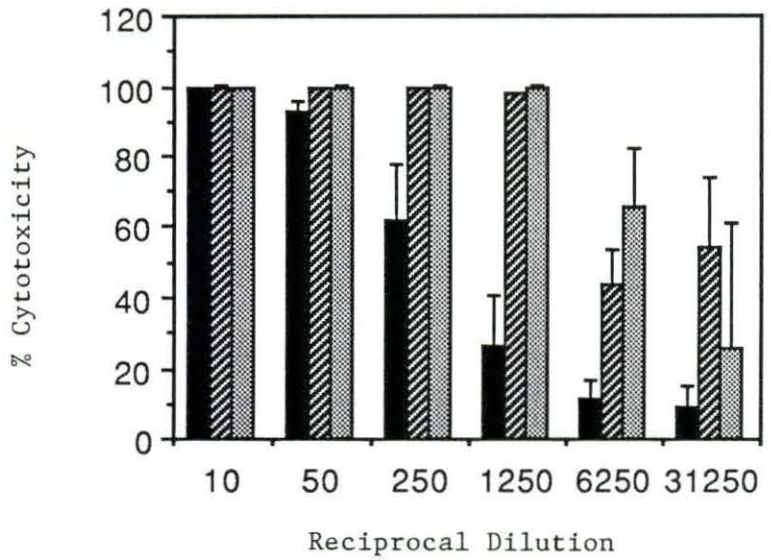
Confirmation that cytotoxicity was attributable to TNF was determined by using anti-TNF rabbit antisera, which was able to block the cytotoxicity of supernatants from E. coli K235 (0.1 ug LPS, 48 hr stimulation, all dilutions) and M. bovis 62L (5.0 ug LPS, 48 hr stimulation all dilutions) by up to 87% (data not shown).

Interleukin 1 Assay

Preliminary studies showed that while supernatants from BALB/c/ByJ PEC tended to decrease in Il-1 production over a 24-72 hr incubation period, the Il-1 activity of supernatants from macrophages stimulated with Moraxella LPS generally increased over the same incubation period. Maximum Il-1 activity for supernatants from PEC treated with 5 ug of M. bovis 62L LPS was seen at 72 hr and was only somewhat greater than of supernatants from RPMI 1640-treated PEC. Somewhat more substantial Il-1 activity was seen with supernatants from PEC treated with 5 ug of M. bovis ATCC LPS for 72 hr. Supernatants from PEC treated with 0.5 or 5.0 ug M. ovis or M. phenylpyruvica LPS for any time period did not contain Il-1 activity beyond that of the supernatants from RPMI 1640-treated PEC. Figure 14 shows the results of the Il-1 assay using supernatants from PEC treated for 72 hr with 5 ug of each of the Moraxella LPS preparations.

Figure 12. TNF assay with M. bovis strains
The cytotoxicity of supernatants from BALB/c/ByJ PEC incubated with 5.0 ug of M. bovis 62L (striped) or M. bovis ATCC 10900 (shaded) LPS or 0.1 ug E. coli K235 (solid) LPS for 48 hr against TNF-sensitive L929 fibroblasts is shown. Supernatants from BALB/c/ByJ PEC incubated with RPMI only for 48 hr showed less than 20 % cytotoxicity at all dilutions.

Figure 13. TNF assay with non-M. bovis strains
The cytoxocity of supernatants from BALB/c/ByJ PEC incubated with 5.0 ug M. ovis ATCC 33078 (striped), M. phenylpyruvica ATCC 23333 (shaded) LPS, or 0.1 ug E. coli K235 (solid) LPS for 48 hr against TNF-sensitive L292 fibroblasts is shown. The cytotoxicity of supernatants incubated with RPMI only for 48 hr was less than 20 % at all dilutions.


