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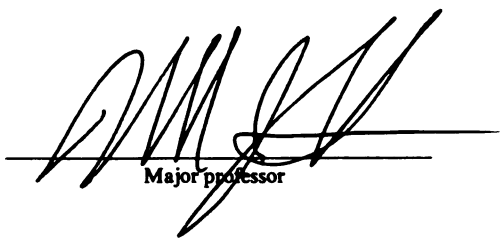

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BIOACTIVITIES OF BOVINE ANTI-COLIFORM ANTIBODIES ELICITED BY J5 VACCINATIONS

By

Anantachai Chaiyotwittayakun

A DISSERTATION

Submitted to
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ABSTRACT

BIOACTIVITIES OF BOVINE ANTI-COLIFORM ANTIBODIES ELICITED BY J5 VACCINATIONS

By

Anantachai Chaiyotwittayakun

Five healthy Holstein steers were immunized 12 times with a J5 bacterin at time 0, 30 days later, and every 2 weeks for 10 subsequent immunizations. Sera were collected from pre-immunization and on days 4, 6, 11, and 13 post immunizations, and assayed for relative contents of anti-J5 IgM, IgG_1, and IgG_2 antibodies by ELISA. Selected sera from pre- and 6-day-post-3^{rd}, 6^{th}, 9^{th}, and 12^{th} immunization were used to determine isotype specific endpoint antibody titers and cross-reactivity against Gram-negative bacteria isolated from clinical disease cases. Immunization number and day post immunization significantly ($P < 0.0001$) influenced anti-J5 *E. coli* antibody responses for all 3 isotypes. Two immunizations were required to increase ($P < 0.005$) serum anti-J5 *E. coli* IgM antibody levels above pre-immunization. However, 5 immunizations were required to significantly ($P \leq 0.02$) increase anti-J5 IgG_1 and IgG_2 antibody levels and detect isotype switching. Anti-J5 antibody titers for all isotypes were significantly ($P < 0.005$) higher than background after 6 immunizations. Cross-reactivity of isotype specific anti-J5 antibodies to all Gram-negative bacteria in ELISA was significantly ($P < 0.05$) increased with hyperimmunization. Anti-J5 IgM and IgG_2 antibodies were more cross-reactive to

Serratia spp., *E. coli 487* and *K. pneumoniae* than other bacteria tested. Anti-J5 IgG_1 antibodies tended to cross-react with *E. coli 487* and *Pseudomonas spp.* more than other bacteria tested. However, only low to undetectable cross-reactivity of isotype specific anti-J5 antibodies with purified LPS or Lipid A was observed, which was similar to the result for *S. aureus* used as a negative control in ELISA.

Western blot analysis showed that the anti-J5 IgG_1 and IgG_2 recognized at least two predominant antigen clusters with molecular masses of 8-10.5 and 34-39 kDa. An identical pattern of 34-39 kDa antigen recognition by IgG_2 antibody was observed in commercial fetal bovine serum and in J5 antisera from the pre-, 3rd, and 9th immunization collections. However, the 8-10.5 kDa proteins appeared to be the target antigens in the J5 bacterin because the specific recognition of these antigens by IgG_1 or IgG_2 antibodies was only observed when J5 hyperimmune sera were used in the assays. Western blotting also revealed strong recognition of LPS by anti-J5 IgG_1 antibodies. However, there was little or no recognition of Lipid A or *S. aureus* proteins by any antiserum or isotype. Protein A enriched IgG_2 antibody from the hyperimmune serum significantly ($P<0.001$) enhanced neutrophil phagocytosis of GFP-J5 *E. coli* compared to no antibody or enriched IgG_1 antibody, especially with incubation times from 120 to 150 minutes. Thus, results from this work indicated that J5 hyperimmunization elicited high levels of cross-reactive serum IgG_2 antibodies that increasingly recognized small (8-10.5 kDa) and larger (34-39 kDa) antigens on most Gram-negative bacteria and promoted effective opsonization of *E. coli* for enhanced neutrophil phagocytosis of the bacteria. Increasing the number of exposures of the bovine immune system to J5 bacterin elicits antibodies that should protect animals from a variety of Gram-negative infectious diseases.

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KEY TO SYMBOLS OR ABBREVIATIONS

Ab(s) Antibody(-ies)

Ag(s) antigen(s)

ANOVA analysis of variance

APC antigen-presenting cell

CD cluster of differentiation

cfu colony-forming unit

C_H heavy chain constant

DC(s) Dendritic cell(s)

ELISA enzyme-linked immunosorbent assay

Fab fragment antigen-binding of immunoglobulin carrying one antigen-binding site

Fc fragment crystallizable, heavy-chain constant regions of immunoglobulin

FI Fluorescent intensity

GFP green fluorescent protein

Ig immunoglobulin

IL interleukin

INF-γ Interferon-gamma

KDO 2-keto-3deoxyoctonate

LPS lipopolysaccharide

MAbs monoclonal antibodies

MHC major histocompatibility complex (i.e., class I and II)

MLP murine lipoprotein

MWCO molecular weight cut-off

"O" oligosaccharide

OD optical density

OMPs outer membrane proteins

OmpA outer membrane protein A

PAL peptidoglycan-associated lipoprotein

PMN Polymorphonuclear neutrophil

PS(s) polysaccharide(s)

SASS saturated-ammonium sulfate sera

SAS-PAGE Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TD thymus-dependent

TI thymus-independent

T_H1 T helper cell type 1

TNF tumor necrosis factor (i.e. TNF-α)

udg uridine diphosphate galactose

V_H heavy chain variable

V_λ λ light chain gene locus

INTRODUCTION

Core antigen technology has been demonstrated to be a potentially useful tool for enhancing antibody protection against diseases caused by Gram-negative bacteria in humans and animals. In cattle, commercial bacterins developed from the J5 mutant strain of *E. coli* have been used extensively as part of routine coliform mastitis prevention programs. Nonetheless, mastitis remains one of the most costly diseases in U.S. animal agriculture for most dairy enterprises (DeGraves and Fetrow, 1993, Reneau, 1993). In well-managed dairy herds, clinical mastitis caused by pathogens of environmental origin, such as coliforms and streptococci, remains a significant problem. In particular, coliform organisms are the most important cause of severe mastitis that can result in agalactiae, premature culling, or death. Although therapy, including use of antibacterials may ameliorate the detrimental effects of coliform mastitis, many cases do not respond. Additionally, therapy often requires milk to be discarded for variable periods following treatment, and raises food safety and public health concerns regarding potential resistance to antibacterials (White, 1999).

Therefore, preventive alternatives that can help to decrease the incidence of new intramammary infections, reduce clinical severity, and reduce the need for therapy, are in the best interests of dairy cattle, producers, processors, and consumers. However, success of the J5 vaccine varies, and cows in herds using this technology still suffer from clinical coliform mastitis. Additionally, the immune mechanisms induced by J5 bacterins in dairy cattle in response to coliform infections are still unclear. The biological relevance of antibody responses to this vaccine has not been well determined. Thus, it is

time to consider why the vaccines have limited success, and how to improve their efficacy from development of new applications or technology.

Therefore, we have developed unique resources in the form of anti-coliform antisera and enriched specific antibody from these sera to begin the study of the potential immune basis of core-antigen vaccine protection against gram-negative bacteria, including those that cause mastitis in dairy cows. Existing J5 *E. coli* core antigen technology was used to elicit these antibodies in hyperimmunized steers based on literature that supports the idea that such antibodies may cross-react with a variety of coliform bacteria and their endotoxins (Tyler et al., 1992; Tomita et al., 1995).

We expect that results of this study should provide some answers regarding what may constitute humoral immune protection against mastitis-causing coliforms in J5 vaccinated cattle. The goal of this work was to increase the knowledge of the diversity of coliform antigens recognized by isotype-specific anti-J5 *E. coli* antibodies, the biological roles of these antibodies, and the potential to improve humoral immune responses against coliform bacteria using an alternative vaccination protocol for an existing J5 bacterin vaccine. With this knowledge, novel vaccination protocols and technologies aimed at preventing coliform infections could be developed and improved.

Overall goal

The overall goal of the study was to better understand humoral immune responses elicited by available J5 *E. coli* bacterin vaccine. This will promote future development of more effective vaccination protocols and vaccine preparations for controlling mastitis-causing coliforms and other diseases of cattle caused by Gram-negative bacteria. We believe results of this study have: 1) addressed a significant economic problem of the dairy industry; 2) addressed a significant gap in knowledge about humoral immunity against mastitis-causing coliforms; 3) advanced available vaccination protocols aimed at preventing coliform infections; and 4) opened the door of discovery to novel vaccine technologies and other strategies for enhancing control of disease caused by coliforms and other Gram-negative pathogens.

Main Hypothesis

The hypothesis of the current study was that repeated exposure of the bovine immune system to Rc mutant J5 *E. coli* bacterin would result in highly mature antibodies that cross-react in isotype-specific fashion with a variety of antigens on heterologous Gram-negative bacteria for improved bacterial recognition and clearance by bovine neutrophils.

Questions regarding the hypothesis

1. Does repeated exposure of the bovine immune system to a bacterin of the Rc mutant J5 *E. coli* elicit a mature antibody response in hyperimmunized cattle?

   Objectives in response to Question 1

   1.1 To determine if a mature antibody response occurs during hyperimmunization of cattle.

   1.2 To determine isotypes of serum antibody responses and titers during hyperimmunization of cattle.

2. Do maturing anti-J5 *E. coli* antibodies cross-react with a variety of Gram-negative bacteria and antigens on the bacteria in an isotype-specific fashion?

   Objectives in response to Question 2

   2.1 To determine the cross-reactivity of bacterial recognition by maturing isotype-specific anti-J5 *E. coli* antibodies.

   2.2 To determine the diversity of bacterial antigen recognition by maturing isotype-specific anti-J5 *E. coli* antibodies.

3. Do IgG_2 antibodies from hyperimmune serum promote better phagocytosis of mastitis-causing coliforms by bovine neutrophils?

   Objectives in response to Question 3

   3.1 To purify IgG_2 antibodies from J5 bacterin-derived hyperimmune serum for subsequent biological testing.

   3.2 To test the effectiveness of the enriched IgG_2 antibody to opsonize *E. coli* for enhanced neutrophil phagocytosis.

CHAPTER 1

REVIEW OF LITERATURE

J5 Escherichia coli bacterin

J5 *Escherichia coli* bacterin is a core antigen technology developed from *E. coli* 0111:B4 (strain J5), which is a genetically stable rough mutant based on colony morphology (Tyler et al., 1990b). This technology was convincing because, apparently, Gram-negative core antigens posses marked chemical, structural, and immunological homology across bacterial species, genera, and groups (Tyler et al., 1990b). Rough mutant bacteria are designated by the capital letter R, followed by a subscript, a, b, c, d, e (**Figure 1**, Tyler et al., 1990b). Like a variety of mutant bacterial strains lacking specific enzymes for complete somatic chain assembly, the J5 strain which is an Rc mutant, lacks uridine diphosphate galactose (udg)-4-epimerase, which is required for complete synthesis of the oligosaccharide side chain portion of the LPS molecule (**Figures 1 and 2**, Elbien and Heath, 1965; Tyler et al., 1990b). As a result, the bacteria lack O polysaccharide chains and have exposed core protein and lipid A antigens. These also make the J5 *E. coli* cell wall structure different from wild type *E. coli* (smooth Gram-negative bacteria) (**Figures 1 and 2**).

Basic structure of Gram-negative bacteria

In wild type Gram-negative bacteria, structures of the cell wall play important roles as virulence factors, which subsequently cause pathophysiological changes in the host. The cell wall components of Gram-negative bacteria consist of (1) an inner cytoplasmic membrane; (2) a peptidoglycan layer; and (3) an outer membrane consisting

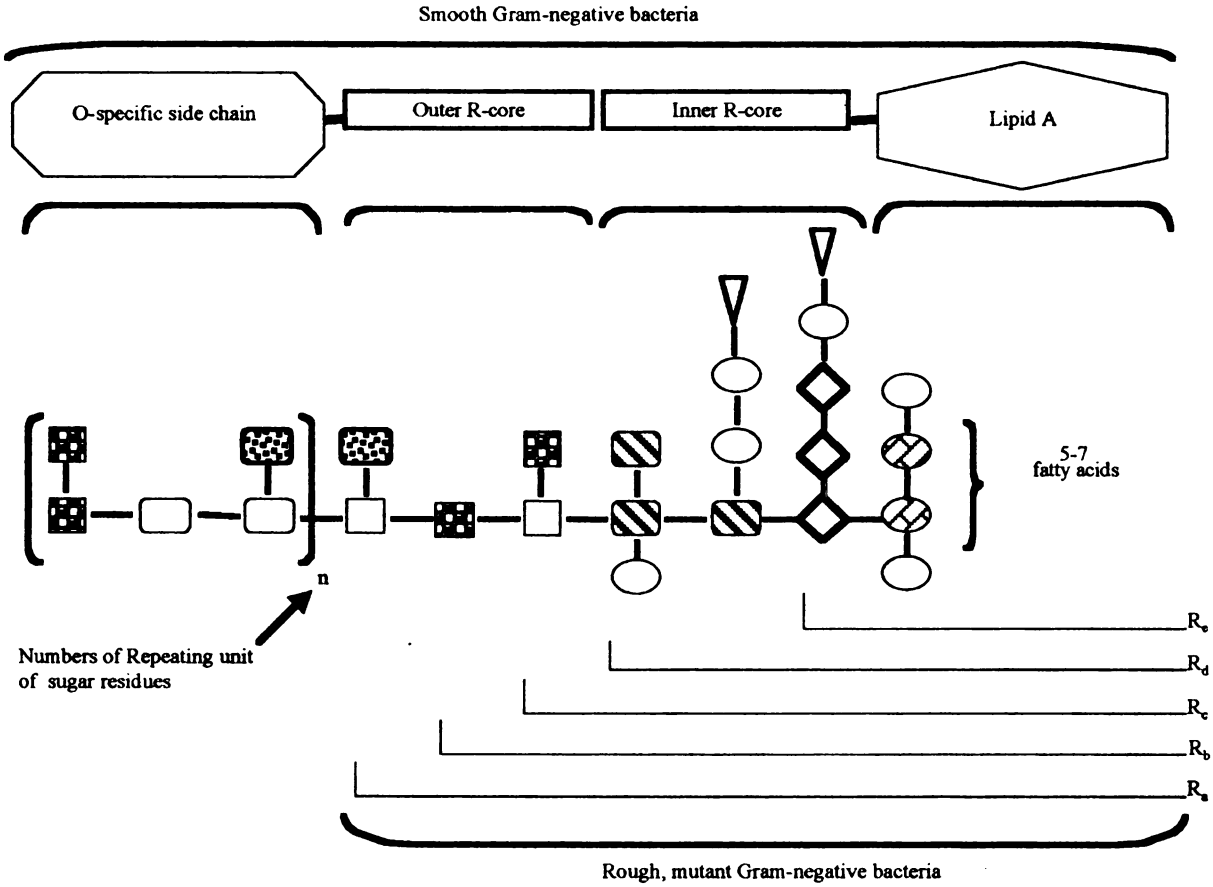


Figure 1 General structure of the Lipopolysaccharide (LPS) structures of smooth and rough strains of Gram-negative bacteria. In smooth Gram-negative bacteria, LPS consists of the "O" antigen side chain, R-core polysaccharide and Lipid A. "**n**" represents number of repeating units of sugar residues in the O-specific side chain. Rough Gram-negative bacteria lack "O" antigens and consist of Lipid A and some R-core antigens. These are termed "R" mutants, with the various mutations given as subscripts (a, b, c, d, or e). Ra indicates the complete core, Rb to Re indicate the incomplete core structures. (Adapted from Appelmelk et al., 1993; Tyler et al., 1990)

of two layers: (a) a phospholipid protein layer, and (b) an outer lipopolysaccharide (**LPS**) layer, which is incomplete in mutant Gram-negative bacteria including J5 *E. coli* (**Figure 3**, Kremer et al., 1990; Tyler et al., 1990b). The inner cytoplasmic membrane contains enzymes for synthesis of basic chemical units of the peptidoglycan, the phospholipid protein and the LPS layers (Kremer et al., 1990). The peptidoglycan layer maintains the bacterial shape and is relatively thinner in Gram-negative bacteria (5-20% of the cell wall) than in Gram-positive bacteria (as much as 90% of the cell wall) (Bergquist and Pogosian, 2000; Kremer et al., 1990).

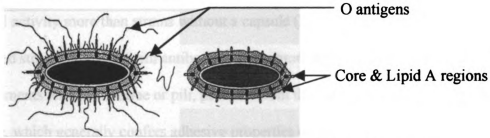


Figure 2 Schematic drawings of Wild type *Escherichia coli* (left) showing O-polysaccharide chains, and J5 *E. coli*, Rc mutant (right) showing exposed core and Lipid A regions.

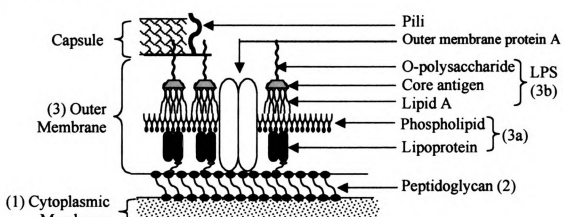


Figure 3 Schematic model of the cell wall of Gram-negative bacteria (adapted from Kremer et al., 1990). The cell wall components consist of 3 main layers, including (1) an inner cytoplasmic membrane; (2) a peptidoglycan layer; and (3) an outer membrane consisting of two layers: a) a phospholipid-protein layer and b) an LPS layer. Some bacteria also have an outer membrane protein A layer, that readily binds the Fc region of IgG antibodies, and pili that extend away from the surface of the bacteria and facilitate adhesion to host cells and tissues.

Bacterial outer membranes are in direct contact with the environment unless the cells process an additional capsular polysaccharide, or slime layer, which is found in a number of *E. coli* strains (Bergquist and Pogosian, 2000; Kremer et al., 1990). However, the biochemical structure of capsular antigens and their role in the pathogenesis in bovine mastitis are not clear (Sanchez-Carlos et al., 1984). Capsules may inhibit activation of the alternative pathway of the complement system (without involvement of immunoglobulins) (Stevens et al., 1978), thus decreasing susceptibility to lysis and phagocytosis. It is well established that strains with a heat-labile capsule resist serum bactericidal activity more than strains without a capsule (Hill, 1981). Opsonization of encapsulated strains of *E. coli* with antibodies is also more difficult (Hill et al., 1983b). Protein filaments termed fimbriae or pili, protrude from the outer membrane and through the capsule, which generally confers adhesive properties of bacteria (reviewed by Kremer et al., 1990). However, *E. coli* pili do not seem to play a significant role in the pathogenesis of coliform mastitis, as opposed to mastitis caused by *Staphylococcus aureus* and *S. agalactiae* (reviewed by Kremer et al., 1990). There is another surface structure of *E. coli* called curli, which may be involved in adherence during wound colonization or colonization of fibronectin-coated surfaces (Olsen et al., 1989), and this may be problematic in cows with damaged teats.

Lipopolysaccharide, also termed endotoxin, is composed of a variable oligosaccharide region (**O or somatic antigens**) covalently linked to a highly conserved core polysaccharide [N-acetylglucosamine, 2-keto-3deoxyoctonate (**KDO**), heptose and glucose residues] and lipid A (**LA**) regions (**Figures 2** and **3**, Elbien and Heath 1965; Jann and Jann, 1987; Raetz, 1993). With combinations of hexose molecule numbers,

composition, and linkage, O-specific antigens can vary widely and specifically for each specific Gram-negative organism (Elbien and Heath 1965; Raetz, 1993). LPS is a key virulence factor released from the Gram-negative bacteria upon cell growth and death. Toxicity of LPS varies, depending on Lipid A-associated proteins and the organism, and is dose-dependent (Frost, 1984; Giri, et al., 1984; Lohuis et al., 1988b). Generally, as dosage increases, latency time decreases, the peak effect becomes more pronounced, and the duration of the effect protracted (Lohuis et al., 1988b).

Natural antibodies elicited against O polysaccharides do well to protect animals against homologous Gram-negative bacterial strains but are not useful in protection against heterologous strains. This is a problem for protection against coliform mastitis because the causative pathogens represent a diverse population of species and antigens. Because the lipid A component of LPS is so well conserved across Gram-negative bacteria, antibodies elicited against this core antigen logically should confer cross-reactive protection against most Gram-negative bacteria. Indeed, this is the basis for vaccinating cows with mutant forms of Gram-negative coliforms for protection against mastitis. One such vaccine strain commonly employed is J5 *E. coli*.

Core antigens elicit protective immunity against Gram-negative bacterial diseases

Core antigens (i.e. J5 bacterin) have been used as immunogens (active immunization) in both humans and animals to enhance immune resistance against Gram-negative bacteria that are responsible for many diseases, including neonatal coliform septicemia, salmonellosis, and coliform mastitis. Evidence suggests that this bacterin also elicits cross-reactive serum antibodies that protect against sepsis caused by heterologous Gram-negative bacterial infections, for example *E. coli, Klebsiella, spp.*,

and *Pseudomonas aeruginosa*, (Bhattacharjee et al., 1994; Ziegler et al., 1973, 1975, 1982; Baumgartner et al., 1985; Calandra et al., 1988;). The relative risk of developing septic shock was about 6 times greater in untreated surgical patients who became infected with *E. coli, Klebsiella, P. aeruginosa, Enterobacter sp.* or *Proteus sp.*, than in surgical patients who received plasma taken from donors immunized with the endotoxin glycolipid core of *J5 E. coli* (Baumgartner et al., 1985). As in the Gram-negative infection model for humans, antibodies elicited by heat-killed J5 *E. coli* in experimentally *Haemophilus*-infected mice provided partial protection, with 80% survival in treated mice as compared to 28% survival in untreated mice (Marks et al., 1982). IgG and J5 LPS-specific IgG antibodies affinity purified from hyperimmune serum of rabbits immunized with heat-killed J5 *E. coli* significantly protected neutropenic rats against lethal consequences of *Pseudomonas aeruginosa* infection as compared to rats not administered antibody treatment (Bhattacharjee et al., 1994). While these antibodies elicited by J5 *E. coli* vaccination provided some cross-protective activity against existing Gram-negative infections, they did not prevent new infections with Gram-negative bacteria (Baumgartner et al., 1985). This suggests that anti-J5 antibodies have a short half-life in vivo. In cattle, success of the J5 vaccine varies, and many cows in herds using this technology still suffer from clinical diseases (Hill, 1991; Tomita, et al., 2000). Additionally, the immune mechanisms and the biological relevance of the antibody response induced by J5 vaccination in dairy cattle are still unclear.

Bovine antibody response and mastitis

As shown in **Figure 4**, bovine antibodies (**Abs**) are composed of two heavy (dark grey) and two light (white) chains. These chains combine to form two **antigen binding**

sites (Fab) and one **Fc region** of the full monomeric antibody molecule (**Figure 4**). Antibodies, which are the specific product of the adaptive immune response (humoral immunity), are found in the fluid compartment of blood and in extracellular fluid (Janeway et al., 2001). Bovine antibodies exist as six isotypes called immunoglobulin (**Ig**) M (IgM), IgG_1, IgG_2, IgG_3, IgA, and IgE, of which relatively little is known of IgG_3 and IgE (reviewed by Burton et al., 2002, **Figure 4**). Unlike humans, cattle lack IgD (Nassens, 1997). Molecular weight characteristics of bovine IgM, IgG_1, IgG_2, and IgA are given in **Table 1**.

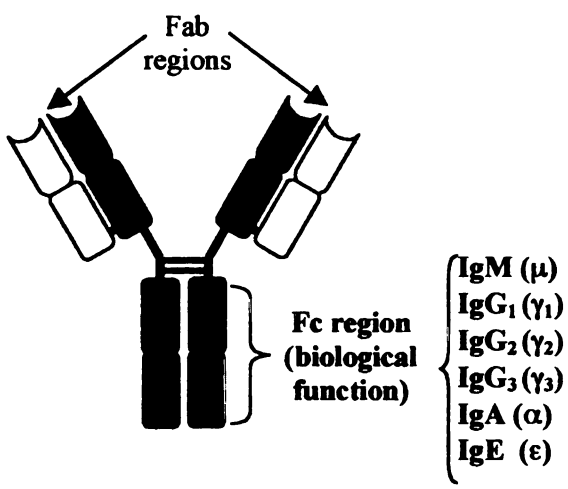


Figure 4 Monomeric antibody molecules are composed of two heavy (dark gray) and two light (white) chains. These chains combine to form two Fab and one Fc region of the full antibody molecule. The Fab are encoded by VDJ gene segments in immunoglobulin genes and undergo editing during immune responses for improved affinity of binding with antigen. The Fc region determines an antibody isotype and is responsible for the biological functions of antibodies. Isotype switching from IgM to IgG_1 and IgG_2 occurs in response to repeated antigen exposure and increases the biological repertoire of antibodies for more effective clearance of pathogens (figure from Burton et al., 2002).

Bovine IgA antibodies are usually dimerized (**Figure 5**) and are present in very low concentrations in bovine serum and milk (**Table 2**; reviewed by Butler, 1981; 1983; Tizard 1996). This is in contrast to non-ruminant species for which IgA constitutes the major milk immunoglobulin, and reflects the fact that the bovine mammary gland is part

of the peripheral immune system rather than the mucosal immune system (Harp et al., 1988; Kehrli and Harp, 2001, reviewed by Burton et al., 2002).

Bovine IgM occurs predominantly as a pentamer (**Figure 5**) and in relatively modest concentrations in serum and milk (**Table 2**; reviewed by Butler, 1981; 1983; Tizard, 1996). However, because of its ability to bind with 5 to 10 antigens and to fix complement, IgM is thought to be a highly effective antibody for neutralizing toxins (**Figure 6a**), complement fixation, and also may opsonize bacteria for efficient clearance via tissue macrophages and blood neutrophils (reviewed by Kremer et al., 1990). However, given that concentrations of IgM in milk are low, the contribution of this antibody class to clearance of mastitis pathogens may be relatively small. Instead, antibody protection against inflammatory pathogens may depend on IgG_1 and IgG_2 (reviewed by Burton et al., 2002; Burton and Erskine, 2003).

IgM **IgG** **IgA**

Figure 5 Antibody molecules are synthesized as monomers, such as is shown for IgG (middle), but IgM and IgA molecules are usually found in serum as multimers [either as pentameric IgM (left) or dimeric IgA (right)].

Monomeric IgG_1 and IgG_2 antibodies (**Figure 5**) are much smaller than IgA or IgM antibodies (**Table 1**) and are found in relatively high and almost equivalent concentrations in bovine serum (**Table 2**; Tizard 1996; Butler, 1981; 1983). These, along with IgM, are the main classes of antibodies of the peripheral immune system. IgG

antibodies predominate over all other isotypes in serum, accounting for approximately 90% of the total serum immunoglobulin (**Table 2**, McGuire and Musoke, 1981). However, the IgG_1 to IgG_2 ratio in non-mastitic bovine milk is 7:1 as compared with an IgG_1 to IgG_2 ratio of 0.7:1 in serum (Guidry et al., 1980b). This is due to selective and active transport of IgG_1 into milk of healthy mammary glands (Butler, 1998). However, after intramammary bacterial challenge, the IgG_1:IgG_2 ratio in milk reverses to 1:16, leading some to hypothesize that IgG_2 must be the most important antibody isotype in defense against intramammary infections in dairy cows (Burton and Erskine, 2003).

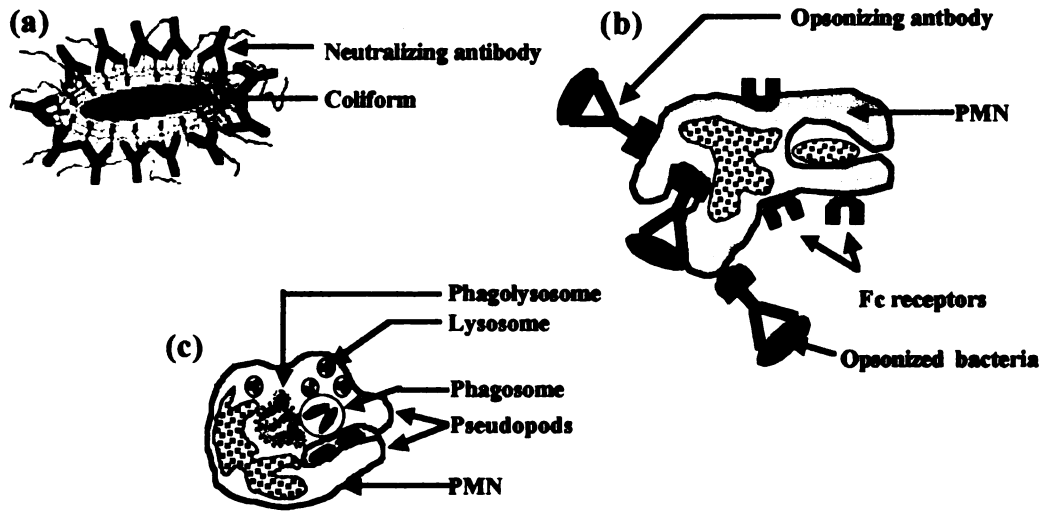


Figure 6 Antibodies effectively block pathogens from interacting with host cells and may have varying degrees of toxin neutralization capacity (**a**) or opsonization capacity (**b**). In cows, bacteria opsonized with IgG_2 antibody leads to rapid receptor-mediated phagocytosis by neutrophils resulting in pathogen degradation through activation of the respiratory burst (generating toxic ROS) and through phagosome-lysosome fusion (**c**). (adapted from Burton et al., 2002).

Table 1 Molecular size characteristics of the major bovine antibody isotypes. N/A = not available. (adapted from Butler, 1981)

Antibody isotype	Molecular Weight (kDa)		
	Intact molecule	Heavy chain	Light chain
IgM	1,000	61.8-76	22.5-26
IgG_1	146-163	56-59	22.5-26
IgG_2	146-150	54-59	22.5-26
IgA:			22.5-26
in colostrum	385-430	61	N/A
in respiratory tract	430 & 642	62.7	N/A

Table 2 Normal serum and milk (> 2 weeks postpartum) concentrations of the major antibody classes (isotypes) in dairy cows (from Burton et al., 2002).

Antibody isotype	**Serum levels (mg/ml)**	**Milk levels (mg/ml)**	**Main biological functions (via Fc region)**
IgM	3.05 (6.0 – 4.30)	0.86 (0.37 – 1.5)	Agglutination, complement fixation, opsonization blocking/neutralization
IgG_1	11.20 (6.00 – 15.10)	0.58 (0.33 – 1.20)	Complement fixation, blocking/neutralization
IgG_2	9.20 (5.00 – 13.50)	0.055 (0.037 – 0.06)	Opsonization, blocking/neutralization
IgA	0.37 (0.06 – 1.00)	0.08 (0.05 – 0.110)	Agglutination, blocking/neutralization

Substantiating this hypothesis, IgG_2 antibodies are well known opsonins for bovine neutrophils, the key cellular defense against intramammary bacteria (Burton and Erskine, 2003; **Figure 6b, 6c**). Freshly migrated neutrophils express high levels of surface Fc receptors that are specific for pathogen-bound IgG_2 antibodies (reviewed by Burton and Erskine, 2003). Thus, rapid leakage of blood-derived IgG_2 antibodies into milk shortly after infection must play a significant role in subsequent neutrophil clearance of the intramammary pathogens (reviewed by Burton et al., 2002; Burton and Erskine, 2003).

Passive transudation of IgG_2 is highly associated with neutrophil recruitment into the gland and significantly increases the opsonic activity of milk (reviewed by Burton et al., 2002; Burton and Erskine, 2003). Interestingly, the selective transfer of blood IgG_1 into milk may be suppressed during the acute phase of the mammary inflammatory response (Lascelles, 1979) possibly explaining why researchers observe relatively greater increases in milk IgG_2 compared to IgG_1 in 4 to 12 hours post infection, to the extent that the normal IgG_1/IgG_2 ratio can become reversed (reviewed by Burton and Erskine, 2003). It has also been shown that return to active IgG_1 transport post acute inflammation is accompanied by the formation of immune complexes in milk, which appear to inhibit neutrophil phagocytosis (Targowski and Clucinski, 1985), possibly by blocking IgG_2 Fc receptors and competing with IgG_2 for binding sites on pathogens (Targowski and Klucinski, 1985; Tao et al., 1995). Virtually nothing is known about if or how anti-J5 *E. coli* IgG_2 antibodies mediate defense against mastitis-causing coliforms (reviewed by Burton et al., 2002; Burton and Erskine, 2003)

IgG_1 is able to fix complement by the classical pathway (C3b molecules) through its Fc portion (McGuire and Musoke, 1981). Together with serum-derived complement, IgG_1 impedes the growth of serum-sensitive coliform bacteria (Carroll, 1974; Carroll et al., 1969). Although IgG_2 had been thought as a non-complement-fixing IgG subclass, studies demonstrated bovine IgG_2 fixed bovine complement in vitro, but was much less effective than IgG_1 (reviewed by Burton and Erskine, 2003). The ability of IgG_1 to fix complement (Butler, 1981; 1983) may be important to neutrophil phagocytosis in non-mammary tissues but its role in mammary defense against mastitis-causing coliforms is not at all clear. Despite the fact that mammary neutrophils have been demonstrated to bind more complement than blood neutrophils (DiCarlo et al., 1996), complement concentrations in normal milk are extremely low (reviewed by Burton et al., 2002; Burton and Erskine, 2003). Endotoxin-induced mammary inflammation has been shown to increase the levels of complement detected in bovine milk (Rainard et al., 1998), but milk itself has also been shown to mask the inflammatory activity of activated complement (Colditz and Maas, 1987). Thus, enhanced IgG_1 levels in milk, subsequent to the active inflammatory response to infection, have the potential to actively reduce the effectiveness of diffused IgG_2 antibodies in neutrophil-mediated bacterial clearance. Both IgG_1 and IgG_2 may be effective blocking or neutralizing antibodies (**Table 2**).

Maturation of bovine antibody

Generally, maturation of the antibody response is the process of development of highly specific antibodies by T-cell-dependent antigen-activated B cells. This process includes affinity maturation of the Fab regions by editing of the variable (VDJ) region of the heavy chain gene segments, and switching of gene segments in the constant (Fc)

region (**Figure 7**, Bonhomme et al., 2000; Butler, 1998; Kaattari et al., 2002). This process is considered an integral part of adaptive immune responses that usually occurs in the germinal centers of secondary lymphoid organs including the spleen and lymph nodes (Bonhomme et al., 2000; Butler, 1998; Kaattari et al., 2002; Liu et al., 1992). The maturation process ultimately improves affinity of antibodies produced by some B cell clones for their corresponding antigens (Bonhomme et al., 2000; Butler, 1998; Kaattari et al., 2002). Memory B cells stimulated by the same antigens (**Figure 8**) produce mature antibodies with higher affinity as compared to naïve B cells. This continued or repeated stimulation of memory B cells also leads to isotype switching, mediated in large part by T-cell derived cytokines (e.g. IL-4 for IgG_1 and IgE, IFN-(fir IgG_2) present in the responding germinal center of lymph nodes draining the affected tissue.

Studies in mice demonstrated that antibody maturation occurs following multiple exposures with the same antigen (Furukawa et al., 1999). In human antibodies, affinity maturation rapidly generates multiple variants to a broader range of epitopes that react with tumor associated antigens, and that display greater than 100-fold higher affinity and distinct specificities than the first antibodies produced (Pancook et al., 2001). It was also determined that trout are capable of generating new, higher affinity antibodies relatively late in the antibody response, which then become dominant (Kaattari et al, 2002).

In cattle, maturation of the antibody response is not completely understood but appears to share marked similarity to that of sheep (Aitken et al., 1999; Lei Parng et al., 1996). For example, antigen-stimulated B cells rely on somatic hypermutation and gene conversion to generate diversity in the Fab-encoding VDJ (Heavy chain) and VJ (light chain) regions of antibody genes in ruminants (Aitken et al., 1999; Butler, 1998; Lei

Parng et al., 1996). Somatic hypermutation occurs as point mutations accumulate when cells rapidly proliferate. Gene conversion is a nonreciprocal transfer of genetic information from one locus to another. Indeed, Lei Parng et al. (1996) have proposed that the pseudogenes occurring in the λ light chain gene locus (V_λ), which are the predominant light chains expressed in bovine antibodies, may serve to diversify the light chain repertoire through gene conversion. Because bovine immunoglobulin genes possess an extremely limited number of heavy chain variable (V_H) region gene segments and gene families, the generation of diversity in heavy chain gene segments relies mostly on somatic rearrangement. Consequently, cattle are unable to establish a diverse primary antibody repertoire from the process of heavy chain gene rearrangements, a paradigm that departs significantly from the murine/human system (Aitken et al., 1999; Butler, 1998). Thus, naïve B cell clones in cattle may express very similar or identical VDJ gene combinations (and thus Fab regions in antibody molecules). Subsequently, antigen-activated and proliferating B cells undergo variable-region somatic hypermutation and gene conversion in the spleen and lymph nodes under the influence of cognate B cell-T helper cell recognition of specific antigens. In this case, the antigen-specific T cell-derived cytokines present in the germinal center will determine if and what isotype switching occurs (Estes, 1996; Butler, 1998).

In summary, maturation of the antibody response involves editing of the variable (VDJ) region of heavy chain gene segments, and constant region gene switching (isotype switching, **Figure** 7). VDJ region editing modifies the sequences of Fab regions of antibody molecules, ultimately improving antibody affinity produced by some B cell clones for specific antigens (**Figure 7**, Butler, 1998). Isotype switching enables

antibodies with the same or affinity matured Fab regions to use different heavy chain constant region genes. This permits alteration of the biological functions (Fc region, **Figure 4**) of the antibodies to given antigens, thus expanding the capabilities of the humoral immune response (Estes, 1996). Thus, maturation of the antibody response should improve its overall effectiveness in clearing infections and toxins that cause clinical disease, including coliform mastitis. It is important to understand if the antibody response to J5 *E. coli* vaccination is capable of maturation in vaccinated dairy cattle. This knowledge would have significant implications for the efficacy of this vaccine to protect cows against a large variety of gram-negative bacteria that cause clinical mastitis as well as other diseases.

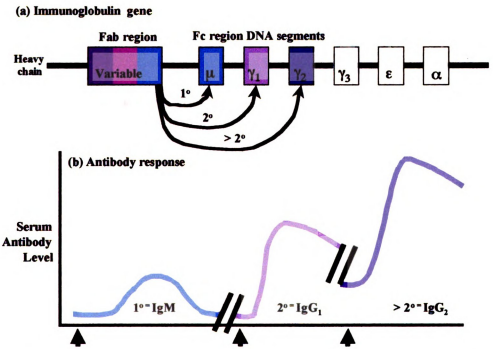
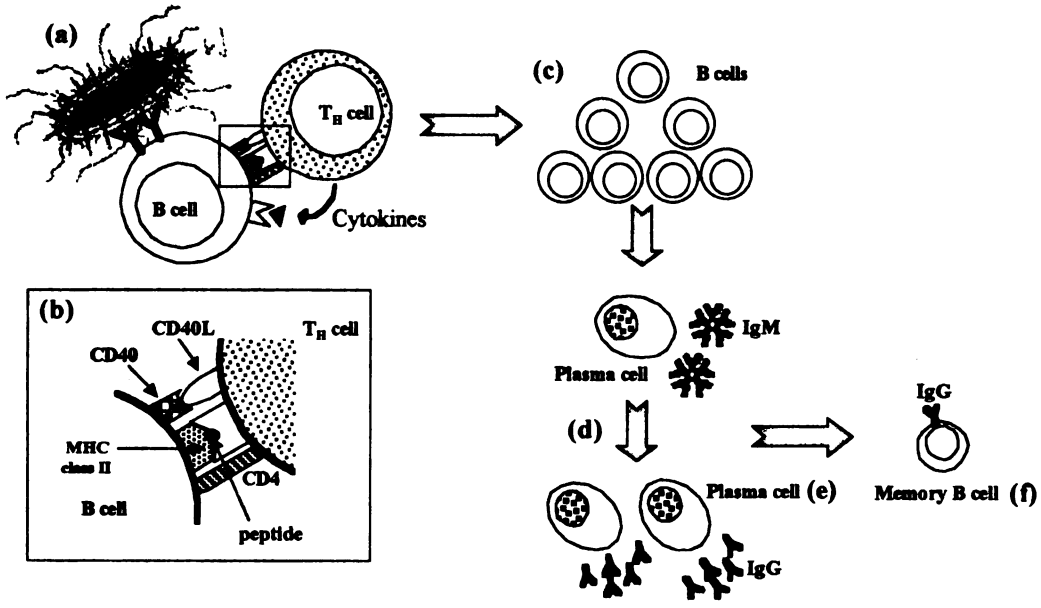


Figure 7 Repeated exposure of the immune system to the same antigens on bacteria promotes isotype switching to occur in B cells. Panel (a) shows switching of Fc region gene segments in bovine immunoglobulin genes after the immune sytem has been exposed to one (1°), two (2°), or more (> 2°), doses of bacterin (arrows). Panel (b) shows the corresponding changes in serum isotype-specific antibody levels following the first (1°), second (2°), or more (> 2°) doses of bacterin (arrows).

Figure 8 B cell activation with subsequent immunoglobulin isotype switching requires collaboration between B cells and helper T cells (T_H) that recognize the same pathogen. In panel (a), membrane-bound antibody on the surface of a specific B cell recognizes a pathogen and binds to it. The B cell then internalizes the pathogen and processes it into small pieces, some of which are peptides that bind to the antigen binding groove of MHC II molecules for surface presentation to nearby $CD4^+$ T_H cells (a and b). When the T_H cell recognizes the peptide presented in MHC II molecules by the B cell, the T_H cell responds by secreting a variety of cytokines (a) that cause clonal expansion of the B cell (c), isotype switching from IgM to IgG antibody subclasses (d), and secretion of large amounts of antigen and isotype specific antibodies from differentiated plasma cells (e). Some B cells so activated will also differentiate into memory cells that can respond rapidly to the same antigen upon subsequent exposures (f).



Coliform mastitis

Coliform mastitis is one of the most costly diseases in animal agriculture in the U.S., with costs from decreased milk production, discarded milk, therapeutic costs, mortality, and culling for dairy enterprises (DeGraves and Fetrow, 1993; Reneau, 1993). Studies in California, Ohio, and Pennsylvania have determined that the incidence of clinical mastitis in herds with low somatic cell counts averages 22 to 50 cases per 100

cows milking per year (Erskine et al., 1988; Hogan et al., 1989a; Gonzalez et al., 1990). In the Pennsylvania study, the proportion of clinical mastitis cases attributable to coliform bacteria (lactose-fermenting, Gram-negative bacilli of the family *Enterobacteriaceae*, including the genera *Escherichia*, *Klebsiella* and *Enterobacter*) was significantly higher in low somatic cell count (LSCC, ≤ 150,000 cells/ml) herds (43.5 ± 3.5%, n = 12) than in high somatic cell count (HSCC, ≥ 700,000 cells/ml) herds (8.0 ± 3.4%, n = 6) (Erskine et al., 1988). In the other studies, coliforms were isolated from 30 to 46% of the clinical cases of which *E. coli*, *Klebsiella spp.*, and *Enterobacter spp.* accounted for 14.6%, 2.6%, and 2.8% of the cases, respectively (Bartlett et. al., 1992; Gonzales et al., 1990; Hogan et al., 1989a; Smith et al., 1985).

Furthermore, coliforms are the predominant cause of both clinical mastitis and severe, life threatening, mastitis in well-managed dairy herds (Anderson et al., 1982; Erskine et al., 1988; Hogan et al., 1989a; Gonzalez et al., 1990; Erskine et al., 2002). Studies have isolated coliforms as the causative agent in 58.9% (56/95) and 55% (56/104) of severe clinical cases (Erskine et al., 2002). In addition, almost 30% of clinical coliform cases result in severe mastitis with abnormal milk, swelling of quarter, and systemic signs (review by Eberhart et al., 1979; Hogan et al., 1989a). However, it is suspected that when no bacteria are isolated on culture from milk samples collected from clinical mastitis cases, that the causative agent is often a coliform infection already eliminated by the cow's immune defenses (Bartlett et al., 1993).

Severe coliform mastitis may result in a total cessation of the lactation or in death. In an Illinois study, approximately 14% (6/42) of acute coliform cases resulted in death of the cow, as opposed to no death among 59 acute cases caused by Gram-positive

bacteria (Anderson et al., 1982). Similarly, 25% (14/56) of severe coliform mastitis cases died or were culled as compared to no loss cows in severe clinical mastitis caused by other pathogens (Erskine, 2002). Unlike *S. aureus*, coliforms do not colonize the teat canal and are minimally invasive of the mammary tissue (Frost and Wanasinghe, 1976). Thus, chronic intramammary coliform infections are uncommon (Eberhart, 1977; Jain, 1979). Typically, only one-fourth of coliform infections exceeded 30 days in duration (Smith, 1983).

Increased incidence of coliform mastitis is associated with the early postpartum period, bedding materials, warm humid weather, and increasing parity (Bramley, 1985; Erskine et al., 1988; Hogan et al., 1989a; 1989b; Smith et al., 1985). Slow migration of blood polymorphonuclear neutrophil (**PMN**) into the mammary gland and impaired oxidative burst activity are considered the important immune risk factors for severe coliform mastitis during the early postpartum period (reviewed by Dosogne et. al., 2002). The susceptibility of individual cows to severe coliform mastitis appears to be associated with the degree of neutropenia or impairment of blood PMN function (Dosogne et. al., 2002). Lymphocyte function including proliferation and antibody response has also been found to be impaired during early lactation, but a relation with the severity of coliform mastitis has not been clearly established (reviewed by Dosogne et. al., 2002).