120
100
80
60
40
20
0
% Cytotoxicity
10
50
250
1250
6250
31250
Reciprocal Dilution

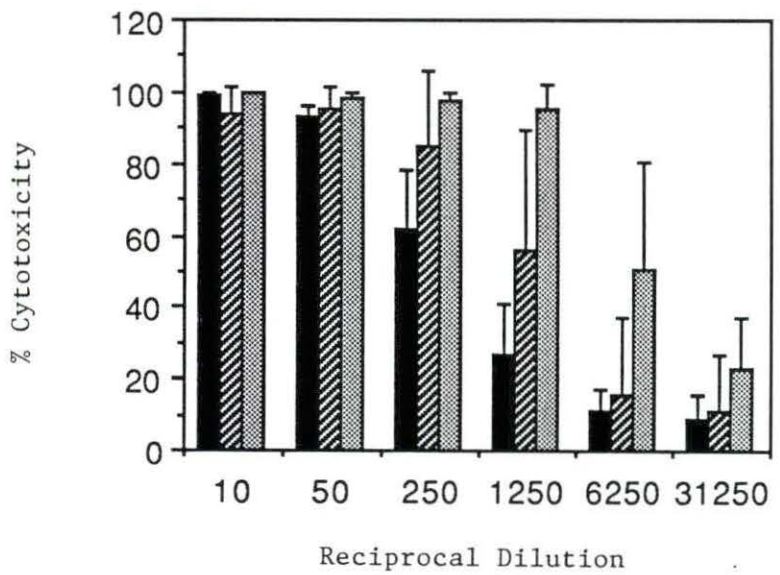
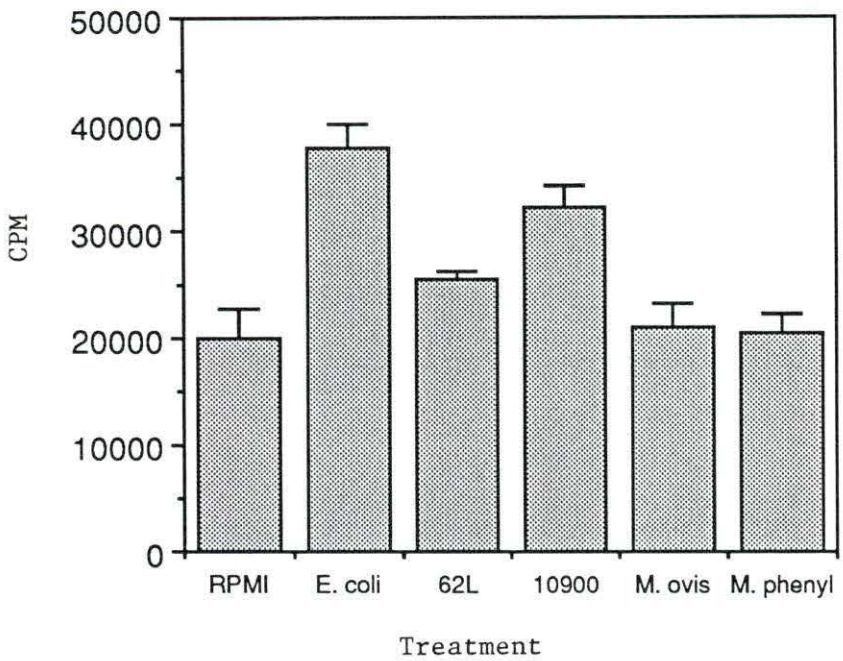

120
100
80
60
40
20
0
% Cytotoxicity
10
50
250
1250
6250
31250
Reciprocal Dilution

Figure 14. Interleukin-1 assay
The mean CPM +/- SD for triplicate cultures of Il-1-dependent D10.G4.1 cells incubated with supernatants (1:10 dilution) from BALB/c/ByJ PEC treated with 5 ug Moraxella LPS for 72 hr is shown.


50000
40000
30000
20000
10000
0
CPM
RPMI
E. coli
62L
10900
M. ovis
M. phenyl
Treatment

Lethality in Galactosamine-sensitized Mice

The lethal dose required to kill 50 % of the mice (LD_{50}) for Moraxella LPS was found to be approximately 500 times that of the LD_{50} for E. coli K235 LPS (0.1 ug).