Inflammatory and systemic response to intramammary invading pathogens

The teat streak canal is a physical barrier that normally prevents intramammary infection in cows. However, when breached, bacteria gain easy entry into the duct system and mammary epithelium. This initiates a nonspecific innate immune response resulting in inflammation. Inflammation is the leakage of serum proteins (including

antibodies) from blood into the infected tissue, with subsequent massive recruitment of blood-derived neutrophils. Rapid inflammatory responses must occur if the neutrophils are to successfully clear the infecting pathogen before it proliferates and causes tissue damage and clinical disease. However, the inflammatory response must also be controlled to avoid excess tissue damage from reactive oxygen species and enzymes elaborated by infiltrating neutrophils. Antibodies are key in facilitating rapid and controlled inflammation.

Inflammation at the site of infection is initiated by the response of resident macrophages to common components of bacterial cell walls, such as LPS or lipoproteins of coliforms. This induces rapid production and secretion of various macrophage cytokines and tissue chemokines that cause effusion of blood components through the capillaries, and recruit large number of neutrophils to the site. In dairy cattle, neutrophils play a pivotal role as the first line of cellular immune defense against intramammary pathogens. Blood-derived neutrophils gain access to the mammary parenchyma and milk through the process of migration and chemotaxis. The destruction of infecting bacteria by phagocytosis uses both oxygen-dependent and oxygen-independent killing mechanisms (**Figure 6c**; Burton and Erskine, 2003; Burvenich et al., 1994; Hill et al., 1978). This mammary inflammatory response, like all inflammatory responses, results in five hallmark clinical signs: swelling, redness, pain, heat, and abnormal milk (Burton and Erskine, 2003; Lohuis et al., 1988b; Tizard, 1996).

Systemic responses to intramammary infection are also noted, especially during severe coliform mastitis. These include fever, increased concentrations of serum cortisol, fibrinogen, complement, haptoglobin, and ceruloplasmin, transient decreases in serum Fe

and Zn, and mobilization of leukocytes out of the blood (neutrophils) and lymph nodes (mononuclear leukocytes) into the mammary gland. These systemic changes are in large part caused by cytokines, especially IL-1, IL-6, and TNF-α, that escape from the inflamed mammary gland (Bishop et al., 1976), and are observed with both natural coliform infections and when animals are treated with intramammary doses of LPS (Conner et al., 1986; Erskine et al., 1989; Erskine et al., 1993; Jackson et al., 1990; Lohuis et al., 1990; Shuster et al., 1992). Released eicosanoids following LPS exposure are also responsible for many pathophysiologic changes, including shock, pulmonary hypertension, abortion, and diarrhea (Tyler and Cullor, 2002).

Coliform mastitis versus core antigen technology

The dairy industry has adopted core-antigen vaccine technology to promote the prevention of coliform mastitis. Currently, commercially available core-antigen vaccines for mastitis include J5 BACTERIN™ (Pharmacia Animal Health), Mastiguard™, J-VAC™ (Merial Ltd.), Endovac-Bovi® (Re-mutant *Salmonella typhimurium* bacterin-toxoid. IMMVAC Inc.), and Master Guard® J5 (Agri Laboratories). Typically, these vaccines are administered subcutaneously into dairy cows one to three times prior to the early lactation period, the time of the lactation cycle that is believed to have the greatest risk for new intramammary infections (Clark, & VanRoekel, 1994; González et al., 1989; Hogan et al., 1992a; 1992b; 1995, 1999). However, disparity exists for both the recommended vaccination protocols and the antibody responses elicited by various vaccines and vaccination protocols.

Historically, the first 60 days of lactation has been the period of peak occurrence of clinical coliform mastitis. Evidence from early research trials indicated that a three-

immunization protocol can significantly reduce the incidence, severity and duration of clinical coliform mastitis (Clark & van Roekel, 1994; Gonzalez *et al*, 1989; Hogan *et al*,1992a; 1992b; 1995). Studies in two commercial California dairy herds reported that the incidence rate of Gram-negative mastitis was lower in cows vaccinated by 3 doses of whole cell bacterin of J5 *E. coli* plus Freund's incomplete adjuvant (2.6%) than in unvaccinated cows (12.8%) during the first three months of lactation (Gonzalez et al., 1989). Only 20% of cows vaccinated with this J5 bacterin (prepared from the heat-killed *Escherichia coli* J5 Rc mutant at a 5 ml dose emulsified with 1 ml of Freund's incomplete adjuvant) had IMI caused by Gram-negative bacteria as compared to % of non-vaccinated controls (Hogan, et al., 1992a). As compared to placebo, cows vaccinated by commercial J5 bacterin at drying off, 30 days later, and 48 hours after calving and challenged by intramammary infusion of *E. coli* 727, had reduced bacterial numbers in milk from infected quarters and reduced local signs and shortened duration of mild clinical mastitis compared to non-vaccinated controls (Hogan *et al.*, 1995). However, there was no effect of vaccination on systemic signs, dry matter intake, and somatic cell count in this study (Hogan et al., 1995). Two commercially available vaccines J5 Bacterin and J-VAC were compared in another study (Tomita et al., 2000). Here, serum IgG, IgG_1 and IgG_2 antibody titers to *E. coli* J5 whole cell antigens were higher in vaccinated cows around 2-6 weeks after calving than in unvaccinated control cows. Also, both serum and milk IgG_1 and IgG_2 antibody titers elicited by J5 Bacterin were consistently higher than those elicited by J-VAC (Tomita et al., 2000). Nonetheless, neither vaccine prevented coliform mastitis, though all vaccinated cows tended to have a reduced severity of disease compared to unvaccinated cows (Tomita et al., 2000). It is

possible that the increased antibody titers achieved by vaccination were not high enough or mature enough to provide effective humoral immune protection against the infecting bacteria.

Although much of the attention of J5 vaccination protocols has focused on the prevention of coliform mastitis in the early part of lactation, a field trial of six Michigan dairies reported that the highest incidence of severe clinical coliform mastitis was between the 3rd and 5th months of lactation, even when herd J5 vaccination programs were employed (Erskine, 2002). In other studies, cows immunized with J5 according to these protocols still had clinical coliform mastitis and systemic disease, especially in the period between calving and 180 days in milk (Hogan et al., 1992a; 1999). As was observed in the study of Tomita, et al. (2000), this suggests that the anti-coliform antibody response elicited by dry period and peripartum J5 vaccinations may be short-lived and (or) ineffective in today's high producing cows. A potential practical limitation of active immunization using J5 bacterin may be the shorter half-life of core-antigen specific IgG antibodies (7.6 days) in the serum of cattle as compared to total serum IgG (22.7 days; Douglas et al., 1989).

Low titers (< 1:240) among cows of naturally occurring serum anti-J5 *E. coli* IgG_1 antibodies have been shown to be negatively correlated with mastitis incidence, resulting in 5.33 times higher risk of disease than cows with titers > 1:240 (Tyler et al., 1988). Similarly, titers in response to J-5 immunization were negatively correlated with severity of clinical outcome following experimental challenge using heterologous *E. coli* (Hogan et al., 1992a). In another experimental challenge trial, vaccination using J5 bacterin elaborated significantly higher antibody responses in serum and milk, higher

serum IgG titers, and less depression of milk production and dry matter intake than was observed in non-vaccinated cows (Hogan et al., 1992b). However, differences in antibody titers between vaccinated and control cows were not impressive in that study despite the statistically significant differences highlighted. This could be why J5 vaccines are not effective in preventing coliform mastitis in some cows.

In an attempt to improve antibody responses to J5 vaccination, researchers have considered different routes of administration. For example, Hogan et al. (1997) demonstrated that intramammary infusion of J5 enhanced serum and milk IgG and IgM antibody titers as compared to the systemic route of administration. Total milk IgG titers in cows administered intramammary vaccination were higher (13.1) than in cows receiving subcutaneously administered bacterin (10.1) at calving. This may have been due to the combination of active transfer of IgG_1 from serum and local production of IgG_1 from B cells present in the mammary gland parenchyma. This was substantiated by the finding that serum IgG titers were higher in intramammarily vaccinated cows than in cows vaccinated subcutaneously (Hogan et al., 1997). However, antibodies elicited by J5 intramammary infusion may not have been high enough or of the protective isotypes because they did not prevent infection or reduce the severity of coliform mastitis (Smith et al., 1999). Also, rectal temperatures were increased in the intramammarily vaccinated cows but not in the subcutaneously vaccinated cows (Hogan et al., 1997). Therefore, the intramammary route of J5 vaccination is not more efficacious in preventing or reducing signs of clinical coliform mastitis than the subcutaneous route.

The induction of cross-reactive antibodies is most likely responsible for successful applications of J5 vaccine programs when these do work well. However, there

is a paucity of research available that describes the biological activities of anti-J5 *E. coli* antibodies, including isotype-specificity of pathogen and antigen recognition, and biological functions of each responding antibody isotype. The purpose of the current study was to begin to characterize the bioactivities of bovine ant-coliform antibodies elicited by J5 vaccination. The hypothesis of the study was that repeated exposure of the bovine immune system to a bacterin of Rc mutant J5 *E. coli* would result in highly mature serum antibody responses, producing in particular a strong IgG_2 response that would cross react with increasingly diverse antigens on a variety of heterologous Gram-negative bacteria. This would result in enhanced bacterial opsonization for effective phagocytic uptake by bovine neutrophils.

CHAPTER 2

ISOTYPE SPECIFIC SERUM ANTI-COLIFORM ANTIBODIES CROSS-REACT WITH GRAM-NEGATIVE BACTERIA

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Abstract

This study was conducted to determine response profiles, titers, and cross reactivity for Gram-negative bacteria of isotype specific anti-J5 *E. coli* antibodies elicited by J5 hyperimmunization in healthy Holstein cattle. Five steers were used. Immunizations were administered at time 0, 30 days later, and every 2 weeks for 10 subsequent immunizations. Blood samples were collected pre-immunization and on days 4, 6, 11, and 13 post each immunization, harvested for sera, and assayed for relative contents of anti-J5 *E. coli* IgM, IgG_1, and IgG_2 antibodies by ELISA. Anti-J5 *E. coli* antibody response profiles were recorded as optical densities of appropriately diluted sera. Selected sera from pre-immunization and 6 days post-3rd, 6th, 9th, and 12th immunization were also assayed for isotype specific end-point titers. Those selected sera were then used to determine cross-reactivity against a variety of Gram-negative bacteria, most of these isolated from clinical mastitis cases. Repeated measurement analysis showed that immunization number and day post immunization significantly ($P < 0.0001$) influenced anti-J5 *E. coli* antibody responses for all three isotypes. Two immunizations

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were required to increase ($P \leq 0.05$) mean serum anti-J5 *E. coli* IgM and IgG_1antibody above pre-immunization levels. However, five immunizations were required to detect significant IgG_1 to IgG_2 isotype switching, and to increase anti-J5 *E. coli* IgG_2 antibody above pre-immunization levels ($P \leq 0.02$). Anti-J5 antibody titers of all isotypes tended to increase through 12 immunizations, and were significantly higher than pre-immunization levels after 6 immunizations ($P < 0.005$). Cross-reactivity of isotype specific anti-J5 antibodies to all Gram-negative bacteria was increased ($P < 0.05$) in hyperimmunized steers. Anti-J5 IgM and IgG_2 antibodies tended to cross-react with *Serratia spp.*, *E. coli 487* and *K. pneumoniae* more than with other bacteria. Anti-J5 IgG_1 antibodies tended to cross-react with *E. coli 487* and *Pseudomonas spp.* more than with other bacteria. However, no cross-reactivity of anti-J5 antibodies with either LPS or Lipid A was observed, which was similar to the result for *S. aureus*. Results of our study suggest that multiple dose immunizations with J5 *E. coli* bacterin may be required to elicit high levels of serum IgG_1 and IgG_2 antibodies that strongly cross-react with mastitis causing Gram-negative bacteria. Our results also suggest that LPS and Lipid A core antigens in the J5 vaccine might not be the key antigens stimulating high IgG_1 and IgG_2 antibody titers in vaccinated cattle.

Key words: humoral immunity, dairy cattle, core antigen, cross-reactivity

1. Introduction

Active immunization of dairy cattle, with *Escherichia coli* J5 bacterin, has been used by dairy producers as a method to enhance immune resistance against Gram-

negative bacteria, including mastitis-causing coliforms. The J5 bacterin is derived from a mutant stain of *E. coli* (0111:B4; Rc mutant), which lacks the "O" antigen capsular portion of the cell wall but retains core lipopolysaccharide (LPS) and Lipid A antigens that are highly conserved across all Gram-negative bacteria (Cullor, 1991b; Gonzalez et al., 1989; Tyler et al., 1990b). Immunization of cattle with bacterins of J5 *E. coli* was previously shown to elicit serum antibodies with cross-reactivity to a wide variety of Gram-negative bacteria (Tyler et al., 1990a; 1992). However, some researchers have claimed that J5 bacterin is a poor immunogen, eliciting poor serum and milk antibody responses in vaccinated cattle (reviewed by Kehrli and Harp, 2001). Others have reported relatively good IgM and IgG antibody responses in serum and milk of vaccinated cows (Tyler et al., 1991, 1992; Tomita et al., 1995). Nevertheless, the biological relevance of these antibody responses has not been well determined, leaving a paucity of data on the mechanism(s) by which J5 vaccination protects dairy cattle from disease caused by Gram-negative bacteria, especially coliforms. This is apparent by the lack of knowledge of the magnitude of isotype-specific antibody responses, diversity of antigens recognized, and the biological roles of isotype-specific serum antibodies elicited by J5 vaccination. There is also little data available exploring the potential of improving humoral immune responses against coliform bacteria using vaccination protocols or vaccine preparations different from those currently recommended and available.

We hypothesized in the current study that cattle hyperimmunized with J5 *E. coli* bacterin would mount strong humoral immune responses, inducing IgG_1 and IgG_2 antibodies that are cross-reactive with a wide variety of Gram-negative bacteria. The objectives were to determine response profiles for isotype specific anti-J5 *E. coli*

antibodies in steers immunized 12 times with J5 *E. coli* bacterin; to determine titers of isotype specific anti-J5 *E. coli* antibody in sera collected pre-immunization and on 6 days post-3rd, 6th, 9th, and 12th immunization, and to determine the cross reactivity of antibodies contained in those selected sera with a variety of Gram-negative bacteria.

2. Materials and Methods

2.1 Animals

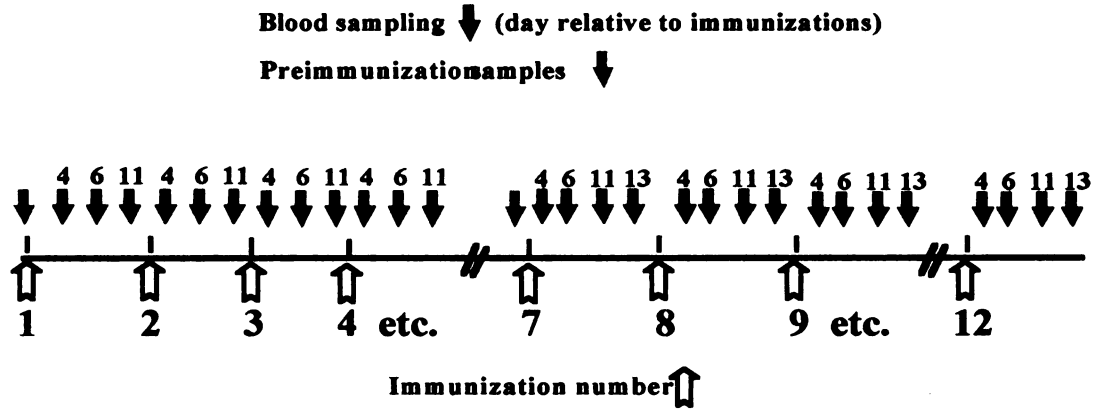
Five healthy, 5-month old Holstein donor steers were used for this study. Steers were owned by a private dairy in Michigan, and housed and cared for at the farm for the duration of the study. Animals were group-housed with free access to water and feed. In preparation for entry into our study, steers were immunized twice for BVD, IBR, PI_3 BRSV and 5 serovars of *Leptospira* (Triangle 9; Fort Dodge Animal Health, Fort Dodge, IA), and dewormed (Ivermec, Merk & Co., Inc., Rahway, NJ) at 3 to 4 months of age (before the trial). All steers were deemed healthy at the start of the study, and remained healthy throughout the study.

2.2 J5 Immunization schedule

All steers were started on the J5 hyperimmunization protocol, bled and immunized on the same days (see below). A total of 12 immunizations were administered to each steer by subcutaneous injection of 5 ml of a commercially available J5 *Escherichia coli* bacterin (Pharmacia Animal Health, MI). The first immunization was administered at study time 0 (immunization number 1, **Figure 9**). The second immunization was administered 30 days later. The subsequent 10 immunizations

(immunization numbers 3 through 12) were administered every two weeks, with an 8-week rest period between immunization numbers 6 and 7 (**Figure 9**).

Figure 9 Schedule of immunizations (white arrows) and blood sampling (blue and black arrows). Blood was collected from each steer immediately before the first and seventh immunizations (preimmunization samples), on days 4, 6, and 11 post immunization for immunization numbers 1 through 6, and on days 4, 6, 11, and 13 post immunization for immunization numbers 7 through 12.



2.3 Blood collections

Blood was collected from each steer immediately before the first immunization (pre-immunization), on days 4, 6, and 11 post immunization for immunization numbers 1 through 6, and on days 4, 6, 11, and 13 post immunization for immunization numbers 7 through 12 (**Figure 9**). One-hundred and fifty milliliters of blood were collected from jugular veins into 50-ml sterile conical tubes using sterilized 14G, 3.75-cm needles. Samples were allowed to clot overnight at 4°C and were then centrifuged at 2400 × g [(GS-6R BECKMAN Centrifuge (Beckman Instrument, Inc., Schaumburg, IL)] for 30

min at 4°C. Sera were harvested and stored in sterile 50-ml tubes at -20°C until assayed by ELISA.

2.4 Preparation of phenol-killed whole J5 Escherichia coli for use in ELISA

J5 *E. coli* Rc mutant, donated by Dr. James Cullor (University of California, Davis, CA), was plated on 5% sheep blood agar and incubated overnight at 37°C. The plate was then checked for culture purity. Single colonies from a pure culture plate were selected and inoculated in 15 ml of Trypticase Soy Broth (**TSB**) in an orbital shaker [120 rotations per minute (**rpm**); J.T Baker, Phillipsburg, NJ] for 18 hr at 37°C. In ordewr to establish purity of the inoculum, a 0.01 ml aliquot was collected from the inoculum and plated on 5% sheep blood agar for isolation of individual colonies, and incubated overnight at 37°C. The inoculum was stored at 4°C during this incubation, and if the culture provided a pure culture, the inoculum was transferred into 1 liter-TSB and incubated with shaking @ 120 rpm for 18 hr at 37°C. This preparation was then stored at 4°C while purity was assured by culture, and then bacteria were killed by adding 15 ml of a 99% liquid phenol solution (Sigma; St. Louis., MO) per liter of the bacterial preparation. This solution was incubated with shaking for 1 hr at 37°C and 120 rpm. The phenol-killed whole cell pellets were recruited by centrifugation at 1000 × g for 12 min at 4°C. The pellets were washed twice by suspension in sterile 0.9% NaCl solution and centrifugation (1000 × g for 12 min at 4°C). The pellets were suspended in sterile 0.9% NaCl solution to reach 13% optical transmission (determined by Beckman DU 650 Spectrophotometer, BECKMAN COULTER, Inc., Fullerton, CA), which represented approximately 1×10^9 cfu of killed bacteria/ml. This was the antigen used to coat wells of ELISA plates (see below) ands was stored in 12 ml aliquots at -20°C.

2.5 Isotype specific anti-J5 E. coli antibody response profiles

Serum anti-J5 *E. coli* IgM, IgG_1, and IgG_2 antibody responses were determined using a J5 *E. coli* ELISA protocol modified from Tyler et al. (1990a) according to the methods of Burton et al. (1993). Briefly, one hundred microliters of J5 E. coli antigen per well were used to coat wells of 96-well ELISA plates (ProBind Flat Bottom plates, Fisher Scientific). The plates were covered and left to incubate on a flat surface at room temperature for 15 hours, after which unbound antigen was removed by six washes using 200 µl/well of biowash solution (0.9% saline containing 0.05% Tween 20). Test sera were prepared for ELISA using separate 96-well, U-bottomed, polystyrene microtiter plates (Becton Dickinson and Company, Flankin Lakes, NJ). Individual samples were placed in the top well of each column and doubly diluted down the 8 rows of the column, starting with a 1:50 dilution in row A and ending with a dilution of 1:6400 in row H. Serum dilutions were made using a diluent, which was PBS, pH 7.3 with 0.5% Tween 20. A positive control serum consisting of pooled steer sera from blood samples collected following the 5^{th} immunization was similarly diluted down rows of one column of each plate. The negative control in this assay was fetal bovine serum (heat-inactivated, endotoxin $\leq$ 10 EU/ml, Life Technologies, Rockville, MD) and was diluted in the same fashion in one column of each plate. Samples contained in each well of the U-bottomed dilution plates were then transferred (100 µl/well) to washed ELISA plates using a multi-channel pipetter (Costar Corporation, Cambridge, MA). Additional controls in this assay included quadruplicate wells of 1:400 dilutions of the positive and negative control sera,

duplicate blank wells, duplicate wells that received only initial J5 coating, and duplicate wells that received all reagents except test sample.

Once all samples were delivered to appropriate wells, ELISA plates were sealed and incubated at 37 °C for 45 min, washed 6 times (as above) to remove unbound antibodies, and 100 µl/well of detection antibodies (Horseradish peroxidase-conjugated sheep anti-bovine IgM, IgG_1, or IgG_2, BETHYL Laboratories, Inc., Montgomery, TX) diluted to 1:25,000 with the diluent were added to wells of appropriate plates. Plates were again sealed and incubated for 30 min at 37°C, washed 6 times to remove unbound detection antibody, and 125 µl/well of substrate (hydrogen peroxide-azino-*bis*-3-ethylbenzthiazoline sulfonic acid, Sigma Chemical Co., St. Louis, MO) added to all wells except the duplicate blank wells and duplicate J5 antigen only well for 45 min at 37°C. Relative antibody concentrations in samples were recorded as optical density (OD) following spectrometric analysis at a dual wavelength of 405-450 nm using an ELISA plate reader (Benchmark, Bio-RAD Laboratories, Hercules, CA). The plate reader was blanked against the 2 blank wells of each plate. In our ELISA assays, the greatest and most repeatable differences in IgG_1 ODs between the positive and negative control sera occurred at the 1:400 dilution. All samples in a plate were repeated if OD values of the positive and negative control sera were not within pre-established ranges ($0.99 \leq OD \leq 1.10$ and $OD \leq 0.09$, respectively). Mean antibody response was reported from statistical analysis of test sample OD at serum dilutions of 1:100 for IgM and of 1:400 for IgG_1 and IgG_2. Isotype specific anti-J5 *E. coli* antibody responses based on ODs of these selected dilutions of test sera were parallel to the response profiles obtained from OD of one

doubling dilution above and below the selected dilution (correlation coefficients between sample dilutions within isotype > 0.90; $P < 0.0001$).

2.6 Isotype specific anti-J5 E. coli antibody titers in selected serum samples

The previously described ELISA was used to determine isotype specific anti-J5 *E. coli* antibody endpoint titers in selected sera (pre-immunization, and 6 days post 3rd, 6th, 9th, and 12th immunizations). These sera were doubly diluted down columns of each plate in duplicate, including 1:4, 1:8, 1:16,..., and 1:131,072 dilutions. Pooled hyperimmune steer sera (7th immunization) and fetal bovine serum (heat-inactivated, endotoxin ≤ 10 EU/ml, Life Technologies, Rockville, MD) served as positive and negative controls, respectively, and were doubly diluted down 2 columns in the same fashion as test sera. End-point titers of the test and control sera were recorded as $\log_2$ of the last dilution to yield an OD < 0.10, which we considered as zero titer because the FBS negative control always had an OD < 0.10.

2.7 Antibody cross-reactivity assay

The cross-reactivity of isotype-specific antibodies from the titered sera was determined by ELISA, basically as described for the profiling of isotype specific anti-J5 antibodies. However multiple bacterial solutions (approximately 1×10^9 cfu/ml each) in addition to the J5 *E. coli* (Rc mutant) were prepared for well coating of the ELISA plates. *Escherichia coli McDonald 487, Pseudomonas spp., Serratia spp., Klebsiella pneumoniae, Salmonella newport, Salmonella typhimurium,* J5 *E. coli* and *Staphylococcus aureus* were cultured and killed with phenol to make antigen solutions as previously described. *S. newport and S. typhimurium* were donated by the Animal Health Diagnostic Laboratory, East Lansing MI. The other bacteria originated from milk

cultures of cows with clinical mastitis (bacterial stocks available through the Mastitis Research Laboratory, Michigan State University; Department of Large Animal Clinical Sciences. The ELISA plates were coated using 100 µl/well of each bacterial solution. Similar preparations of J5 *E. coli* and *S. aureus* were used as antigen positive and negative controls, respectively. LPS (Sigma-Aldrich Co., St. Louis, MO, 10 µg/ml) and lipid A [monophosphoryl from *S. minnesota* (Re 595), Sigma-Aldrich Co., St. Louis, MO, 10 µg/ml] were also used as antigens in some wells and were prepared as described in Freudenburg (1989). The sera from pre-immunization, and 6 days post-3rd, 6th, 9th, and 12th immunizations were then diluted at 1:50 (for IgG_2 and IgM) or 1:200 (for IgG_1) for determining isotype specific cross-reactivity of the antibodies contained in the various sera and to quantify relative concentrations (as OD) of the isotype specific cross-reactive antibodies.

2.8 Statistical analysis

For statistical analysis of the antibody response profiles, immunization number and sample day nested within immunization number were included in a mixed model as fixed effects along with assay day, assay plate nested within assay day, and animal as random effects. In addition, autoregressive order one covariance structure based on Schwarz' Bayesian model-fit criterion was specified in the REPEATED statement of SAS to model covariance structure within subject. Statistical differences in mean OD over days following each immunization, and specific differences between the mean OD for immunizations 1, 2, and 3 versus the pre-immunization mean OD or the ODs of all other immunizations were determined at a significant level $P < 0.05$ using LSMEAN and ESTIMATE statements of SAS with the Tukey-Kramer adjustment factor.

Repeated measures analysis of variance was used to analyze anti-J5 *E.coli* IgM, IgG_1, and IgG_2 antibody end-point titer data and isotype specific cross-reactivity data sets. The analyses were carried out utilizing the MIXED procedure of SAS software (SAS Institute, Cary, NC). Overall significance of effects in the model included number of immunizations for the isotype specific anti-J5 E. *coli* antibody titer data, the interaction of "BACTERIA" and immunization number, and BACTERIA for cross-reactivity OD data, and was assessed by the Type III F-test. Statistical significance of adjusted mean comparisons of effects declared relevant by the t-test was determined by the Tukey-Kramer multiple comparison procedure. The significance level was set at _ = 0.05.

3. Results

3.1 Hyperimmune response profiles

The Least Squares Means (LSMean ± SEM) reported in **Figures 10, 11, and 12** were derived from the mean ODs of samples collected after each immunization. Steer did not contribute significantly to variation in mean post immunization antibody responses for any isotype studied ($P > 0.10$). However, immunization number and day post immunization significantly influenced all antibody response curves ($P < 0.0001$). The following trends were observed for the day post immunization effect (data not shown). There was no difference between sampling days following the first 3 immunizations and for the 8^{th} to 12^{th} immunizations. For all other immunizations, highest ODs were observed at 11 days post immunization (4^{th}, 5^{th} and 6^{th}), or at 6 and 11 days post 7th immunization. Adjusted differences in mean OD within immunization over days post immunization showed that only two doses of J5 bacterin were required to

significantly ($P \leq 0.05$) increase serum anti-J5 *E. coli* IgM antibody above pre-immunization levels (**Figure 10**). Similarly, it took two J5 doses to elicit increases in anti-J5 *E. coli* IgG_1 antibodies pre-immunization levels ($P = 0.004$), and this isotype continued to increase in serum through the 8th immunization, after which the IgG_1 attained a plateau ($P \leq 0.02$, **Figure 11**). For IgG_2, five doses of J5 vaccine were required to achieve isotype switching, and increase serum anti-J5 *E. coli* IgG_2 antibodies above pre-immunization levels ($P < 0.02$; **Figure 12**).

3.2 Isotype specific anti-J5 E. coli antibody endpoint titers

Pre-immunization sera contained varying amounts of naturally occurring anti-J5 antibodies for all three isotypes (**Figure 13**). These anti-J5 antibody endpoint titers increased through 12 immunizations for all three isotypes, and were significantly higher ($P < 0.005$) than pre-immunization following administration of the 6th dose of J5 bacterin (**Figure 13**). It is noteworthy that three doses of vaccine were not sufficient to increase anti-J5 *E. coli* IgM, IgG_1, or IgG_2 titers because this is the number of doses recommended by the vaccine manufacturer.

3.3 Isotype specific antibody cross-reactivity

As evidenced by increased OD values, cross-reactive IgM antibodies in sera of the test steers were high after 6 immunizations for *Klebsiella app., Pseudomonas, and Serratia app.* ($P < 0.01$), after 9 immunizations for *S. typhimurium* and *E. coli* 487, ($P < 0.01$), and after 12 immunizations for *S. Newport* ($P < 0.01$) and Lipid A ($P < 0.05$) as compared to corresponding pre-immunization titers (**Figure 14**). Anti-J5 IgG_1 significantly increased after the 6th immunization for all bacterial antigens ($P < 0.01$) except for *E. coli* 487, which only required 3 immunizations ($P < 0.001$), and *S. newport*,

which required 9 immunizations ($P < 0.05$, **Figure 15**). IgG_2 antibody titer increased significantly after the 6^{th} immunization for all bacterial antigens compared to pre-immunization titers.($P < 0.01$). Also, the IgG_1 and IgG_2 antibodies in test sera were more likely to cross-react with *E. coli 487* and *Pseudomonas spp.* than *Salmonella* bacteria ($P < 0.05$, **Figure 15 & 16**). Low cross-reactivity for LPS, Lipid A, and *S. aureus* was observed for all three isotypes, especially as compared with cross-reactivity to Gram-negative bacteria ($P < 0.001$, **Figure 14-16**).

4. Discussion

In the current study, J5 hyperimmunization of dairy cattle achieved increased isotype specific immune responses, titers, and cross-reactivity of anti-J5 antibodies with multi-heterologous Gram-negative bacteria compared to no immunization and the manufacturer's recommended three immunizations. Therefore, results of this work suggest that more doses of J5 bacterin than currently recommended may be required to induce strong cross-reactive anti-J5 IgM, IgG_1, and especially, IgG_2 serum antibody responses in dairy cattle.

Generally, antibodies of the IgM isotype are the first to be secreted from B cells in response to primary exposure to thymus-dependent (**TD**) antigens (Janeway et al., 2001). With successive exposures to the same antigen, IgM-to-IgG_1 isotype switching occurs in the responsive B cell clones, effectively shutting down their IgM secretion. It is now known that this classic humoral immune response occurs via a specific class of B cells that are characterized by lack of surface CD5 expression (called B-2 cells). However, a second class of CD5-expressing B cells ($CD5^+$, B-1 cells) has been identified and secretes

predominantly IgM antibodies in response to repeated stimulation by thymus-independent (**TI**) antigens (Haas and Estes, 2000; Hayakawa et al., 1984; Kantor 1991; Mond et al., 1995). TI antigens characteristically possess highly repetitive epitopes, such as those that occur in capsular polysaccharides of Gram-negative bacteria (Mond et al., 1995).

Because core lipopolysaccharide antigens are exposed in the J5 strain of *E. coli*, it is very possible that the sustained IgM responses to J5 hyperimmunization observed in the current study resulted from preferential activation of $CD5^+$ B cells. $CD5^+$ B cells exist in cattle and have even been shown to account for up to 30% of adult peripheral blood lymphocytes during certain infections (Buza et al., 1997; Haas and Estes, 2000; Meirom et al., 1993; Nassens and Williams, 1992). Antigen-activated bovine $CD5^+$ B cells do not undergo significant IgM-to-IgG_1 isotype switching, eventually leading to very high serum IgM antibody titers (Hayakawa et al., 1984; Janeway et al., 2001; Nassens and Williams, 1992). So, while some B cell clones obviously switched from IgM to IgG_1 production in our J5 hyperimmunized steers, other B cell clones that may have been $CD5^+$ also continued to produce increasing amounts of IgM with each successive J5 immunization.

In the current study, evidence exists for isotype switching (from IgM to IgG_1 and IgG_2) after repeated exposure of the bovine immune system to Rc mutant J5 *E. coli* bacterin. Theoretically, strong IgG antibody responses result when $CD5^-$ B cells are continuously exposed to TD antigens in the context of major histocompatibility complex (MHC) class II antigens on antigen presenting cells (Haas and Estes, 2000; Janeway et al., 2001). Specific interaction between the antigen-presenting cells (APCs) and $CD4^+$ T-

helper cells (T_H2) via CD40:CD40L ligation causes the T_H2 cells to secrete potent stimulatory cytokines (IL-4, IL-5, and IL-6) that promote isotype switching in $CD5^-$ B cells from IgM to IgG_1 (Haas and Estes, 2000; Janeway et al., 2001; McGuire et al., 1987). This humoral response may have occurred in our J5 hyperimmunized steers because we observed significant serum IgG_1 antibody responses after the 5^{th} immunization, even as the IgM response continued to increase. In addition, CD40:CD40L ligation must have occurred in some APC and T_H1 $CD4^+$ cells, with subsequent release of proinflammatory IFN-γ from the T cells, because there was also isotype switching to IgG_2 after the 5^{th} exposure to J5 bacterin. IFN-γ has been shown to be the major cytokine for IgM to IgG_2 switching in bovine $CD5^-$ B cells (Estes, 1996).

However, peak anti-J5 *E. coli* IgG_2 antibody responses were not achieved until the 11^{th} exposure to J5 bacterin, at which time the IgG_1 response had plateaued. Interestingly, the IgM response continued to increase beyond immunization number 11. While it is unknown what components of the J5 bacterin are recognized best or most quickly by responsive B and T cells, the early and continued increase in the serum IgM response may suggest that the TI-LPS antigens may have rapidly stimulated $CD5^+$ B cells production of IgM. In contrast, bacterial proteins (TD antigens) may have required more exposures and time to be properly processed and presented in MHC II molecules of APC for T_H cell-induced production of IgG_1 and IgG_2 by $CD5^-$ B cells.

If the scenario just described was true, we would have expected to observe significant cross-reactivity of the serum IgM antibodies with LPS and (or) Lipid A during ELISA. However, this was not the case. In fact, antibodies of all 3 isotypes reacted weakly only with LPS or Lipid A, and only after 12 J5 immunizations. Instead, the

antibodies contained in the test sera demonstrated increasing cross-reactivity with whole (killed) Gram-negative bacteria (but not with *S. aureus*). We also observed considerable variation in binding preference of the three antibody isotypes across bacteria. For example, IgG_1 and IgG_2 antibodies recognized *E. coli* 487 better than IgM antibodies, which preferentially cross-reacted with *Serratia spp.* However, there were some pre-existing antibodies against Gram-negative bacteria, which was previously reported (Gonzalez et al., 1989). This might be explained through the natural exposure of the animal to Gram-negative bacteria in its environments, and especially in the digestive tract. Also, in an agreement with a previous study (Tyler et al., 1991), the polyclonal antibodies in hyperimmune serum did not recognize a Gram-positive isolate, *S. aureus*.

There is controversy over how LPS influences the humoral immune response. Most J5 vaccine studies have focused attention on anti-LPS and anti-Lipid A IgG antibodies in immune serum suggesting that the levels of these are relatively high following immunization and thus that these antibodies are responsible for cross-protective against heterologous Gram-negative pathogens (Aydintug et al., 2001; Baumgartner et al., 1987; Pollack et al., 1989; Tyler et al., 1991; 1992). Our IgM, IgG_1 and IgG_2 cross-reactive antibody data do not support this notion because we found little cross-reactivity of antibodies, even in hyperimmunized serum, with LPS or Lipid A. Hellman and colleagues (1997) suggested that solid-phase assays, such as the ELISA, may not be the best approach to study anti-LPS antibody. However, our results on antibody cross-reactivity with LPS is in agreement with results of previous ELISA studies that IgG elicited from *E. coli* J5 bacterin binds only weakly to LPS from

heterologous, smooth, Gram-negative bacteria in solid-phase assays (Siber et al., 1985; Warren et al., 1987).

5. Conclusion

Mean anti-J5 *E. coli* IgG_1 and IgG_2 serum antibody responses elicited by three J5 immunizations were not impressive. In contrast, J5 hyperimmunization (five or more vaccinations) elicited strong and sustained serum IgG_1 and IgG_2 antibody responses. In this steer model of hyperimmunization, at least 5 injections of the manufacturer's recommended dose of J5 bacterin were required to successfully elevate anti-J5 *E. coli* IgG_1 and IgG_2 antibody levels above background (pre-immunization) levels and to initiate IgM to IgG_1 or IgG_2 isotype switching. Even so, it took 8 to 10 immunizations to elicit maximum anti-J5 *E. coli* IgG_1 and IgG_2 antibody responses, the latter which could be sustained during the subsequent 11^{th} and 12^{th} immunization periods. Anti-J5 *E. coli* IgM antibody responses rose sharply from background after only 2 immunizations and, quite unexpectedly, continued to increase over the remaining 10 immunization periods. Importantly, the levels of cross-reactivity for heterologous Gram-negative bacteria also increased for each antibody isotype with increasing immunizations, but responses to LPS and Lipid A were always low.

Therefore, results of our study suggest that repeated exposure of the bovine immune system to Rc mutant J5 *E. coli* bacterin results in antibodies of all isotypes that preferentially recognize non-LPS antigens shared by a diverse group of Gram-negative bacteria. The slow, but continuous increase in cross-reactive serum IgG_2 antibodies with increasing doses of administered J5 bacterin would suggest that J5 vaccine use protocols

be modified to obtain this response in target animals because IgG_2 is the main opsonic and cytophilic isotype for bovine neutrophils recruited to sites of Gram-negative infections.

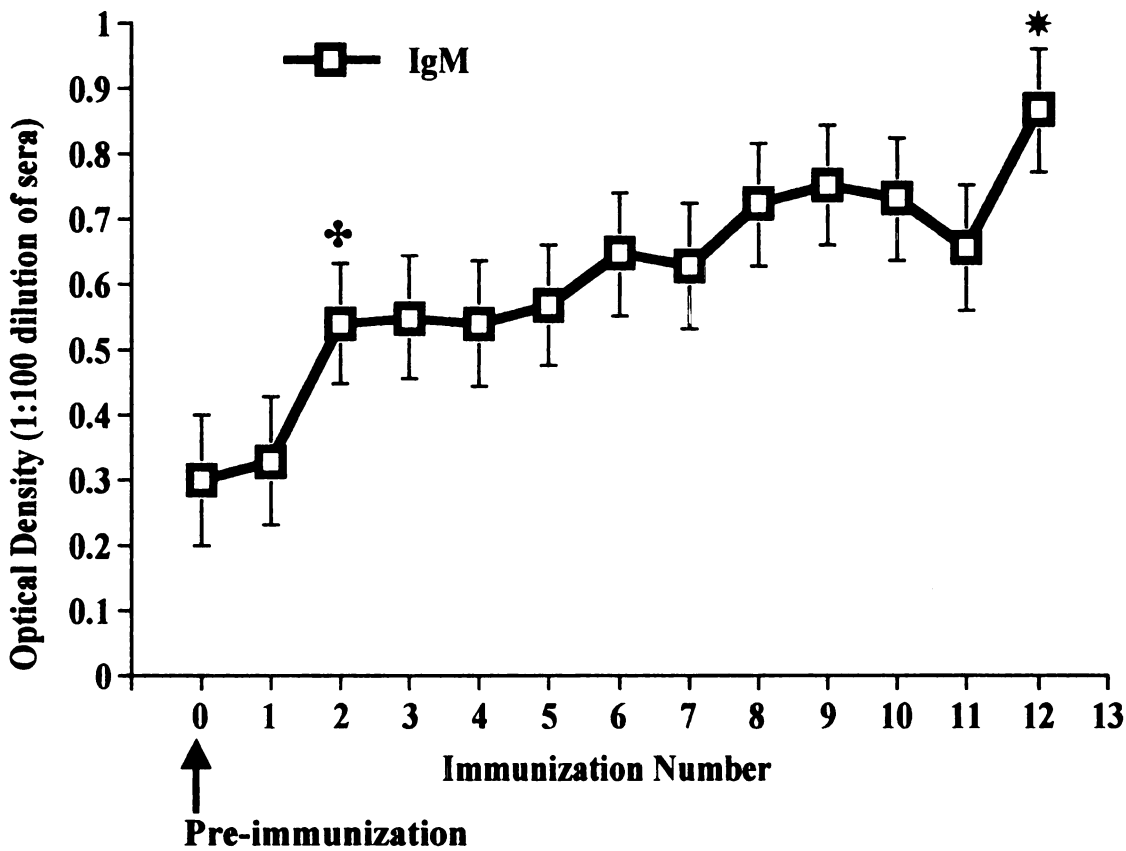


Figure 10 Mean serum anti-J5 *E. coli* IgM antibody response profile of five Holstein steers immunized 12 times with a J5 bacterin. Each data point is an immunization number LSMean from sera collected either on days 4, 6, and 11 post immunization (first six immunizations) or on days 4, 6, 11, and 13 post immunization (last six immunizations), expressed as optical density (± SEM) when sera were diluted 1:100. Immunization number ($P < 0.0001$) and day within immunization number ($P = 0.018$) significantly influenced the mean anti-J5 *E. coli* IgM antibody response. [* Indicates the point at which mean optical density was significantly higher than the pre-immunization mean ($P \leq 0.05$). ✱ Indicates the mean optical density was significantly different than that at the 3rd immunization ($P \leq 0.03$)].

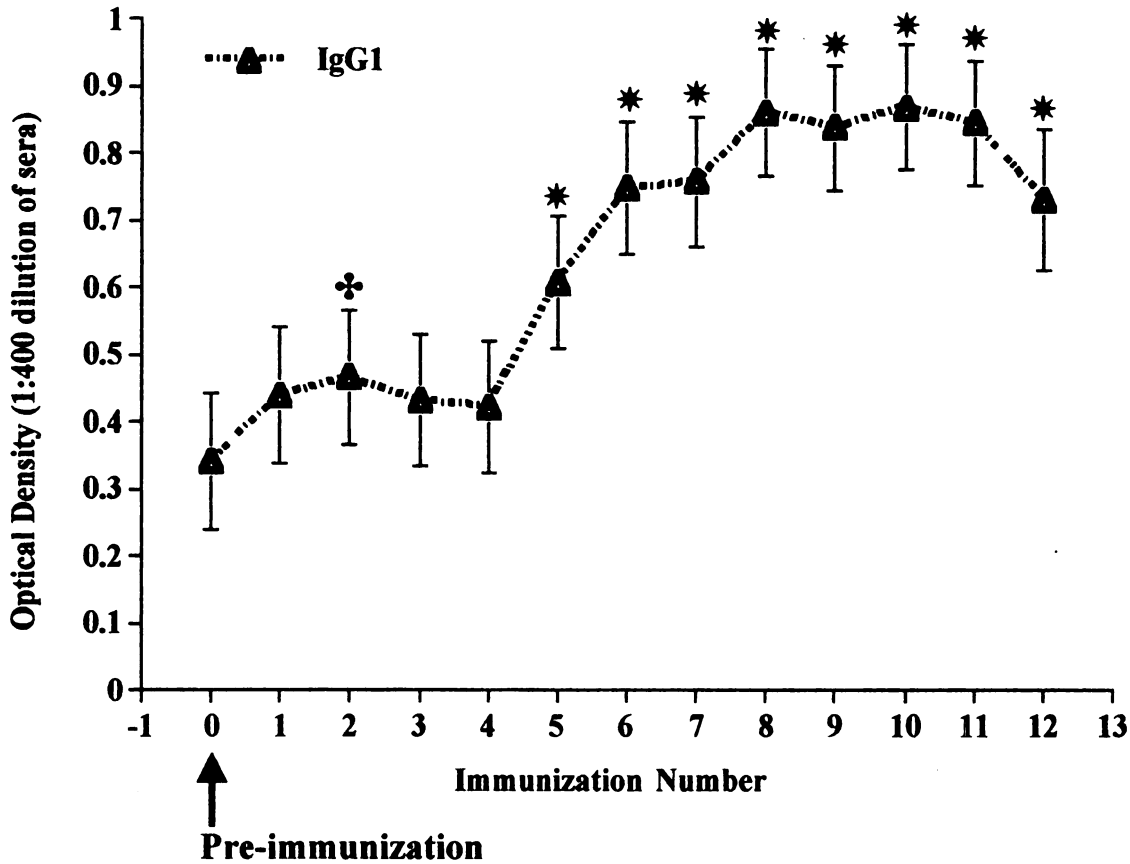


Figure 11 Mean serum anti-J5 *E. coli* IgG_1 antibody response profile of five Holstein steers immunized 12 times with a J5 bacterin. Each data point is an immunization number LSMean from sera collected either on days 4, 6, and 11 post immunization (first six immunizations) or on days 4, 6, 11, and 13 post immunization (last six immunizations), expressed as optical density (± SEM) when sera were diluted 1:400. Immunization number ($P < 0.0001$) and day within immunization number ($P = 0.011$) significantly influenced the mean anti-J5 *E. coli* IgG_1 antibody response. [* Indicates the point at which mean optical density was significantly higher than the pre-immunization mean ($P = 0.004$). ✱ Indicates mean optical densities that were significantly higher than optical density at the 3rd immunization ($P \leq 0.02$)].