Peritoneal Exudate Cell Toxicity

The results of the PEC toxicity assay indicate that Moraxella LPS is moderately toxic for elicited BALB/c/ByJ PEC at doses of 5 and 50 ug, although a few cultures exhibited greater sensitivity to LPS (e.g., M. bovis ATCC 10900 at 5 ug and M. ovis at 50 ug). In addition, E. coli K235 LPS was not toxic at 50 ug for elicited PEC, although slight toxicity was seen using 5 ug of this LPS. With a few exceptions, all the LPS were non-toxic for resident PEC (e.g., M. ovis at 50 ug). These results are summarized in Table 4.

Table 2. Peritoneal exudate cell toxicity

Treatment	Dose[b]	% Viability of BALB/c/ByJ PEC[a] resident[c]	elicited[d]
PBS control	-	n.d.[e]	65.7
E. coli K235	50.0	61.6	61.1
	5.0	67.6	48.7
	0.5	65.7	57.3
M. bovis 62L	50.0	66.9	49.6
	5.0	70.2	63.0
	0.5	70.1	48.6
M. bovis ATCC 10900	50.0	56.9	42.4
	5.0	63.9	29.6
	0.5	69.9	64.7
M. ovis ATCC 33078	50.0	18.9	35.5
	5.0	62.4	62.0
	0.5	54.1	68.4
M. phenyl-pyruvica ATCC 23333	50.0	57.9	51.0
	5.0	59.6	54.5
	0.5	44.1	54.1

[a] Viability determined by counting 5000 cells per sample.

[b] ug LPS added in 50 ul RPMI.

[c] Obtained by peritoneal lavage of unstimulated mice.

[d] Obtained by peritoneal lavage of mice stimulated by i.p. injection of 2 ml 3.0 % thioglycollate 3 days prior to peritoneal lavage.

[e] n.d. = not determined.

DISCUSSION

Compositional Analysis and SDS-PAGE Profiles

The absence of detectable protein by chemical and spectrophotometric analysis, and Coomassie blue staining, as well as the absence of detectable nucleic acids indicates that relatively pure preparations of lipopolysaccharide were obtained from these four Moraxella strains by the hot phenol-water method of extraction employed here (Table 1). The per cent hexose determined colorimetrically for these organisms, particularly M. bovis ATCC 10900, is low compared to some strains of Salmonella or E. coli (Wilkinson, 1977). In addition, the KDO content of these LPSs was found to be quite high, especially for M. bovis 62L and M. phenylpyruvica ATCC 23333 (about 10 % for each strain) and contradicts an earlier report by Adams et al., who did not detect any KDO in M. osloensis. However, a somewhat more recent report examining Neisseria gonorrhoeae stated that this organism's LPS contained 9.0 % KDO (Karkhanis et al., 1979) using the same method employed for the present work (Karkhanis et al., 1978). As the genus Moraxella is in the family Neisseriaceae it may be reasonable to assume that similarly high amounts of KDO exist in both genera; Adams' group also notes that the strains used in their work differ from true neisserias, while resembling so-called false neisserias such as N. (Branhamella) catarrhalis.

The variability in sugar composition can be attributed to the microheterogeneity within a particular LPS preparation (Morrison and Ulevitch, 1978) and to variation from preparation to preparation because

of incompletely synthesized LPS molecules (Wilkinson, 1977). The high variability found in the sugar composition of M. phenylpyruvica may be due to phase variation with respect to oligosaccharide chain length, as has been found with Coxiella burnetii (Vishwanath and Hackstadt, 1988). In addition, it has been demonstrated, using monoclonal antibodies, that the lipooligosaccharide phenotype is unstable for N. gonorrhoeae (Schneider, et al., 1988). These authors believe that within a population of gonococcal cells from a single strain there exist several variants, each of which produce a phenotypically different lipooligosaccharide (LOS) and which may interconvert. Although the proportion of each LOS would reflect the relative proportion of variants within that population producing each LOS, the authors state that populations of variant cells large enough to permit extraction of analyzable material could contain revertants which produce unique LOS molecules (Schneider et al., 1988). Thus, it is possible that M. phenylpyruvica may contain similar revertants, resulting in high variability of LPS composition.