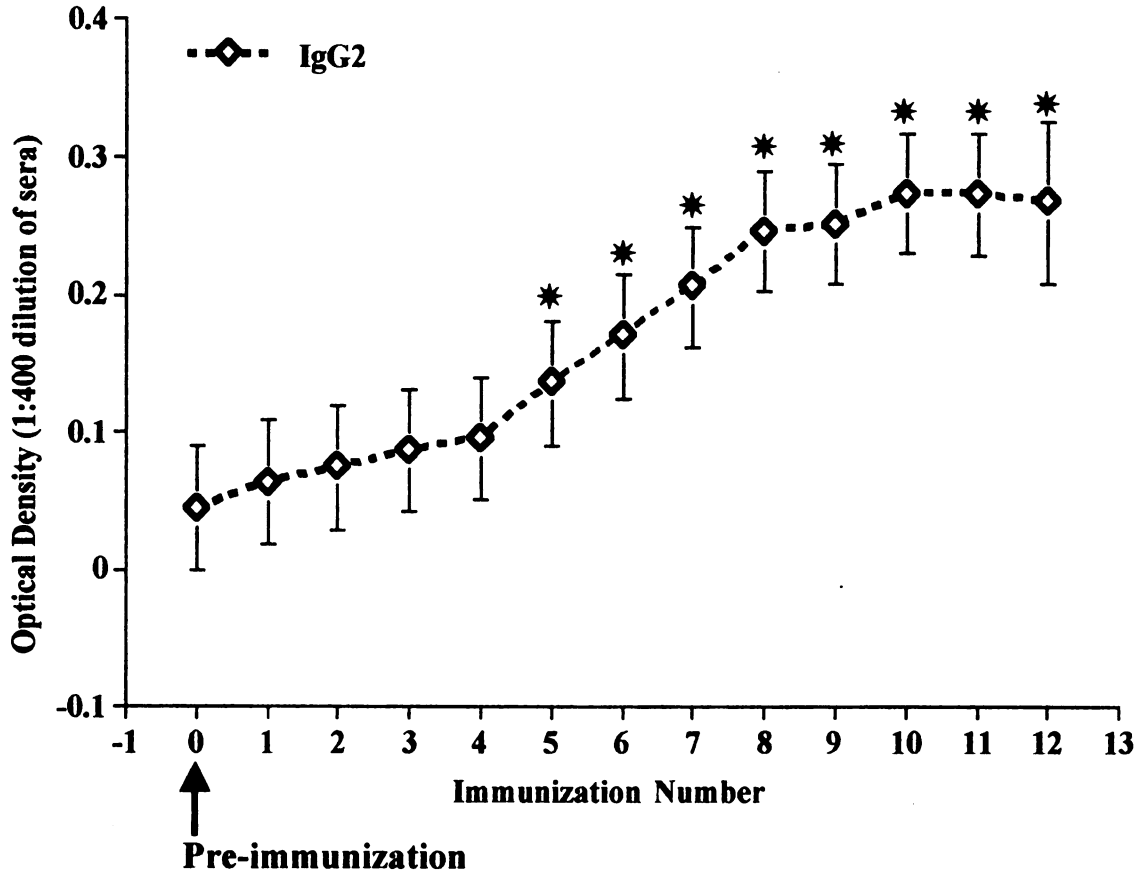


Figure 12 Mean serum anti-J5 *E. coli* IgG_2 antibody response profile of five Holstein steers immunized 12 times with a J5 bacterin. Each data point is an immunization number LSMean from sera collected either on days 4, 6, and 11 post immunization (first six immunizations) or on days 4, 6, 11, and 13 post immunization (last six immunizations), expressed as optical density (± SEM) when sera were diluted 1:400. Immunization number ($P < 0.0001$) and day within immunization number ($P < 0.0001$) significantly influenced the mean anti-J5 *E. coli* IgG_2 antibody response. [✱ Indicates mean optical densities that were significantly higher than the optical density at 3rd immunization ($P \leq 0.001$)].

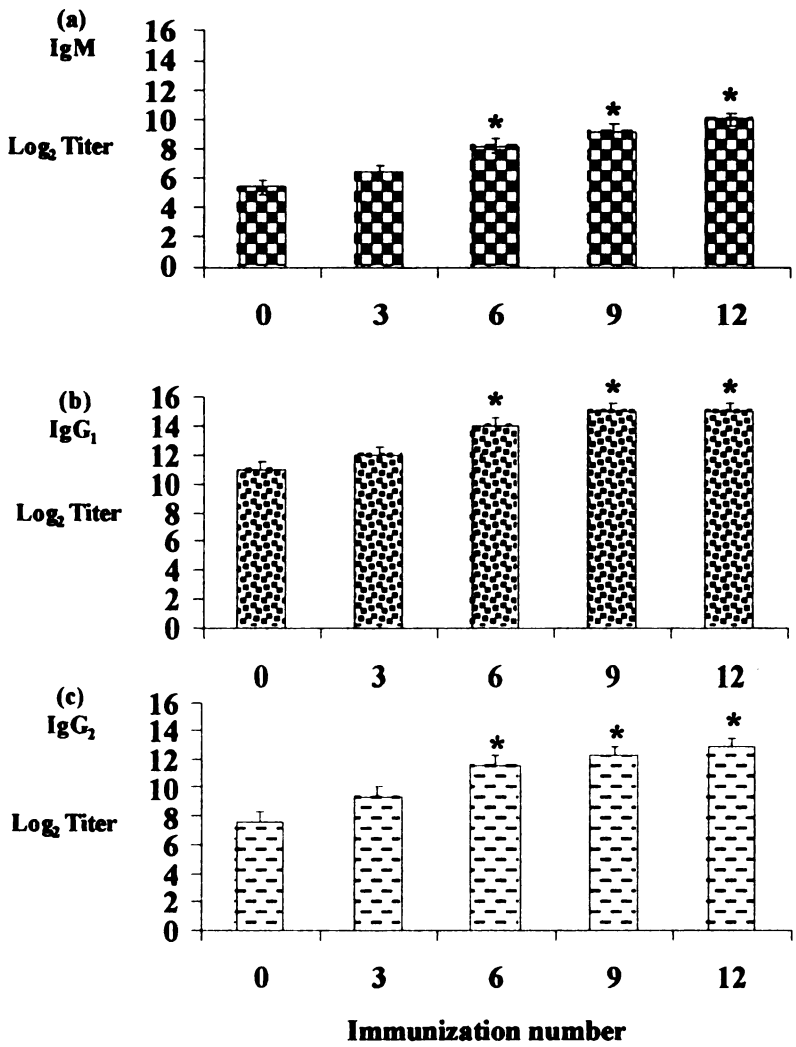


Figure 13 Mean (± SEM) isotype-specific anti-J5 *E. coli* antibody titers of selected hyperimmune serum samples from five Holstein steers. Titers are expressed as $\log_2$ of endpoint titer. (a) IgM; (b) IgG_1; (c) IgG_2. * Mean titer is different from the pre-immunization titer at $P < 0.005$.

Figure 14 Mean (± SEM) cross-reactivity of anti-J5 *E. coli* IgM antibodies contained in selected sera (at 1:50 dilution) from pre-, 3rd, 6th, 9th, and 12th immunizations to heterologous Gram-negative bacterial antigens, LPS and Lipid A. Data were presented as OD. Each panel represents anti-J5 IgM antibody against test Gram-negative bacterial antigens compared to J5 *E. coli* as a positive control and *S. aureus* as a negative control. Test Gram-negative bacteria include: panel **(a)** *S. typhimurium, Serratia spp.*, and *S. newport*; panel **(b)** *E. coli* 487, *Pseudomonas spp.*, and *Klebsiella spp.;* and panel **(c)** LPS (from *E. coli* 111:B4) and Lipid A (from *S. minnesota*, Re mutant). Mean OD significantly higher following immunizations; **a** ($P < 0.01$), **b** ($P < 0.05$). Mean OD significantly higher than LPS, Lipid A and *S. aureus*, * ($P < 0.001$).

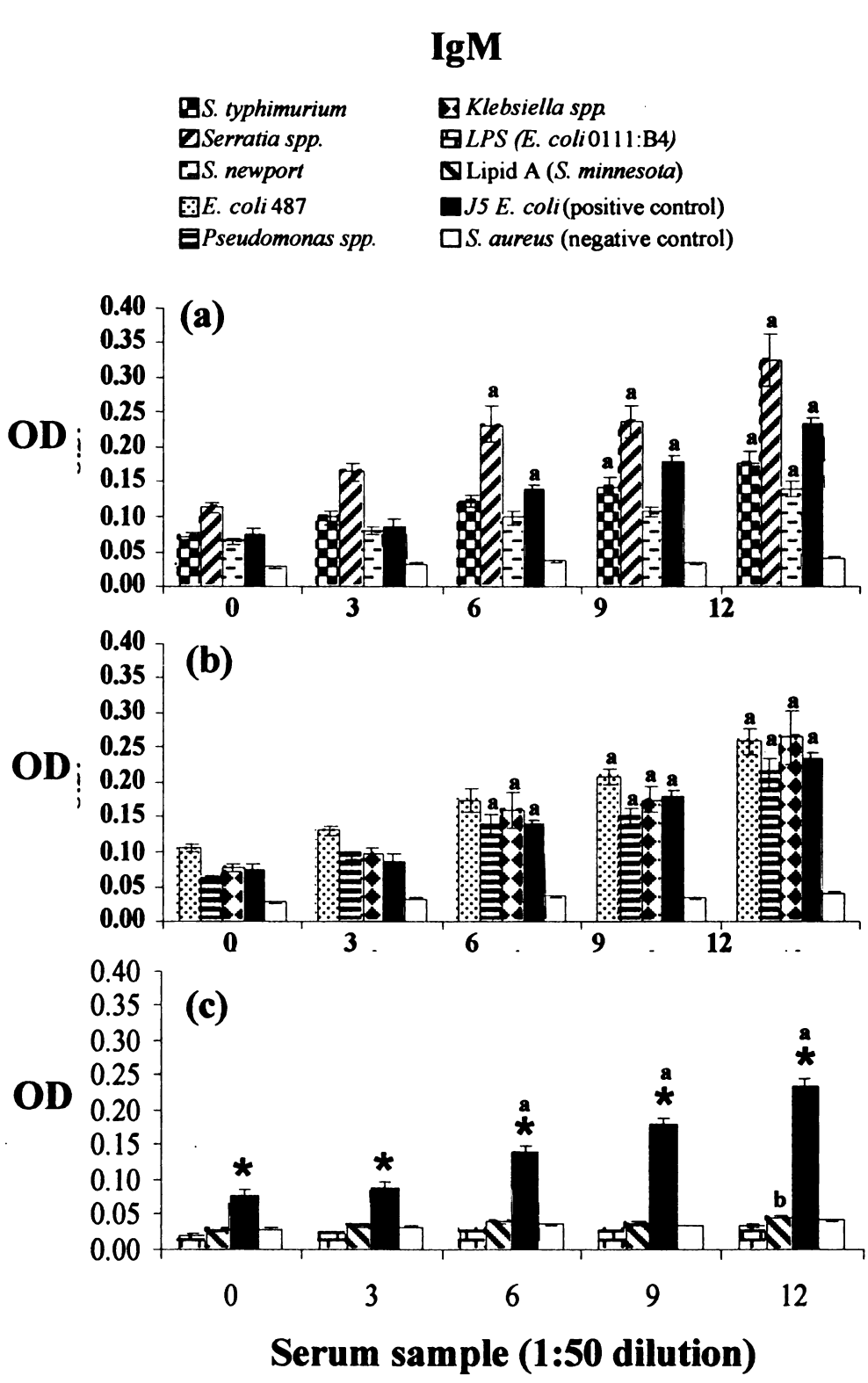

IgM
S. typhimurium
Serratia spp.
S. newport
E. coli 487
Pseudomonas spp.
Klebsiella spp.
LPS (E. coli 0111:B4)
Lipid A (S. minnesota)
J5 E. coli (positive control)
S. aureus (negative control)
(a)
(b)
(c)
OD
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
3
6
9
12
Serum sample (1:50 dilution)

Figure 15 Mean (± SEM) cross-reactivity of anti-J5 *E. coli* IgG_1 antibodies contained in selected sera (at 1:200 dilution) from pre-, 3rd, 6th, 9th, and 12th immunizations to heterologous Gram-negative bacterial antigens, LPS and Lipid A. Data were presented as OD. Each panel represents anti-J5 IgG_1 antibody against test Gram-negative bacterial antigens compared to J5 *E. coli* as a positive control and *S. aureus* as a negative control. Test Gram-negative bacteria include: panel **(a)** *S. typhimurium*, *Serratia spp.*, and *S. newport*; panel **(b)** *E. coli* 487, *Pseudomonas spp.*, and *Klebsiella spp.;* and panel **(c)** LPS (from *E. coli* 111:B4) and Lipid A (from *S. minnesota*, Re mutant). Mean OD significantly higher following immunizations; **a** ($P < 0.01$), **b** ($P < 0.05$). Mean OD significantly higher than LPS, Lipid A and *S. aureus*, * ($P < 0.001$).

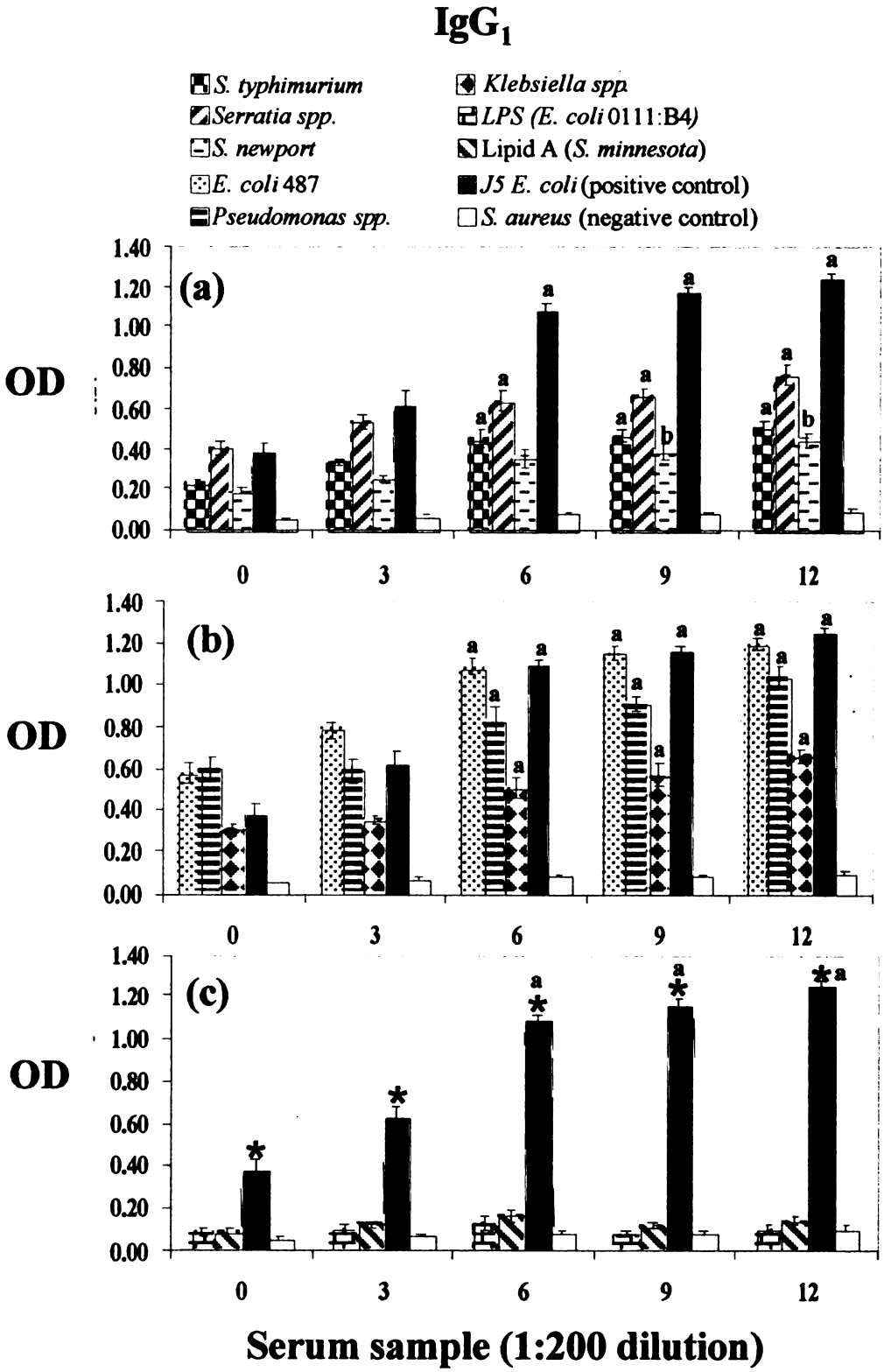
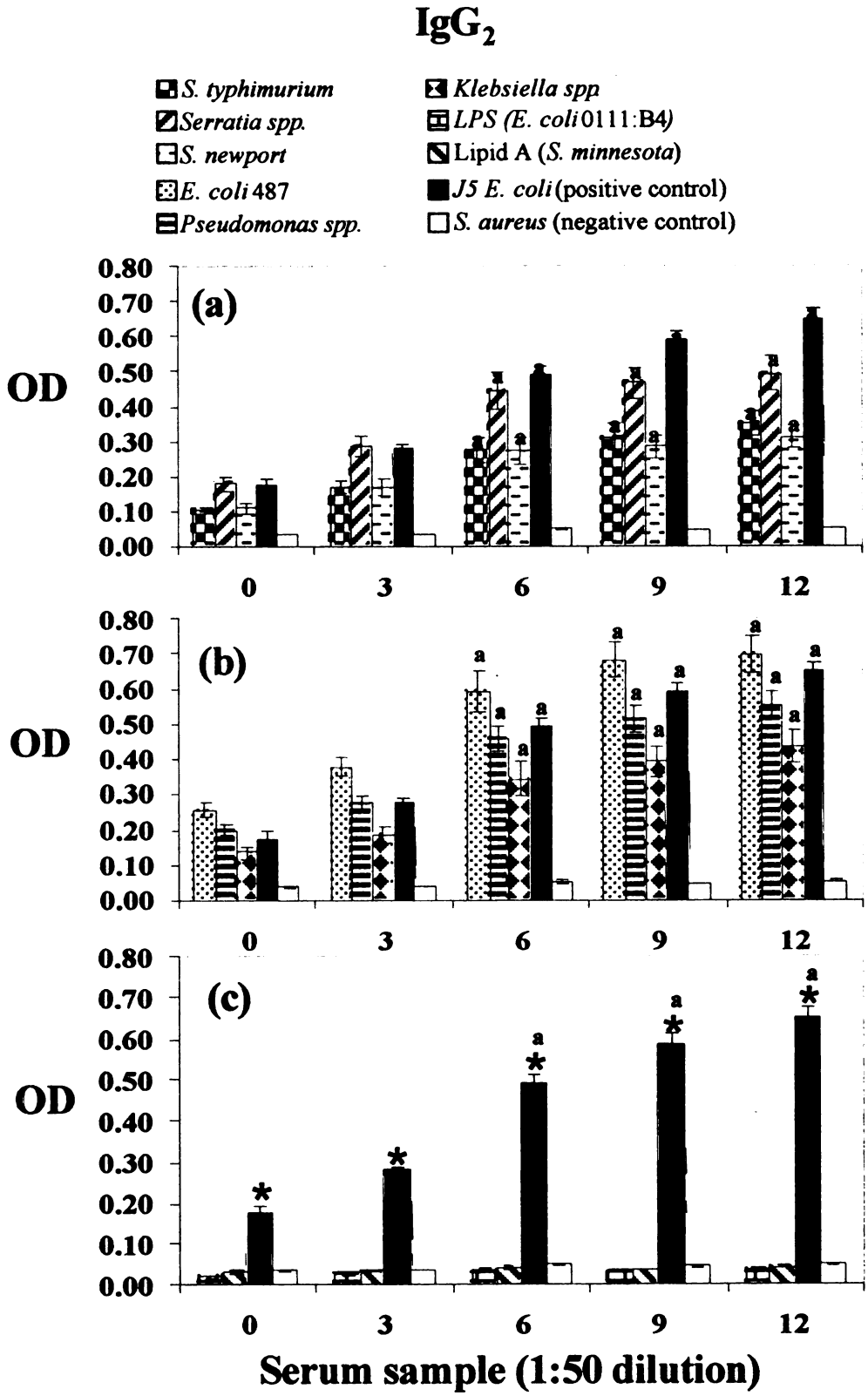

IgG1
S. typhimurium
Serratia spp.
S. newport
E. coli 487
Pseudomonas spp.
Klebsiella spp.
LPS (E. coli 0111:B4)
Lipid A (S. minnesota)
J5 E. coli (positive control)
S. aureus (negative control)
(a)
(b)
(c)
OD
OD
OD
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
3
6
9
12
Serum sample (1:200 dilution)

Figure 16 Mean (± SEM) cross-reactivity of anti-J5 *E. coli* IgG_2 antibodies contained in selected sera (at 1:50 dilution) from pre-, 3rd, 6th, 9th, and 12th immunizations to heterologous Gram-negative bacterial antigens, LPS and Lipid A. Data were presented as OD. Each panel represents anti-J5 IgG_2 antibody against test Gram-negative bacterial antigens compared to J5 *E. coli* as a positive control and *S. aureus* as a negative control. Test Gram-negative bacteria include: panel **(a)** *S. typhimurium*, *Serratia spp.*, and *S. newport*; panel **(b)** *E. coli* 487, *Pseudomonas spp.*, and *Klebsiella spp.;* and panel **(c)** LPS (from *E. coli* 111:B4) and Lipid A (from *S. minnesota*, Re mutant). Mean OD significantly higher following immunizations; **a** ($P < 0.01$), **b** ($P < 0.05$). Mean OD significantly higher than LPS, Lipid A and *S. aureus*, * ($P < 0.001$).


IgG2
S. typhimurium
Serratia spp.
S. newport
E. coli 487
Pseudomonas spp.
Klebsiella spp
LPS (E. coli 0111:B4)
Lipid A (S. minnesota)
J5 E. coli (positive control)
S. aureus (negative control)
(a)
(b)
(c)
OD
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
3
6
9
12
Serum sample (1:50 dilution)

CHAPTER 3

GRAM-NEGATIVE BACTERIAL ANTIGEN RECOGNITION BY ISOTYPE SPECIFIC ANTI-J5 *E. coli* ANTIBODY

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Abstract

We conducted this study to determine the cross-reactivity and diversity of bacterial antigen recognition by serum antibodies following multiple-dose administration of J5 bacterin in steers, and determine if enriched anti-J5 *E. coli* IgG_1 and IgG_2 antibodies opsonized *E. coli* for improved neutrophil phagocytosis. Lysates were prepared from heterologous Gram-negative bacteria by sonication and used to determine isotype specific antibody recognition of various proteins and LPS. Anti-J5 *E. coli* IgG_1 and IgG_2 bound distinctively to Gram-negative bacterial protein antigens of a few key sizes, the most predominant groups being of molecular masses 8-10.5 and 34-39 kDa. IgG_2 recognized these proteins more strongly than IgG_1, whereas there was little recognition of them by IgM antibodies. Therefore, the 8-10.5 kDa antigens may be the targets of T and B cell recognition during immunization of cattle with J5 vaccines. We also observed modest interaction between IgG_1 in hyperimmune serum and purified LPS. However, there was little or no cross-reactivity of our antisera or enriched IgG_1 and IgG_2 antibodies to Lipid A or *S. aureus*. Also only enriched IgG_2 antibodies enhanced phagocytosis of GFP-

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transformed J5 *E. coli* by bovine neutrophils compared to no antibody controls. Thus, multiple J5 immunizations appear to enhance IgG_2 antibodies that increasingly recognize small proteins in heterologous Gram-negative bacteria and enhance neutrophil phagocytosis of *E. coli* in vitro.

Keywords: antigens, humoral immunity, vaccine, phagocytosis

1. Introduction

Cell wall components of Gram-negative bacteria are considered to play an important role as virulence factors, which subsequently cause pathophysiological changes in the host (Kremer et al., 1990; Tyler et al., 1990b). Most researchers have been focusing on two cell wall components, including LPS and Lipid A regarding specific host immune response following immunization with J5 *E. coli* bacterins. Studies reported relatively high antibody responses (both monoclonal and polyclonal antibody) against LPS and Lipid A antigens in the vaccine, which is thought to be the key response that provides protection against a variety of Gram-negative pathogens in immunized animals (Aydintug et al., 2001; Baumgartner et al., 1987; Pollack et al., 1989; Tyler et al., 1991; 1992). However, other evidence suggests that antisera containing IgG antibodies bind only weakly to LPS from multiple heterologous Gram-negative bacteria (Siber et al., 1985; Warren et al., 1987). In addition, our recent work has shown that serum from J5 hyperimmunized steers contains only low or undetectable levels of anti-LPS and anti-Lipid A IgM, IgG_1 and IgG_2 antibodies (Chapter 2).

It is known that LPS and Lipid A are not the only core antigens contained in the cell walls of Gram-negative bacteria, but that other components including lipoprotein, peptidoglycan, and outer membrane proteins are also shared across bacterial isolates (**OMPs**). Currently, these bacterial antigens are believed to play a role in the pathogenesis of Gram-negative sepsis (Hellman et al., 1997; 2000a). Recent studies in humans and mice reported conserved gram-negative bacterial OMPs including outer membrane protein A (OmpA), and peptidoglycan-associated lipoprotein (PAL) (Hellman et al., 1997; 2000a). These OMPs are released into human blood circulation during sepsis as a complex with LPS, which is most likely to occur during rapid bacteria growth and may play a role in the pathogenesis of Gram-negative sepsis (Hellman et al., 1997; 2000a). OMPs are also recognized by anti-J5 IgG polyclonal antibody elicited by heat-killed *E. coli* J5 (Hellman et al., 1997; 2000a; 2000b; 2001). OmpA (i.e., *Klebsiella pneumoniae*) is also used as a carrier protein for some polysaccharides (**PS**) to increase the immunogenicity of PS and enhance protective antibodies against lethal bacterial infection (Libon et al., 2002; Rauly et a., 1999). Thus, use of OMPs in vaccines might contribute toward improved efficacy for host immunity against Gram-negative bacteria. If true, it may be possible to improve J5 bacterins currently used to actively immunize dairy cows against mastitis-causing coliforms.

However, studies in cattle regarding isotype-specific anti-J5 antibodies and their recognition of specific Gram-negative antigens have not been conducted. For our study, we have used serum antibodies from J5 hyperimmunized steers to examine which protein and polysaccharide antigens from Gram-negative bacteria are recognized. Based on work of Chapter 2, our hypothesis was that repeated exposure of the bovine immune system to

Rc mutant J5 *E. coli* bacterin would result in highly mature IgG_2 antibodies that cross-react with a variety of protein antigens on heterologous Gram-negative bacteria, and that these antibodies would enhance neutrophil recognition and phagocytosis of Gram-negative bacteria. Therefore, the current study was conducted: 1) to determine the cross-reactivity and diversity of bacterial antigen recognition by maturing isotype specific antibodies in J5 hyperimmune serum; and 2) to determine if the IgG_2 fraction of hyperimmune serum opsonizes *E. coli* better than the IgG_1 fraction for improved neutrophil phagocytosis of coliform bacteria.

2. Materials and Methods

2.1 Test and control samples

Test samples included various pre-immune and immune test sera, IgG_1 and IgG_2 antibodies affinity purified from immune test sera, and fetal bovine serum as a negative control (GibcoBRL, Life-technologies). Previously described in Chapter 2, test sera were harvested from blood samples collected from five healthy Holstein steers that were subcutaneously administered multiple doses of J5 bacterin over time (Pharmacia-Animal Health). The sera were pooled by immunization number (pre-immunization, 3rd, 6th, and 9th immunization) and stored at -20 °C until use.

2.2 Protein A purification of serum IgG_1 and IgG_2 antibodies

2.2.1 Ammonium sulfate precipitation

Hyperimmune sera collected from the five Holstein steers vaccinated 9 times using J5 *E. coli* bacterin were prepared for Protein A column chromatography by a modification of the method of Harlow and Lane (1999) (**Figure 17**). Briefly, one liter of

Figure 17 Protein A chromatography isolation of IgG_1 and IgG_2 from pooled serum of J5 *E. coli* hyperimmunized steers. An Affi-Prep Protein A column was used in conjunction with a pH gradient elutant to produce two eluted fractions of IgG_1 and IgG_2 (see Figure 18).

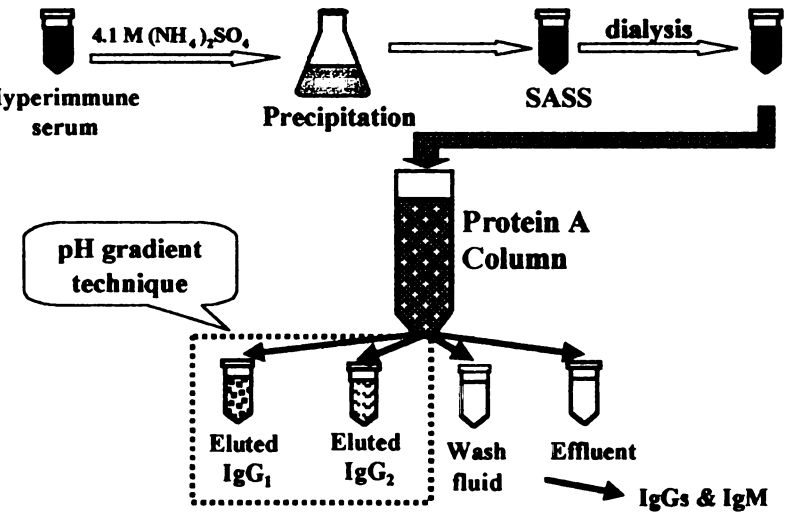
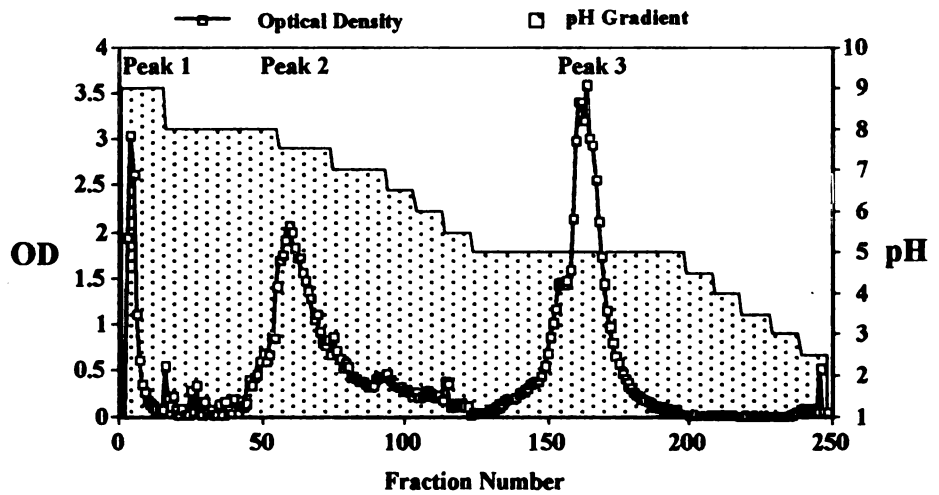


Figure 18 Elution pattern of affinity purified immunoglobulins from an Affi-Prep Protein A column using a pH gradient from 8.0 to 2.0. One-milliliter fractions were collected, neutralized immediately by adding 1 M Tris pH 9.0, and protein concentrations (μg/ml) determined a optical density at $\lambda = 280$ nm. Peak 1 was the effluent. Peak 2 eluted between pH 8.0 and 7.5 and peak 3 at pH 5.0, and were shown by subsequent Western blot analysis (see Figure 25) to be IgG_1 and IgG2, respectively.



the pooled hyperimmune serum was centrifuged at 3210 × g (GS-6R BECKMAN Centrifuge, Beckman Instruments, Inc., Schaumburg, IL) at 4 °C for 15 minutes to remove debris. Protein concentration of the supernatant was determined using the Warburg-Christian program by spectrophotometry (Model DU® 650, Beckman Coulter, Inc., Fullerton, CA). Seven hundred milliliters of 4.1 M saturated ammonium sulfate, pH 7.0 was gradually added to the serum and the proteins were allowed to precipitate overnight at 4°C. The suspension was then gently stirred for 5 minutes and centrifuged at 3210 × g at 4 °C for 25 minutes. After discarding the supernatant, the pellet was reconstituted with sterile Milli-Q water to a final volume of 30 to 50% of the starting serum, and termed saturated-ammonium sulfate serum (**SASS**). Subsequently, the SASS was dialyzed for 6 changes every 6 to 8 hours against pre-cooled 0.9 % NaCl at 4 °C using 50,000 molecular weight cut-off (MWCO) dialysis membranes (Spectra/Pro® 6 Membrane, Spectrum Laboratories, Inc.).

2.2.2 Dialysis

Two 1-liter beakers containing 500 ml of Milli-Q H_2O (~18 mΩ·Cm, Milli-Q water system) and 500 ml of 0.9% NaCl were prepared and pre-cooled at 4 °C. Dialysis membrane (Molecular weight cut off (MWCO) = 50,000, Spectra/Pro® 6 Membrane, Spectrum Laboratories, Inc.) was cut to approximately 45.8 to 50.8 cm long for 6 - 8 pieces or as needed, and placed into the beaker containing 500 ml of Milli-Q H_2O at 4 °C for 15 minutes. The membranes were transferred to another beaker containing 0.9% NaCl, allowed to soak for another 15 minutes, and rinsed with 20 ml of Milli-Q H_2O. One end of each dialysis membrane was double-clamped with dialysis clamps. The membrane was filled by the sample (i.e., SASS) approximately half full and secured by a

dialysis clamps at the other end. The membranes containing samples were placed into a 24-liter carboy containing 0.9% NaCl, which had been pre-cooled at 4 °C and stirred with a magnetic stir bar. SASS was dialyzed for 6 changes every 6 to 8 hours and transferred to a graduated cylinder to record volume and determine the amount of protein at 280 nm using Nucleic Acid Module in the Spectrophotometer (Model DU® 650, Beckman Coulter, Inc., Fullerton, CA). The samples were then stored in 50-ml sterile conical tubes at -20 °C for further analysis.

2.2.3 Protein A affinity chromatography

Affinity chromatography was performed at room temperature by using a 25-ml-Affi-Prep Protein A support/Econo column attached to an Econo-Column Flow Adapter and equipped with a Biologic LP system Model 2128 Fraction collector (BioRad Laboratories, Hercules, CA). Prior to loading sample, the column was equilibrated with 200 ml of Protein A MAPS® II binding buffer, pH 9.0 (BioRad Laboratories, Hercules, CA) and then 150-170 mg of total protein from dialyzed SASS (in 2.5 ml) containing 0.725 g of Protein A MAPS® II binding buffer solid [calculated by Equation (**a**), Bio-Rad, Hercules, CA] was loading in the column. The pH of SASS was 9.0 and the ionic strength of the SASS approaches that of the MAPS Binding Buffer to ensure optimal immunoglobulin binding.

$$\frac{\text{29 gm of Protein Maps II solid*}}{\text{100 ml of SASS}} \quad x \quad \text{volume of SASS} \quad \ldots\ldots\ldots\ldots\text{(a)}$$

* This number is based upon using SASS dialyzed into 0.9% NaCl.

Non-specifically bound components were removed by pumping 600-ml of Protein A MAPS® II binding buffer through the column until the absorbance (OD) of the effluent was less than 0.001. After washing the column, a pH gradient of citrate-phosphate buffer system (0.1 M Citric acid and 0.2 M Na_2HPO_4) from pH 8.0 through pH 5.5 was used to elute the specifically bound IgG_1 and IgG_2 antibodies (modified from Schmerr et al., 1985, **Figure 18**). A pH gradient was generated by applying sequentially; 40 ml of pH 8.0, 20 ml each of pH 7.5 and 7.0, 10 ml each of pH 6.5, 6.0, and 5.5, and 75 ml of pH 5.5 citrate-phosphate buffer. The eluted samples were collected in aliquots of 1 ml each, neutralized immediately with 1 M Tris, pH 9.0, and absorbance determined at 280 nm. The highest OD fractions were pooled, concentrated, desalted, and stored at -20 °C for further analysis.

2.2.4 Quality test for purified antibodies

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, modified from Laemmli, 1970) and Western blot analyses (modified from Lee and Shewen, 1996) were used to test the quality of purified IgG_1 and IgG_2 antibodies. A molecular weight marker (Precision Plus All Blue; 10, 15, 20, 25, 37, 50, 75, 100, 150 and 250 kDa; BioRad) was loaded on every gel as a size standard. One microgram each of pooled fraction (2^{nd} or 3^{rd} peak, **Figure 18**) was prepared by adding loading buffer at 1:2 ratio [95% (v/v) of Laemnli sample buffer (6.25 mM Tris-HCl, pH 6.8, 25.0% glycerol, 2.0% SDS and 0.01% Bromophenol Blue, BioRad) containing 5% (v/v) β-mercaptoethanol, (Sigma Chemical Co., St Louis, MO)]. The mixed sample was heat-denatured at 95 °C for 4 min and immediately placed on ice prior to sample loading on pre-casted mini gels (12.0% Tris-HCl, 10-well, 30 µl, 0.57-mm Ready Gel, BioRad,

Hercules, CA). Electrophoresis was conducted at constant 200 volts for 35 min using Mini PROTEAN II cell electrophoresis units, Power/Pac 300 power supplies (both from BioRad), and 10% Tris/Glycine/SDS buffer in milli-Q water, pH 8.3 (Bio-Rad) as electrode buffer.

After electrophoresis was complete, the gel was transferred electrophoretically onto a nitrocellulose membrane (0.45 μm; Pierce Chemical Co., Rockford, IL) at a constant voltage (100 volts, PowerMac 300, Bio-Rad) for an hour using a Mini Transblot apparatus (Bio-Rad Laboratories, Hercules, CA) containing transfer buffer (192 mM Glycine and 25 mM Tris HCl (Bio-Rad Laboratories, Hercules, CA), 20.0% (v/v) Methanol). Non-specific binding sites on the membranes were then blocked with a SuperBlock® blocking buffer (Pierce, Rockford) for 1 hr at room temperature with 0.1% added Polyoxythelene Sorbitan Monolaurate (**Tween 20**). The blocked membranes were probed with the sheep anti-bovine HRPO-conjugated isotype-specific detection antibodies (IgG_1 and IgG_2, heavy chain specific, BETHYL Laboratories, Inc., Montgomery, TX) for 60 min at room temperature. The membranes were then washed 6 times and substrate [SuperSignal West Pico Chemiluminescent Substrate system (Pierce)] added for 5 min before being exposed to x-ray films (Kodak BioMax MS; Fisher Scientific) for 10 seconds. Films were developed (Futura 2000 E Automatic X-Ray film processor; Fischer Industries Inc., Geneva, IL).

2.3 Preparation of bacterial antigens

Lipopolysaccharide (*E. coli* serotype 0111:B4, Sigma-Aldrich Co., St. Louis, MO) and Lipid A (monophosphoryl, *S. minnesota* Re 595, Sigma-Aldrich Co., St. Louis, MO) were both dissolved by vigorous vortexing in physiological saline solution, and

0.05% Triethylamine (TEA) to reach a concentration of 1 mg/ml. Bacteria selected for lysates preparation included *Escherichia coli* McDonald 487, *Pseudomonas spp.*, *Serratia spp.*, *Klebsiella pneumoniae*, *Salmonella newport*, *S. typhimurium*, J5 *E. coli* and *Staphylococcus aureus*. J5 *E. coli* and *Staphylococcus aureus* represented positive and negative controls, respectively. *S. newport and S. typhimurium* were kindly donated by Animal Health Diagnostic Laboratory, East Lansing MI. All other bacteria, except the J5 *E. coli*, were isolated from milk of clinical mastitis cases. As shown in **Figure 19**, bacteria were grown on 5% sheep blood agar overnight at 37°C. Single colonies were inoculated in 15 ml of Trypticase soy broth (J.T. Baker, Phillipsburg, NJ) and incubated while shaking (225 rpm) for 18 hours at 37°C. Purity of culture was again determined by culture on 5% sheep blood agar overnight at 37°C (National Mastitis Council, 1987). The inoculums were centrifuged (8000 × g for 15 min at 4°C) to collect bacterial cell pellets, which were then resuspended in 3.0 ml of lysing buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol (**DTT**), and 5% glycerol. Using a probe sonicator (Sonifier), the bacterial cells were disrupted with multiple short bursts of 15 second pulses with 15 second rests for 1-2 min and kept on ice at all times (Harlow and Lane, 1999; Promega, 1996). Resulting crude lysates were cleared of cell debris by centrifugation (10,000 × g for 15 min at 4°C). Trichloro acetic acid (**TCA**) was added to reconstitute the pellet to a final concentration of 10% (w/v) and placed on ice for 5 min to precipitate proteins (Promega, 1996). The TCA-treated mixture was centrifuged (12,000 × g for 2 min at 4°C) to collected the protein pellets, which were washed twice (12,000 × g for 2 min at 4°C) and resuspended in ice cold 0.9% sterile NaCl. Protein concentrations were determined spectrophotometrically by the specific O.D. (λ = 260, 280 and 320 nm)

reading and using the Warburg-Christian Concentration Assay [Model DU® 650, BECKMAN COULTER, Inc., Fullerton, CA, **Equation (b)**], and stored at –20°C until use.

[O.D. (260.0 nm) x (-757.290)] + [O.D. (280.0 nm) x 1552.00] ..... **(b)**

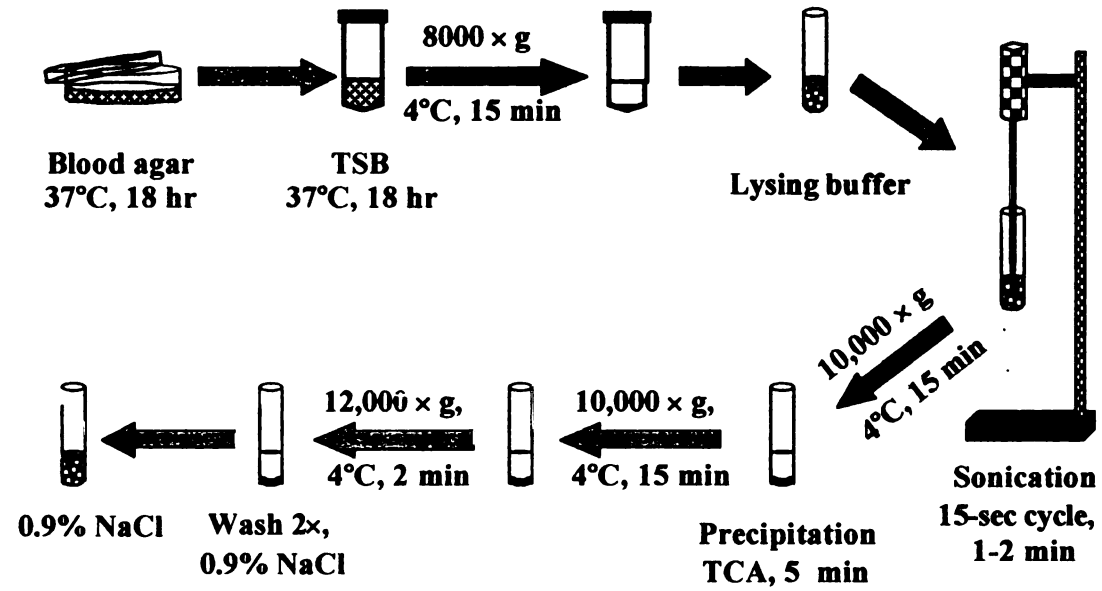


Figure 19 Scheme to illustrate the protocol used for extraction of bacterial proteins for SDS-PAGE. After the various bacterial species were grown on blood agar and propagated in TBS, the cells were pelleted by centrifugation, then lysed and sonicated, and the resulting fragments pelleted and proteins precipitated from them using TCA. Following two centrifugation steps to pellet the precipitated proteins, proteins were washed in normal saline twice and then suspended in the same for use in SDS-PAGE.

2.4 Assays for bacterial antigen recognition by anti-J5 antibodies

2.4.1 SDS-PAGE

SDS-PAGE was used to separate the prepared bacterial protein, LPS, and lipid A antigens by molecular weight. Molecular weight markers (Precision Plus All Blue; 10, 15, 20, 25, 37, 50, 75, 100, 150 and 250 kDa; BioRad) were loaded on every gel as size

standards. Test and control antigens (5 μg each) were prepared by adding loading buffer at a 1:2 ratio [95% (v/v) of Laemnli sample buffer (BioRad) containing 5% (v/v) β-mercaptoethanol, Sigma Chemical Co., St Louis, MO]. The mixed sample was heat-denatured at 95°C for 4 min and placed on ice prior to sample loading on pre-casted Tris-HCl polyacrylamide gels (12.0%, 160×160×0.75 cm). Electrophoresis was conducted at a constant 15 and 30 mA on stacking gel and separating gel, respectively, for 3_ hr at 4°C (PROTEAN II xi cell electrophoresis units, Gibco BRL; (model 4001P programmable power supply, Life Technologies™), and 1M Tris/Glycine/SDS buffer, pH 8.3 as an electrode buffer. Two gels were prepared per sets of samples. One gel was reserved for silver staining (Silver Stain Plus, BioRad) to visualize location and variety of antigens in the samples. The other gel was used for antigen transfer on to nitrocellulose membrane.