The SDS-PAGE profiles (Figure 1) indicate that the M. bovis 62L LPS is smooth while the other three Moraxella LPSs are rough. As the per cent hexose for M. bovis 62L is low, there may be fewer repeating O-polysaccharide units than has been found with strains of the enterobacteriaceae; the polysaccharide side chain of Moraxella LPS may therefore resemble the oligosaccharide moiety of some Neisseria species (Karkhanis et al., 1978; Schneider et al., 1984). The low hexose content and relatively high KDO content of M. bovis ATCC 10900, M. ovis and M. phenylpyruvica are consistent with rough phenotypes or the lack of

repeating oligosaccharide side chain units (Schneider et al., 1984).

Western Blot Analysis

The results of western blot analysis using immunoglobulins prepared from convalescent serum from a gnotobiotic calf infected with M. bovis 62L (Figure 2) show that Moraxella LPS shares some epitopes common to both smooth (e.g., E. coli K235 and S. typhimurium) and rough (e.g., E. coli J5) LPSs from other groups of gram-negative microorganisms. That the immunoglobulins recognized determinants with different relative mobilities is most likely a reflection of the degree of aggregation for each particular LPS preparation (Bradley, 1979), as well as the microheterogeneity of the preparation where some LPS core and lipid A regions may not contain fully synthesized O-side chains (Wilkinson, 1977). In addition, the epitopes recognized are in the fastest migrating region of the LPS, most likely the more conserved lipid A or core regions which may account for the cross-reaction of the immunoglobulin preparation used (Bogard et al., 1987).

The results of western blot analysis using a monoclonal antibody reactive with a M. bovis 62L LPS moiety show that the recognition is species specific and that the monoclonal antibody is recognizing both smooth and rough LPS (Figure 3). The failure of this monoclonal antibody to react with the non-M. bovis strains indicates that there are some interspecies differences among the Moraxella LPSs, as well as intergeneric differences between M. bovis LPS and the LPS of E. coli or Salmonella. Again, the determinants reactive with the monoclonal antibody appear to be

in the lipid A or inner core region indicating that there is a unique epitope present in the M. bovis strains. Of course, the species-specific recognition of this determinant may become useful as a diagnostic tool for the identification of bovine isolates of M. bovis.

Pyrogenicity

The results of the pyrogenicity studies show that the M. bovis strains are indeed capable of provoking a typical biphasic febrile response in rabbits (Figure 4). However, 10 times the amount of E. coli K235 LPS did not produce as great a fever response with the M. bovis strains (e.g., 10 ug for E. coli K235 vs. 100 ug for M. bovis strains). The M. bovis ATCC 10900 LPS did not induce as great a response as the M. bovis 62L LPS, especially for the second peak in temperature increase. This may be a reflection of a lesser ability of M. bovis ATCC 10900 rough LPS to induce so-called endogenous pyrogens, such as Il-1, in vivo compared to M. bovis 62L LPS. However, in vitro studies (see below) do not bear this out, as similar Il-1 and TNF activities are seen with both M. bovis strains. The aggregation state of the preparations used may have influenced the pyrogenic response to some degree. Thus, the LPS from the M. bovis strains is pyrogenic, with M. bovis 62L LPS being more pyrogenic than M. bovis ATCC 10900 but substantially less so than E. coli K235 LPS.

The results of pyrogenicity studies using M. ovis and M. phenylpyruvica LPS show that these strains are also capable of inducing a biphasic febrile response in rabbits (Figure 5). The amount of LPS (25 ug) used for these non-M. bovis strains was 4 times less than that used

for the M. bovis stains and 2.5 times greater than that used for E. coli K235 LPS. The febrile response that was provoked was nearly equal to that produced by the control E. coli K235 LPS and was greater than that induced by M. bovis ATCC 10900. That the non-M. bovis LPS was more pyrogenic than the M. bovis LPS may again be due to interspecies differences in lipid A fatty acid composition or aggregation states which cause M. ovis and M. phenylpyruvica LPS to induce more endogenous pyrogens which mediate the second phase of the fever response; however, LPS from these two strains did not induce Il-1 activity above that caused by stimulation due to adherence (Figure 14) and TNF activity was similar among all four Moraxella species (Figures 12 and 13).

Local Shwartzman Reaction

All the Moraxella LPS preparations were capable of inducing a local Shwartzman reaction in rabbits, using homologous LPS for the priming and provoking doses (Figure 6; Table 2). While small priming doses of E. coli K235 LPS (10 ug) were able to induce lesions with some erythema and slight swelling, larger priming doses of Moraxella LPS were generally required to provoke lesions with substantial swelling and necrosis. Microscopically, no differences in the reaction could be discerned among the various LPSs: stained sections from the lesions consistently showed PMN infiltration, intravascular coagulation, hemorrhage, fibrin deposition and edema (Figures 7-10). However, a dose-dependent relationship existed in terms of the degree of PMN infiltration, hemorrhage, and other lesion pathologies in that lesions from low dose priming sites (10 and 25 ug) had

less hemorrhage, microthrombi and fibrin deposition than lesions from sites of higher priming doses (50 and 100 ug), where there were also more intense multiple foci of PMN accumulation. There appears to be no difference in the ability of smooth or rough Moraxella LPS to provoke a local Shwartzman reaction, thus it would appear that lipid A moieties of these LPSs are equally efficient in activating the coagulative pathways important in the development of the lesions. In addition, TNF and Il-1 levels induced by Moraxella LPSs are similar and these cytokines have been found to induce the local Shwartzman reaction or tissue injury independent of LPS (Beck et al., 1986; Tracey et al., 1986; Beutler and Cerami, 1986).

Splenic Blastogenesis

Moraxella LPS was able to induce LPS-responsive murine splenocytes to undergo blastogenesis (C3H/HeN and BALB/c/ByJ strains). M. bovis 62L and ATCC 10900 LPS were able to stimulate the responder splenocytes to a higher degree than several control LPSs at the same dose for C3H/HeN splenocytes (5 ug; Figure 11). However, only the rough LPSs from M. bovis ATCC 10900, M. ovis, and M. phenylpyruvica were able to stimulate LPS-hyporesponsive splenocytes from C3H/HeJ mice and induced more activity than the control rough LPS from E. coli J5, S. minnesota Rd and B. fragilis at the same dose (5 ug). The ability of these rough Moraxella LPSs to induce moderate blastogenesis, especially at high doses (50 ug), in LPS-hyporesponsive splenocytes may be due to unique or especially potent combination of fatty acid substituents on the diglucosamine

backbone of the lipid A, as recent work emphasizes the importance of fatty acid substituents in the reactivity of synthetic lipid A derivatives (Brade et al., 1988; Kumazawa et al., 1988). Alternatively, these preparations may have serendipitously had the optimum degree of aggregation and bound cations for the induction of a blastogenic response (Galanos and Luderitz, 1984).

Overall, the Moraxella LPS was able to stimulate responsive murine splenocytes to undergo blastogenesis to a degree at least as great as that of E. coli K235 LPS at nearly all doses and rough Moraxella LPS was able to induce modest blastogenesis in hyporesponsive murine splenocytes.

Tumor Necrosis Factor Assay

Supernatants from PEC stimulated with Moraxella LPS for 24 to 72 hr contained greater TNF activity than those from E. coli K235 (Figures 12 and 13), even when considering the modest TNF activity induced from allowing the PEC to adhere, thus activating them (although 5 to 50 times more Moraxella LPS was incubated with the PEC); Supernatants stimulated with RPMI only had moderate activity which was quickly diluted out and which decreased to less than 20 % cytotoxicity by 48 hr, indicating that the stimulus was not sustained in these cultures. However, in PEC cultures incubated with LPS, TNF activity increased from 24 to 48 hr and leveled off thereafter (72 hr). In addition, 5 ug of Moraxella LPS induced more TNF activity than 0.5 ug of Moraxella LPS or 0.1 ug of E. coli K235 LPS (at 48 hr). If peritoneal macrophages take up LPS in a receptor-mediated process as has been proposed (Fox et al., 1987), this

dose-dependent increase in TNF activity may be proportional to the LPS/receptor ratio: more LPS may induce expression of more LPS receptors and subsequently increase the amount of LPS taken into the cell, resulting in increased production of TNF, with the most empirical effects at the level of transcription and translation of the TNF genes (Beutler et al., 1986).