2.4.2 Silver staining procedure

After gel electrophoresis, the gel was submerged in 100 ml of Fixative Enhancer Solution [50% methanol (J.T. Baker), 10% acetic acid, and 10% Fixative Enhancer Concentrate (Bio-Rad, Hercules, CA)], and gently agitated (an orbital shaker, Bellco Glass, Inc., Vineland, NJ) for 20 min at room temperature. The Fixative Enhancer Solution was decanted from the staining vessel, which was replaced by 200 ml of 18-megohm-cm resistivity (Milli-Q) water twice for 10 min each with gentle agitation. About 5 min before the staining step, 5 ml each of Silver Complex Solution (NH_4NO_3 and $AgNO_3$), Reduction Moderator Solution (tungstosilicic acid), and Image Development Reagent (formaldehyde) were orderly added and stirred in 35 ml of Milli-Q water, and followed by 50 ml of Development Accelerator Solution (Na_2CO_3)

immediately before use. The gel was stained with gentle agitation for approximately 5-15 minutes to reach desired staining intensity. The stained gel was then gently agitated in 5% acetic acid to stop the reaction for 15 min and rinsed using 200 ml of Milli-Q water for 5 minutes. The silver stained gel was photographed (Gel Documentation System/Fluoro-S image Analysis System; BioRad) for data presentation.

2.4.3 Western blot analyses

Three gels were used for antigen transfer (Trans-Blot Cell with plate electrode, BioRad) to nitrocellulose blotting membranes (0.45 μm; Pierce, Rockford) at 25 constant volts (PowerMac 300, BioRad) for 6 hr at 4°C, to visualize isotype-specific antibody recognition of antigens by Western blot analyses. The membranes were incubated in a blocking buffer 1 hr at room temperature, with 0.1% added Tween 20. Blocked membranes were probed with test sera (1 mg/ml) diluted 1:1000 in sterile milli-Q water for 60 min at room temperature, and washed six times [BupH Tris Buffer Saline (Pierce) containing 0.05% Tween 20]. The membranes were further probed with anti-bovine HRPO-conjugated isotype-specific detection antibodies (IgM, IgG_1, or IgG_2, heavy chain specific, BETHYL Laboratories, Inc., Montgomery, TX) for 60 min at room temperature. After six more washes, the substrate was added [Super Signal West Pico Chemiluminescent Substrate system (Pierce)] for 5 min before the membranes were exposed to x-ray films (Kodak BioMax MS; Fisher Scientific) for 2 min. Films were developed (Futura 2000 E Automatic X-Ray film processor; Fischer Industries Inc., Geneva, IL) for data presentation. This step was repeated using fetal bovine serum, J5 antisera from pre-, 3rd, and 9th immunizations, and Protein A-enriched IgG_1 and IgG_2 antibody fractions for antigen recognition comparisons.

2.5 Opsonization assay

2.5.1 Preparation of green fluorescent protein-transformed J5 E. coli

Green fluorescent protein (GFP)-transformed J5 *E. coli* was prepared using electroporation as modified from Ausubel et al. (1995). Briefly, J5 *E. coli* were grown on MacConkey agar at 37 °C overnight. Single colonies were picked and inoculated shaking (250 rpm) in 20-ml Luria-Bertani (**LB**) broth at 37 °C overnight. Two milliliters of the inoculum were added in 400-ml LB broth and incubated shaking at 37 °C until the $O.D._{\lambda = 600\ nm}$ was between 0.5 and 1.0. The bacterial pellet was recruited by centrifugation (3,500 × g, for 20 min) and then washed using 200-ml sterile water. The pellet was resuspended in 20 ml of 10% glycerol, centrifuged at 3500 rpm for 15 min, and the supernatant discarded. The remaining pellet was resuspended to a final volume of 2 ml using 10% glycerol, which was equivalent to approximately 1-3 × 10^{10} bacteria/ml. Four hundred microliters of the cell suspension was put into a 1.5-ml ice cold polypropylene tube, and 1 µl of pGFPuv plasmid DNA added (3.3 kb, Clontech Laboratories, Inc., PaoAlto, CA). The suspension was mixed well, and left on ice for 1 min. The suspension was transferred to a 0.2-cm electroporation cuvette, which was placed in a chilled safety chamber and securely sealed by the chamber slide.

The transformation was performed under condition set for 100 ohms and 500 µFD, which produced a 4-5 msec. constant pulse with a 12.5-kV/cm field strength. The cuvette containing transformed bacteria was immediately removed from the chamber and 1 ml of SOC medium added. Transformed J5 *E. coli* (10 µl) were inoculated shaking overnight (~200 rpm, 37 °C) in 15 ml of LB broth containing ampicillin (100 µg/ml). The inoculum was then grown on MacConkey agar at 37 °C overnight and stored in

glycerol at -20 °C until use. The transformed J5 *E. coli* were checked for green florescence of colonies on UV light source (<30 seconds) and visualized using a UV light microscope (**Figure 20**).

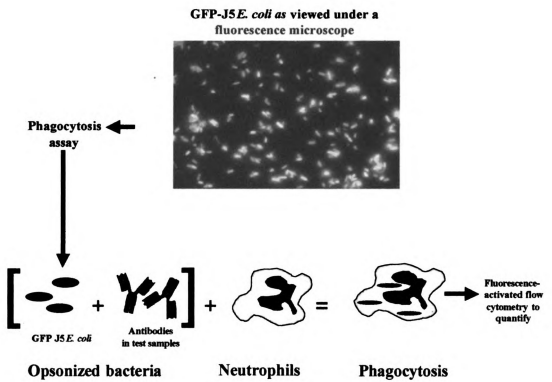


Figure 20 The flow cytometric assay employed in this study to measure neutrophil phagocytosis. Briefly, the J5 E. coli were transformed using a plasmid containing the green fluorescent gene (GFP-J5 *E. coli*) and were used for IgG_1 and IgG_2 opsonizations prior to incubating with neutrophils enriched from blood of three cows for various times. Phagocytising neutrophils fluoresced green upon flow cytometric analysis, and thus could be enumerated.

2.5.2 Opsonization

Regular and GFP-transformed J5 *E. coli* stored in glycerol at -20 °C were grown on Luria agar at 37 °C overnight after confirming culture purity. A single colony from each culture was selected and inoculated separately in 15-ml of Luria broth containing 20 µl of ampicillin (100 µg/ml) at 37 °C and 125 rpm overnight. The inoculum was then

centrifuged at 700 × g, 4 °C for 10 minutes and the supernatant discarded. The bacterial cell pellet was resuspended in 30 ml of sterile PBS, cultured, and washed two more times in 10 ml of sterile PBS, pH 7.2. The final cell suspension was brought to 13.0% transmission (approximately 1×10^9 cfu/ml) with sterile PBS as determined by spectrophotometry (Beckman DU 650 BECKMAN COULTER, Inc., Fullerton, CA) and placed on ice until used. Opsonization treatments included no antibody [GFP-J5 *E. coli* (2.35×10^5 cells) only] and GFP- J5 *E. coli* opsonized with 370 μg each of Protein A-enriched serum IgG_1 or IgG_2, antibody (**Figure 21**) or bovine serum albumin as a protein control [determined by the Warburg-Christian Concentration Assay spectrophotometrically (Model DU® 650, BECKMAN COULTER, Inc., Fullerton, CA)]. Opsonization was performed in 5-ml round-bottomed tubes (Beckton Dickinson) by mixing on an orbital shaker 125 rpm; (Bellco Glass, Inc., Vineland, NJ) at room temperature for 20 min.

2.6 Neutrophil isolation

Neutrophils were harvested from 20-ml blood samples collected from tail veins of three healthy multiparous cows (mid-lactation Holsteins) into vacutainers containing Acid Citrate Dextrose (ACD, Becton & Dickinson). Blood samples were immediately placed on ice and transported to our laboratory. After centrifugation (approximately 700 × g for 20 min at 4 °C), the plasma and top 1/3 of the red cell pack was aspirated and discarded. The remaining red cell pack (containing neutrophils) in each tube was reconstituted in 15 ml of sterile PBS, pH 7.2 and centrifuged at 700 × g, 4 °C for 10 min. Supernatants were aspirated and 12 ml of ice cold hypotonic lysing solution was added for every 3-4 ml of cells to for 1_ min to lyse red blood cells. Immediately after lysis, 6

ml of ice cold Hypertonic restoring solution was added to each tube. The tubes were swirled gently and centrifuged at 500 × g, 4 °C for 10 min to pellet remaining leukocytes (≥ 85% neutrophils, determined flow cytometrically). After aspiration of supernatants, cell pellets were resuspended in 2 ml of sterile PBS/tube, the number of cells per ml determined using an electronic cell counter (Z1 counter, Particle counter, Beckman Coulter), cells resuspended to 2×10^7 cells/ml in sterile PBS, and kept on ice for immediate use in the phagocytosis assay.

2.7 Phagocytosis assay and flow cytometric analysis of phagocytosing neutrophils

The various opsonized bacteria (total volume of 100 µl/well with 2.35×10^7 bacteria/well) were added at 8:1 ratio to prepared bovine neutrophils (2.9×10^6 cells/well) and allowed to undergo phagocytosis for varying hours in on 96-well flat bottom tissue culture plates (Costar, Corning Incorporated, Corning, NY). Incubations proceeded in an orbital shaker (Forma Scientific) at 120 rpm, 39 °C to standardize bacteria-neutrophil contact time across wells for 60, 90 120, and 150 min. After incubations, the contents of each well were treated with 20 µl of Trypsin EDTA (~10%, GibcoBRL, Life Technologies) for 10 min at room temperature to disengage surface-bound bacteria from neutrophils before flow cytometric data acquisition. The contents of each well were then analyzed in duplicate using fluorescence activated flow cytometry (FACSCalibur flow cytometer; Becton Dickinson). For flow cytometric data acquisition, the green fluorescence detector (FL-1) and the side scatter (or granularity) detector (SSC) were set against normal neutrophils (i.e., ones that were not phagocytosing and thus did not fluoresce green). In this situation, all cells fell in the upper left quadrant of SSC-FL-1 density dot plots. Background fluorescence markers were placed on dot plots based on

fluorescence of neutrophils exposed to only regular J5 *E. coli* (with no opsonization). Therefore, any neutrophils containing GFP *E. coli* and fluorescing brighter green than background were considered as phagocytosing cells, and were observed to lie in the upper right quadrant on SSC-FL-1 density dot plots (**Figure 20**). Thus, phagocytosis data were recorded as % phagocytosing neutrophils based on their counts out of a total of 5,000 neutrophils per sample counted.

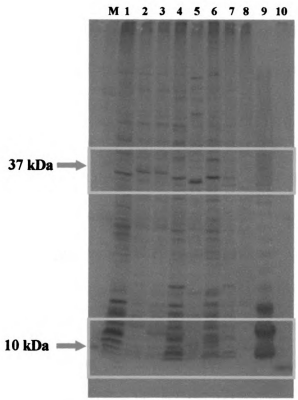
2.8 Statistical analysis

Repeated measures analysis of variance (**ANOVA**) was used to analyze the percentage of phagocytosing neutrophil data set. The analysis was carried out utilizing the MIXED procedure of SAS software (SAS Institute, Cary, NC). Overall significance of effects included in the model (immunization numbers, opsonins, and the interaction of immunization number and opsonins) was assessed by the Type III F-test. Statistical significance of adjusted mean comparisons of phagocytosing neutrophil (%) over the length of incubation by each opsonin declared relevant by the t-test was determined by the Tukey-Kramer multiple comparison procedure. Also, the differences of LSMEAN phagocytosing neutrophil (%) between each opsonin and negative control in each incubation time were determined with the Dunnett adjustment factor. The significance level was set at $P = 0.05$.

3. Results

Using SAS-PAGE followed by silver staining, we were able to visualize multiple complex protein, LPS, and Lipid A antigens from heterologous Gram-negative bacteria

(**Figure 21**). Our Western blot analyses demonstrated Gram-negative antigen recognition by isotype specific serum antibodies. At least two groups of bacterial antigens of approximately 8.5-10.5 and 34-39 kDa were recognized, predominantly by IgG_1 and IgG_2 antibodies (**Figure 22-24**).



M marker
1 J5 *E. coli*
2 *S. newport*
3 *S. typhimurium*
4 *E. coli* 487
5 *Serratia spp.*
6 *K. pneumoniae*
7 *Pseudomonas spp.*
8 *S. aureus*
9 LPS
10 Lipid A

Figure 21 Silver stain of Gram-negative (lanes 1-7) and *S. aureus* (lane 8) bacterial proteins prepared by sonication and protein precipitation, and of commercial LPS (lane 9) and Lipid A (lane 10) separated by SDS-PAGE. A molecular weight marker (kDa) is in lane M. Bacterial proteins (5 μg/lane) were separated by SDS-PAGE (12% gel). The bacterial antigens included J5 *E. coli* (lane 1), *S. newport* (lane 2), *S. typhimurium* (lane 3), *E. coli* 487 (lane 4), *Serratia spp.* (lane 5), K*lebsiella spp.* (lane 6), *Pseudomonas spp.* (lane 7), *S. aureus* (lane 8), LPS (011:B4, lane 9), and Lipid A (*S. minnestoa* Re mutant, lane 10). The highlighted boxes around bands in the 10- and 37-kDa regions (red arrows) were the groups of bacterial antigens recognized by serum antibodies from J5 vaccinated steers in subsequent Western blot analyses (Figure 22-24).

In the 34-39 kDa molecular mass range, the pattern of antigen recognition by antibodies was almost identical across the three antibody isotypes, except that the recognition by IgG_2 antibodies was more intense than for IgM and IgG_1 (**Figure 22**). Similar results were observed when different sources of serum antibodies were used, including those in fetal bovine serum and in J5 antisera from pre-, 3rd and 9th immunizations. For most bacteria, only one molecular mass size of this large antigen was recognized by the antibodies for each bacterial species. In contrast, two antigens from *Salmonella* bacteria appeared to be recognized by the serum antibodies, with one protein of ~36 kDa being the same antigen of this size class recognized in *Klebsiella*. The antigen recognized by serum antibodies in *E. coli* 487 and *Pseudomonas* seemed to be the same size as that recognized in J5 *E. coli* (~35 kDa). There was apparent isotype specific recognition of antigens in *Serratia spp.*, where IgG_1 bound with a 34 kDa protein and IgG_2 bound with a 39 kDa protein (**Figure 22 and 24**). Surprisingly, we observed the same binding pattern of IgG_1 and IgG_2 antibodies from fetal bovine serum to these 34-39 kDa proteins of gram-negative bacteria as from the preimmune, immune, and hyperimmune sera from the J5 vaccinated steers. Therefore, the antibodies recognizing these proteins may be "natural" antibodies.

In contrast to the 34-39 kDa proteins, the intensity of antibody binding to the 8-10.5 kDa proteins increased with increasing number of J5 immunizations and was stronger for IgG_1 than IgG_2 antibodies (**Figure 23**). IgM recognition of this group of small proteins was low. The IgG_1 (and to a lesser degree IgG_2) antibodies of hyperimmune serum also recognized LPS in pure form and in the bacterial lysates (**Figures 23 and 24**). Protein A-enriched G IgG_1 than IgG_2 antibodies had the same

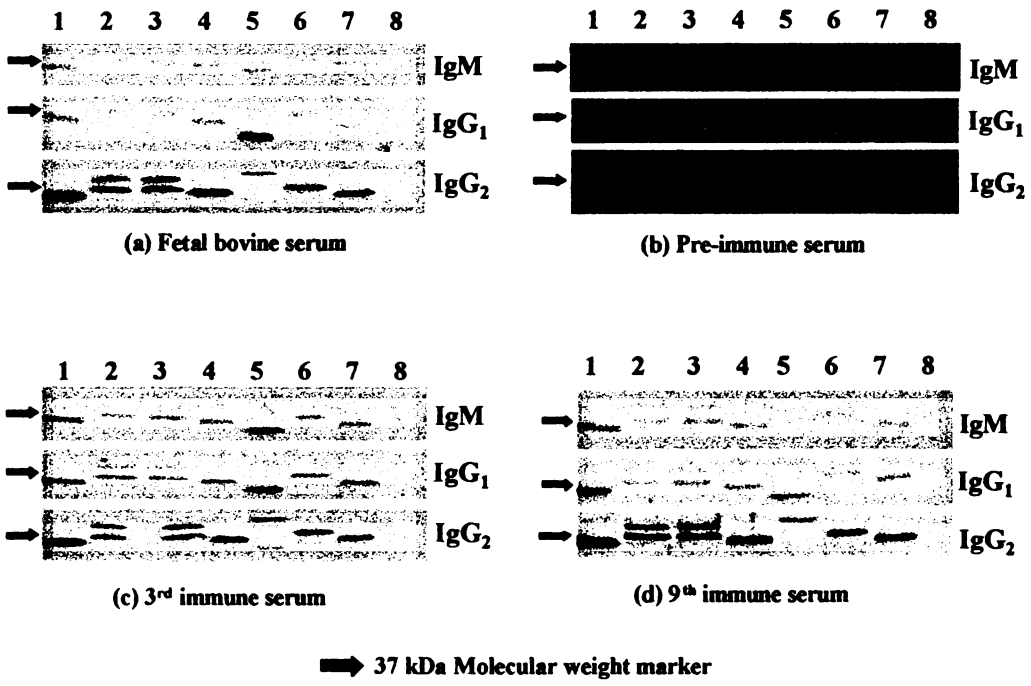


Figure 22 Western blot analyses of Gram-negative bacterial antigens in the molecular mass ranges of 34-39 kDa, recognized by IgM, IgG_1 and IgG_2 antibodies in various sera from non-immunized and J5 *E. coli* immunized cattle. Gram-negative bacterial proteins (5 μg/lane) were separated by SDS-PAGE (12% gel), and transferred to nitrocellulose membranes. The membranes were first probed with fetal bovine serum (**a**) or antisera from pre-immunization (**b**) or following the 3rd immunization (**c**), and 9th immunization (**d**) of steers with J5 bacterin. The membranes were further probed with horseradish peroxidase-conjugated sheep anti-bovine IgM, IgG_1, and IgG_2, heavy chain specific detection antibodies (shown in each panel). Test antigens included J5 *E. coli* (lane 1), *S. newport* (lane 2), *S. typhimurium* (lane 3), *E. coli* 487 (lane 4), *Serratia spp.* (lane 5), *K. pneumoniae*, (lane 6), *Pseudomonas spp.* (lane7), and *S. aureus* (lane 8). The black arrows indicate the position of a 37-kDa molecular weight marker included in each membrane but not shown in the figure.

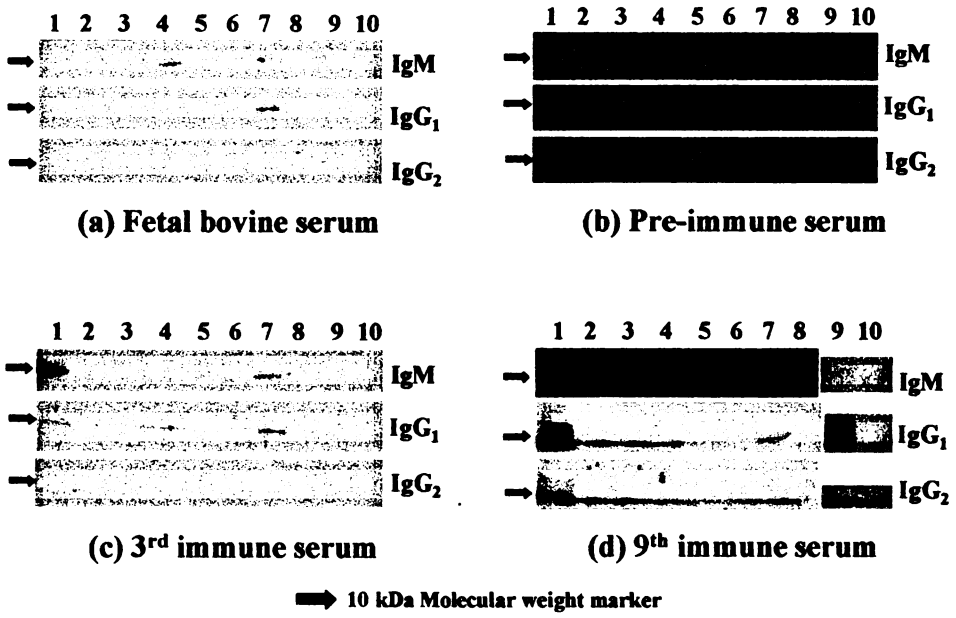


Figure 23 Western blot analyses of Gram-negative bacterial antigens in the molecular mass ranges of 8 to 10.5 kDa, recognized by IgM, IgG_1 and IgG_2 antibodies in various sera from non-immunized and J5 *E. coli* immunized cattle. Gram-negative bacterial proteins, LPS, and Lipid A (5 μg/lane) were separated by SDS-PAGE (12% gel), and transferred to nitrocellulose membranes. The membranes were first probed with fetal bovine serum (**a**) or antisera from pre-immunization (**b**) or following the 3rd immunization (**c**), and 9th immunization (**d**) of steers with J5 *E. coli* bacterin. The membranes were further probed with horseradish peroxidase-conjugated sheep anti-bovine IgM, IgG_1, and IgG_2, heavy chain specific detection antibodies (shown in each panel). Test antigens included J5 *E. coli* (lane 1), *S. newport* (lane 2), *S. typhimurium* (lane 3), *E. coli* 487 (lane 4), *Serratia spp.* (lane 5), *K. pneumoniae*, (lane 6), *Pseudomonas spp.* (lane7), *S. aureus* (lane 8), LPS (011:B4, lane 9), and Lipid A (*S. minnestoa* Re mutant, lane 10). The black arrows indicate the position of a 37-kDa molecular weight marker.

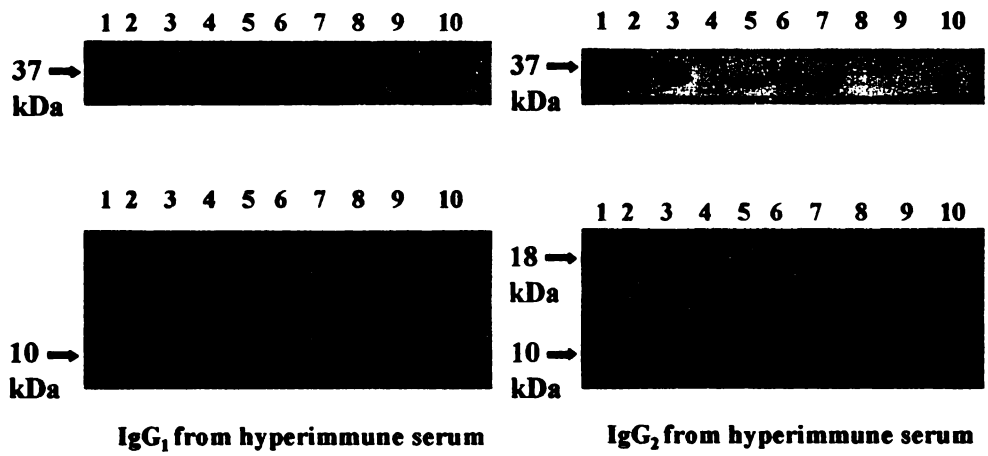


Figure 24 Western blot analyses of Gram-negative bacterial antigens in the molecular mass ranges of 8 to 10.5 kDa and 34 to 39 kDa, recognized by Protein A enriched IgG_1 and IgG_2 antibodies from J5 *E. coli* hyperimmune serum. Gram-negative bacterial antigens (5 μg/lane) were separated by SDS-PAGE (12% gel), and transferred to nitrocellulose membranes. The membranes were first probed with enriched IgG_1 **(a)** or IgG_2 **(b)** antibodies affinity purified from J5 hyperimmune serum (9^{th} immunization) from five Holstein steers. The membranes were further probed with horseradish peroxidase-conjugated sheep anti-bovine IgG_1 (**a**) or IgG_2 (**b**) heavy chain specific detection antibodies. Test antigens included J5 *E. coli* (lane 1), *S. newport* (lane 2), *S. typhimurium* (lane 3), *E. coli* 487 (lane 4), *Serratia spp.* (lane 5), *K. pneumoniae*, (lane 6), *Pseudomonas spp.* (lane7), *S. aureus* (lane 8), LPS (0111:B4, lane 9), and Lipid A (*S. minnestoa* Re mutant, lane 10). The black arrows indicate the position of the 10- and 37-kDa molecular weight markers.

antigen recognition patterns as antibodies in sera, but recognized antigens in *Serratia* and *Klebsiella* more weakly than antigens in other Gram-negative bacteria. Neither purified IgG_1 or IgG_2 in any of the test anti-sera recognized antigens in *S. aureus* (the assay negative control).