As with the local Shwartzman reaction, no differences were observed in the induction of TNF activity between rough and smooth Moraxella LPSs. This result might imply that the lipid A portion of these LPSs is very similar from strain to strain, as the lipid A region has be found to be responsible of the induction of TNF activity (McCartney and Wardlaw, 1985).

Interleukin 1 Assay

In contrast to the high amount of TNF activity found in supernatants from murine PEC stimulated with Moraxella LPS, Il-1 activity from these supernatants was modest at best (Figure 14). The highest Il-1 activity was observed in culture supernatants from PEC treated with the M. bovis 62L and ATCC 10900 LPS, indicating that there is no difference in the ability of smooth or rough Moraxella LPS to induce Il-1. The Il-1 activity of supernatants from PEC stimulated with RPMI only decreased after 24 hr, reflective of the transient stimulation of PEC by adherence. Interleukin 1 activity of the Moraxella LPS-stimulated PEC culture supernatants generally increased over the 72 hr incubation period for the M. bovis strains, while E. coli K235 LPS-stimulated PEC culture

supernatants exhibited the highest Il-1 activity at 48 hr. The Il-1 activity from the M. ovis and M. phenylpyruvica LPS-stimulated PEC culture supernatants increased with increasing the dose of LPS, suggesting that the optimum amount of these LPSs required for stimulating PEC to release IL-1 has not been tested. It may also be that the LPS was somewhat toxic to the PEC cultures during incubation and that Il-1-producing macrophages were killed, since 5 and 50 ug amounts of Moraxella were moderately toxic to murine PEC in toxicity assays (see below). Alternatively, the duration of stimulation of PEC by LPS may also need to be lengthened as recent research on the genetic regulation of Il-1 activity has shown that adherence-stimulated PEC synthesize IL-1 mRNA more quickly than LPS-stimulated PEC (Fuhlbrigge et al., 1987), although E. coli K235 LPS-stimulated PEC had the most Il-1 activity at 48 hr. The high amount of TNF activity induced relative to Il-1 activity by Moraxella LPS suggests that there may be some mechanism controlling increased production of one cytokine at the expense of the other. However, both TNF and Il-1 share a conserved octameric sequence at the DNA level which Beutler and his co-workers believe may be involved in their regulation by LPS (Beutler et al., 1986); thus, other sequences may also be involved in the independent regulation of these cytokines.

Lethal Toxicity in Galactosamine-sensitized Mice

The amount of Moraxella LPS required to kill 50 % of BALB/c/ByJ mice sensitized to the toxic effects of LPS was more than 500 times that required for E. coli K235 LPS (0.1 ug), although death in some Moraxella

LPS-treated mice were seen with as little as 1 ug LPS. After the administration of Moraxella LPS, the mice often showed signs of endotoxemia, such as ruffled fur, ocular exudate and diarrhea but did not succumb to the lethal effects of LPS. Moraxella LPS may be substantially less toxic than E. coli K235 LPS due to its composition, although it is able to induce TNF activity similar to E. coli K235 LPS at comparable doses, and TNF has been recently found to mediate many of the lethal effects of LPS and endotoxin (Beutler and Cerami, 1985; Bauss et al., 1987). The Moraxella LPS is perhaps degraded or detoxified more rapidly than the E. coli LPS in vivo, resulting in less stimulation of the mediators of endotoxin toxicity. For instance, dephosphorylation of LPS by macrophages has recently been shown and lipid A analogs lacking phosphate residues have decreased toxicity and pyrogenicity (Peterson and Munford, 1987).

Peritoneal Exudate Cell Toxicity

Although E. coli LPS has been reported to be toxic for macrophages at 50 ug/ml (Bradley, 1979), no toxicity was observed at this dose for elicited PEC from BALB/c/ByJ mice, although slight toxicity for elicited PEC was observed at 5 ug (Table 4). Moraxella LPS showed modest toxicity at 50 and 5 ug doses, but only for elicited PEC (with the exception of 50 ug M. ovis LPS which was toxic for resident PEC). Resident PEC were relatively resistant to the toxic effects of LPS at all doses, as might be anticipated as they are not as metabolically active and thus less susceptible to the effects of LPS on mitochondria and glucose metabolism (Bradley, 1979).

SUMMARY AND FUTURE DIRECTIONS

Relatively pure preparations of LPS were obtained by a hot phenol-water method of extraction for M. bovis 62L and ATCC 10900, M. ovis ATCC 33078, and M. phenylpyruvica ATCC 23333. The SDS-PAGE profile of M. bovis 62L LPS was smooth while the LPS from the other three Moraxella species was rough. The hexose content for the Moraxella LPSs was low, while the KDO content was high, indicating that these LPS moieties more closely resemble those of Neisseria spp., rather than E. coli. The Moraxella LPS contained epitopes cross-reactive with LPS from enteric gram-negative microorganisms, but M. bovis LPS must contain unique epitopes since a monoclonal antibody reacted with it in a species-specific manner. This monoclonal antibody certainly holds promise as a diagnostic agent for the presence of M. bovis.

Moraxella LPS was pyrogenic, although at doses 2.5 to 10 times greater than the dose required for a similar response to E. coli K235 LPS. Moraxella LPS was also capable of provoking a dermal Shwartzman reaction in rabbits, induced TNF and caused the blastogenesis of LPS-responsive murine splenocytes, all at doses somewhat greater than, but comparable to, E. coli K235 LPS. However, Moraxella LPS was 500 times less toxic than E. coli K235 LPS to sensitized BALB/c/ByJ mice. Little Il-1 activity and moderate PEC toxicity were observed for the LPS from these four species. Western blot analysis with the monoclonal antibody showed that it reacted in a species-specific manner with the M. bovis 62L and ATCC 10900 LPS, as opposed to the cross-reactivity of the bovine immunoglobulins seen among

the Moraxella species, E. coli J5 and K235, and S. typhimurium LPS. Aside from the stimulation of LPS-hyporesponsive C3H/HeJ splenocytes by the rough Moraxella LPSs, no differences in reactivity between rough and smooth Moraxella LPS were detected.

The study of these LPSs could be aided by detailed chemical analysis, which would indicate chemical differences that could account for differences in pyrogenicity and reactivity with antiserum. Knowledge of the chemical makeup would also confirm or refute that Moraxella LPS has oligosaccharide side chain structures similar to those of Neisseria; core and lipid A composition may also have taxonomic value.

As of yet, no clear cut role for M. bovis LPS in pathogenicity has been established, since the LPS from pathogenic M. bovis 62L and nonpathogenic M. bovis ATCC 10900 react very similarly in the biological assays described here. However, examining the effects of each of these LPSs on natural killer cells, examining the release of CETAF from a corneal cell line (e.g., SIRC) and studying serum resistance may further delineate such a role. Additionally, using normal and neutropenic mice to examine the effects of LPS from pathogenic and nonpathogenic M. bovis on the development of corneal lesions may also illuminate any role of M. bovis LPS in pathogenicity.

As previously mentioned, the monoclonal antibody reactive with M. bovis holds promise as a diagnostic tool; in addition, it may be valuable as a protective agent. Previous studies have demonstrated a protective effect of antiserum to the lipid A moiety of E. coli J5 in preventing subsequent infection (Reitschel and Galanos, 1977; Zeigler et al., 1982).

Since the monoclonal antibody appears to be recognizing an epitope in the core or lipid A region of M. bovis LPS, a similar protective effect may also be afforded upon instillation of this monoclonal antibody.

Cattle suffering from the early stages of pinkeye may experience a slight fever. The pyrogenicity noted with the M. bovis species, especially 62L, offers a logical explanation for this fever; thus it may be desirable to include inhibitors of prostaglandin synthesis (e.g., indomethacin or aspirin) in treatment for IBK, as PGE2 has been implicated as mediating the pyrogenic response to LPS (in addition to Il-1).

This work has described the SDS-PAGE profiles and biological reactivity of LPS from several heretofore uncharacterized species of Moraxella. The precise role of this LPS in the pathogenicity of IBK has not been determined, although the LPS from these Moraxella spp. is reactive is most biological assays tested. The species-specific recognition of M. bovis LPS by a monoclonal antibody, however, may yield improvements in the diagnosis of IBK.

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