Protein A affinity purification using the pH gradient technique was successful in isolating nearly pure IgG_1 and IgG_2 antibodies from J5 hyperimmune serum (**Figure 18**). Western blot analysis demonstrated single 50 kDa heavy chains of the antibody molecules from each immunoglobulin fraction collected from the protein A column (**Figure 25**). Minor contamination of IgG_1 antibody was found in the IgG_2 fraction based on the Western blot analysis; however, no IgG_2 antibodies were detected in the IgG_1 fraction (**Figure 25**).

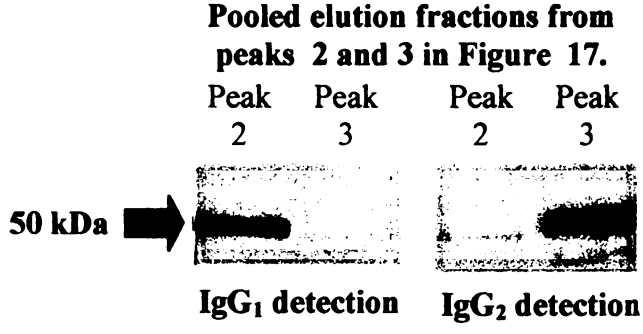


Figure 25 Western blot analysis of IgG_1 (peak 2) and IgG_2 (peak 3) fractions affinity purified from J5 hyperimmune serum using a Protein A column and a pH gradient technique (as shown in Figure 17). One microgram each of pooled fraction was resolved by SDS-PAGE, transferred onto nitrocellulose membrane, and detected with heavy chain specific Horseradish peroxidase-conjugated sheep anti-bovine IgG_1 (left panel), and IgG_2 (right panel) antibodies. Arrow indicates a 50-kDa molecular weight protein, which is the heavy chain of IgG_1 and IgG_2.

The enriched IgG_1 and IgG_2 antibodies were compared against each other and against BSA and no opsonization as opsonins for enhancing neutrophil phagocytosis of *E. coli*. Percentage of neutrophils phagocytosing *E. coli* opsonized with IgG_2 antibodies

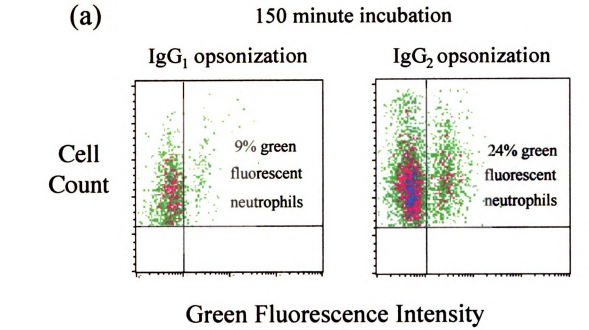
was higher ($P < 0.01$) than IgG_1-BSA-, and no treatment *E. col* at all incubation times studied. In fact, IgG_1 antibodies were ineffective in this assay (**Figures 26 and 27**). The untreated, BSA-, and IgG_1- treated cultures had 9% phagocytosing neutrophils while the IgG_2 cultures had 24% phagocytosing neutrophils by 150 minutes of incubation (**Figure 26 a,b**). There was no incubation time effect on percentage of phagocytosing neutrophils for the no-, BSA-, or IgG_1-opsonin treatments. However, phagocytosis was significantly higher for IgG_2-opsonized bacteria at 90, 120, and 150 minutes of incubation compared to 60 minutes ($P < 0.05$; **Figure 27**).

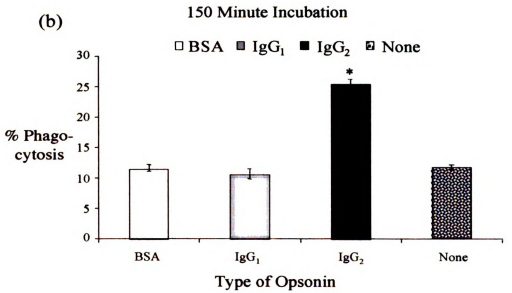
4. Discussion

In this study, we demonstrated antibody maturation in response to repeated exposure of the bovine immune system to J5 *E. coli* bacterin. Maturation was observed as increased recognition through Fab regions of small (8-10.5 kda) proteins in a variety of Gram-negative bacteria, increased isotype switching from IgM to IgG_1 or IgG_2 antibodies, and improved functional activities of the antibodies for neutrophil phagocytosis as part of the serum response to hyperimmunization-induced mature IgG_2 antibodies.

Generally, maturation of the antibody response is the process of development of highly antigen-specific antibodies by T-cell-dependent-antigen-activated B cells. This development includes affinity maturation of the Fab regions of antibodies achieved by hypermutaton and gene conversion of the variable regions of heavy and light chain gene segments, as well as maturation of the Fc regions of the molecule through constant region gene segment switching (isotype switching) (Bonhomme et al., 2000; Butler, 1998;

Figure 26 Flow cytometric analysis of bovine blood neutrophils showed that the cells phagocytosed green fluorescent protein (GFP)-transfromed J5 *E. coli* better when the bacteria were opsonized with enriched IgG_2 versus IgG_1. The enriched immunoglobulins were from pooled sera of 5 Holstein steers hyperimmunized with a commercial J5 *E. coli* bacterin. Neutrophils were from blood collected from the tail veins of 3 mid-lactation Holstein cows. GFP-transformed J5 *E. coli* were incubated with identical amounts (370 μg) of IgG_1 or IgG_2 for 20 minutes at room temperature in an orbital shaker (120 rpm) prior to being added to duplicate cultures of blood neutrophils at 8:1 (bacteria:neutrophils) for 150 minutes at 39°C and 120 rpm. Panel (**a**) shows representative flow cytometric density dot plots for neutrophils of one cow after 150 minutes of incubation with IgG_1-opsonized (left side of panel) and IgG_2-opsonized (right side of panel) GFP-J5 *E. coli*. The Y-axes are side scatter (granularity) of the neutrophils while the X-axes are intensity of green fluorescence associated with the neutrophils. Colored dots lying in the upper right quadrants of these plots are individual neutrophils that have phagocytosed GFP-J5 *E. coli* and thus fluoresced bright green. These represented 9% of all neutrophils in the IgG_1 plot and 24% of all neutrophils in the IgG_2 plot. Data in panel (**b**) show mean (± SEM) phagocytising neutrophils for duplicate neutrophil cultures from the 3 cows at 150 minutes of incubation with bacteria the were opsonized with bovine serum albumin (BSA; white bar), IgG_1 (light gray bar), IgG_2 (dark gray bar), or nothing (stippled bar). *The mean of phagocytising neutrophils was significantly higher for IgG_2 opsonization than for all other opsonization treatments at $P < 0.01$.


(a)
150 minute incubation
IgG1 opsonization
IgG2 opsonization
Cell Count
9% green fluorescent neutrophils
24% green fluorescent neutrophils
Green Fluorescence Intensity


(b)
150 Minute Incubation
BSA
IgG1
IgG2
None
% Phago-cytosis
30
25
20
15
10
5
0
*
BSA
IgG1
IgG2
None
Type of Opsonin

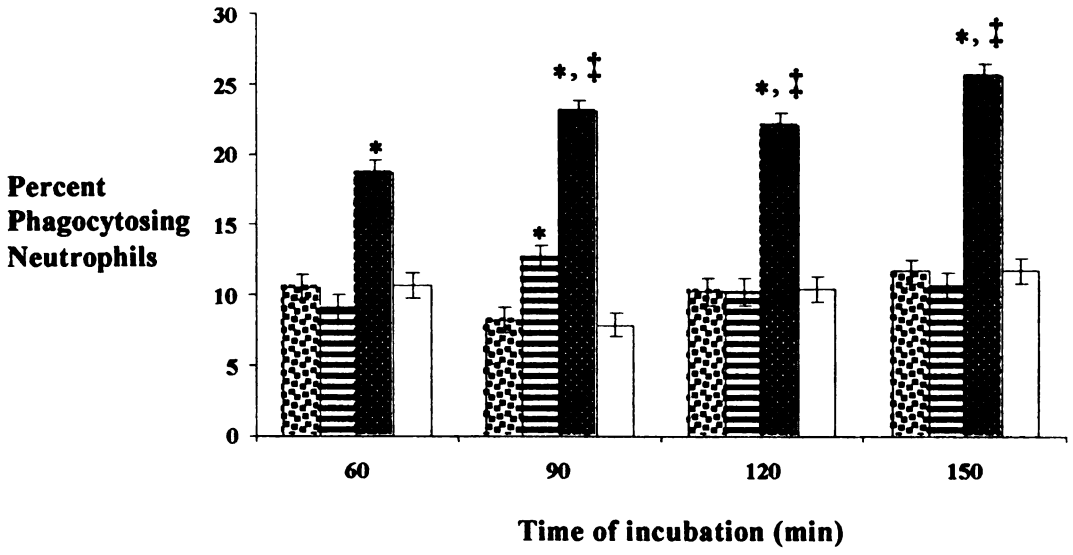


Figure 27 Mean neutrophil phagocytosis of GFP-transformed J5 *E. coli* in the presence of various opsonins; IgG_1 (▤) and IgG_2 (■) (both purified from J5 hyperimmune serum using Protein A affinity chromatography); bovine serum albumin (▦); and no opsonin (□). Data represent the percentage of neutrophils fluorescing green at each incubation time, determined from density dot plots as shown in Figure 26a. * Indicates significant differences within incubation time ($P < 0.01$). ‡ Indicates significant difference from 60 minutes ($P < 0.05$).

Kaattari et al., 2002). This process is an integral part of effective adaptive immune responses and occurs in the germinal centers of secondary lymphoid organs, including the spleen and lymph nodes (Bonhomme et al., 2000; Butler, 1998; Kaattari et al., 2002; Liu et al., 1992).

Isotype switching is largely regulated by T-cell-driven cytokines (i.e. IL-4 for IgG_1 & IgE; IFN-γ for IgG_2 in cattle) and occurs when helper T cells ligate their plasma membrane bound CD40L molecules with CD40 on the B cells (Butler, 1998; Estes, 1996). CD40L-Cd40 binding sends signals to the B cells that they are to acquire a $CD5^-$ phenotype capable of isotype switching from IgM to IgG1 or IgG2 (depending on the cytokines produced by the T cells). The maturation process ultimately improves the affinity and functional diversity of antibodies for bacterial antigens resulting in their clearance (e.g. by neutrophil phagocytosis). For example, VDJ region editing modifies the amino acid sequences of the hypervariable regions of Fab, ultimately improving the affinity of the antibody for its cognate antigen (Butler, 1998). Isotype switching enables antibodies with the same or affinity matured Fab regions to carry out a variety of biological functions, thus expanding the capabilities of the humoral immune response (Estes, 1996). For example, IgM is a strong fixer of complement, IgG_1 is an effective neutralizing antibody, and, in cattle, IgG_2 is a key opsonin for enhanced neutrophil phagocytosis of the infecting bacteria (**Figure 6; Table 2**). Hence, maturation of the antibody response improves its overall effectiveness in clearing infections and toxins that cause clinical disease.

In this study, numerous antigens were extracted from a variety of Gram-negative bacteria with SDS-PAGE and silver stained. We also separated species of LPS on

acrylamide gels and visualized the ladders by silver staining, as previously reported (Cross et al., 1989; Tsai and Frasch, 1982). We had shown in Chapter 2, using ELISA, that antibodies in J5 hyperimmune serum were cross reactive with heterologous Gram-negative bacteria, but had low cross-reactivity with LPS and non-reactive with Lipid A. We expanded results of Chapter 2 in this study by showing which bacterial antigens were bound by the cross-reactive antibodies and by showing that such antigen recognition by IgG_2 antibodies facilitated enhanced *E. coli* phagocytosis by bovine blood neutrophils.

Results of our Western blotting experiments suggested that bacterial antigen recognition by serum antibodies may possess isotype specificity. IgG_1 and IgG_2 bound distinctively to differently sized Gram-negative protein antigens. At least two predominant groups of molecular mass proteins, ranging from of 8-10.5 and 34-39 kDa, were recognized by the IgG_1 and IgG_2 antibodies. Based on the molecular masses of these antibody-recognized antigens, we suspect that they might be outer membrane proteins of the Gram-negative bacterial cell wall, including OmpA, murine lipoprotein (MLP), and perhaps PAL. Evidence from studies employing human and mouse J5 antisera suggested that at least three conserved Gram-negative OMPs may be detected by the serum antibodies. These included OmpA (35 or 37 kDa), PAL (18 or 24kDa), and MLP (5-9 kDa) (Hellman et al., 1997; 1999, 2000a; 2001). OMPs are thought to contribute to the structural integrity of the outer membrane layer of Gram-negative bacteria (Braun and Bosch, 1972; Lazzaroni and Portalier, 1992), and would be clearly accessible for immune system recognition during immunization with J5 *E. coli*. Interestingly, OmpA from *Klebsiella pneumoniae* is used as a carrier protein for some polysaccharide antigens because these are typically poor immunogens. The OmpA

dramatically increases immunogenicity of the polysaccharide PS and induces IgG antibodies during vaccination, which protects mice from lethal bacterial challenges (Libon et al., 2002; Rauly et a., 1999). Therefore, if the proteins recognized by the IgG_1 and IgG_2 antibodies in our J5 hyperimmune serum during Western blotting were such OMPs, these antibodies may be protective against a wide variety of gram-negative bacteria, including those that cause mastitis. However, the ELISA data in Chapter 2 indicated that multiple doses of J5 vaccine are required to elicit high titers of these putatively protective IgG_1 and IgG_2 anti-coliform antibodies.

In addition, the 34-39 kDa antigens identified on our Western blots may not have been the target antigens that increased serum anti-J5 IgM, IgG_1 or IgG_2 antibodies in our hyperimmunized steers (Chapter 2) because an identical pattern of recognition of these proteins was observed for the IgG_2 antibody present in very different sources of test samples, including fetal bovine serum and J5 antisera raised from, pre-, 3rd, and 9th immunizations. These observations were confusing because the fetal bovine serum used in Western blotting was the same as that used for the negative control (no antibody) for ELISAs in Chapter 2. It is possible that our ELISA was not sensitive enough to detect these antibodies recognizing the 34-39kDa antigens, or large antigens that are "hidden" from antibody recognition in phenol-killed whole bacteria, but are exposed in the lysates of these same bacteria, were used for the Western blot analysis. Still, it is difficult to reconcile that such high levels of antigen specific IgG_2 antibodies are present in fetal bovine serum. The dogma is that maternal IgG does not cross the placenta in ruminants because this tissue is syndesmochorial with the uterine epithelium being maintained throughout the entire pregnancy. As a result, it is believed that no prenatal transfer of

maternal antibodies to the fetus occurs. Indeed, using the relatively insensitive RID assay, calves have been shown to be born agammaglobulinaemic or significantly gammaglobulinaemic (Watson et al., 1994). However, our sensitive Western blot analysis suggests that this is not true and that the bovine fetus has serum IgG_1, and especially IgG_2 that cross-reacts with large proteins in heterologous Gram-negative bacteria. Unfortunately, we cannot find any solid evidence to explain our data, which raises the possibility that in cattle, IgG_2 antibodies recognizing some proteins of Gram-negative bacteria are transferred through the placenta. Further investigation of this possibility is required.

The results of this study also suggest that the specific antigen recognition of IgG_1 and IgG_2 antibodies contained in serum of J5 vaccinated steers improved with increasing number of doses. For example, IgG_2 antibody gradually recognized antigens in the 8-10.5 kDa range across Gram-negative bacterial species. Little or no recognition of these antigens was observed when fetal bovine serum and sera from pre-immune, and 3rd immunization were used. This suggested prolonged antigen exposure may be required for bovine T cells and B cells to specifically recognize these particular antigens in the J5 *E. coli* bacterin. If so, it may be possible to improve the J5 vaccine by including an excess of these 8-10.5 kDa Gram-negative bacterial proteins, which may be OMPs. Thius possibility certainly warrants further investigation. It is also possible that addition of detoxified LPS may improve the efficacy of J5 vaccines to prevent coliform diseases including septic shock, because the IgG_1 antibodies in our hyperimmune steer serum specifically recognized LPS during Western blotting.

The patterns of antigen recognition by IgG_1 and IgG_2 serum antibodies observed in this study might be explained through the types of antigen presentation of Gram-negative bacteria by B cells and other antigen presenting cells present in lymph nodes that drained the vaccination site. In the case of IgG_2, it is most likely that the peptide antigens were processed and presented on surface MHC class II molecules (reviewed by Burton and Erskine, 2003). If true, MHC II presentation of the antigens to cognitive T_H1 cells could have activated them to produce IFN-(, which would cause responsive B cells to switch production of IgM or IgG_1 antibodies to IgG_2 (**Figure 7**). As exposure to the same antigen increased, affinity maturation also might have occurred in the IgG_2 antibodies, explaining the increasing recognition of 8-10.5 kDa bacterial proteins by these molecules with increasing number of J5 immunizations. Thus, our hyperimmunization protocol may have shifted the antibody response to a predominately T_H1 response with steadily increasing levels of serum IgG_2 that had increasing affinity for the small bacterial proteins.

In contrast, specific LPS recognition by serum IgG_1 antibodies might be explained through LPS antigen-presentation to T_H2 cells via CD1 molecules on responding B cells. (Howard et al., 1993a; 1993b). Recent studies in human and mice reported that CD1 molecules act as a separate lineage of antigen-presenting molecules and present predominantly non-peptide antigens, including microbial carbohydrate fractions and lipid-containing antigens, especially oligosaccharide, glycolipid, and glycopeptide antigens from a variety of pathogens (Maher and Kronenberg, 1997; reviewed by Porcelli et al., 1996). Cognate T_H2 cells responding to CD1-presented non-specific peptide antigens induce primarily IgM and IgG_1 antibody responses from the antigen-sensitized B

cells (reviewed by Burton and Erskine, 2003). Thus, LPS present in the J5 vaccine may have induced strong serum IgG_1 responses in the hyperimmunized steers of this study through CD1 presentation to T_H2 cells. If true, the J5 vaccine may promote a strong anti-LPS T_H2 response in addition to a T_H1 response.

Finally, we were curious to know if the T_H1-dependent IgG_2 antibodies or the T_H2-dependent IgG_1 antibodies in our bovine hyperimmune serum were capable of opsonizing J5 *E. coli* for enhanced phagocytosis by neutrophils. With Protein A-purified IgG_1 and IgG_2 from hyperimmune serum we clearly demonstrated that only the IgG_2 fraction possessed direct opsonizing activity for bovine neutrophils. This is in agreement with observations of others (Miller, et al., 1988; Butler, 1998), and is logical because bovine neutrophils express receptors that specifically bind the Fc region of pathogen-bound IgG_2, but not IgG_1 (reviewed by Burton and Erskine, 2003).

5. Conclusion

The bovine antibody response elicited by repeated exposure to J5 *E. coli* bacterins can mature into highly effective T_H1-dependent IgG_2 antibody responses when given sufficient antigen stimulation and time to support affinity maturation and isotype switching. As a result, matured IgG_2 antibodies increase their biological activities by developing higher affinity antigen recognition and promoting bacterial clearance through neutrophil phagocytosis.

CHAPTER 4

SUMMARY AND CONCLUSIONS

The current study elucidated new information about the basic biology of serum antibodies induced by vaccinating cattle with J5 bacterin. A three-dose protocol of J5 immunization, recommended by the manufacturer, yielded unimpressive serum IgG_1 and IgG_2 antibody responses, as detected in ELISA using whole cell (killed) J5 *E. coli* as antigen. We determined that anti-J5 antibody titers and cross-reactivity with other Gram-negative bacteria for three key antibody isotypes (IgM, IgG_1, and IgG_2), increased significantly only after 6 doses of the vaccine were administered. In our protocol, J5 bacterin was administered every two weeks. Therefore, our study suggests that frequent and continuing J5 immunization in dairy cattle may be required to sustain high levels of serum IgM, IgG_1, and IgG_2 antibodies that strongly cross-react against heterologous Gram-negative bacteria, including those that cause coliform mastitis.

We also determined that the IgG_1 and IgG_2 antibodies of immune serum bind distinctively to different sizes of Gram-negative bacterial protein antigens and LPS. At least two predominant groups of proteins, of molecular masses 8-10.5 and 34-39 kDa, were recognized by the IgG_1 and IgG_2 antibodies. IgG_2 predominately recognized the larger proteins. According to basic immunology textbooks, we expected to see a trend of enhanced bacterial antigen recognition by maturing IgG_2 antibodies as additional vaccine doses were administered. However, this was not true for our serum IgG_2 antibodies that recognized the larger 34-39 kDa antigens, because the banding patterns on Western blots

were the same in this protein size category regardless if the IgG_2 was from fetal bovine serum, pre-immune, 3rd immunization, or 9th immunization serum from J5 vaccinated steers. In contrast, the 8-10.5 kDa protein antigens were observed as these increasingly immune sera were applied to the Western blots. Therefore, these small antigens may be excellent candidates for improving serum IgG_2 antibody responses in vaccinated cattle because they indicated that the IgG_2 response of J5 immunized steers improved with increasing doses of vaccine. If true, the 8-10.5 kDa proteins in heterologous Gram-negative bacteria increasingly recognized by IgG_2 antibodies in J5 immune serum could be used to improve coliform mastitis vaccines.

This would seem highly relevant because only the Protein A-enriched IgG_2 fraction of our hyperimmune steer serum enhanced neutrophil phagocytosis of opsonized *E. coli*. IgG_1 opsonization was totally ineffective, inducing no greater neutrophil phagocytosis than was achieved using no opsonization or BSA opsonization of the *E. coli*. Our phagocytosis results were not unexpected because Miller et al. (1988) showed that IgG_2 antibodies were the key opsonizing antibodies for bovine neutrophil phagocytosis of *S. aureus*, and bovine neutrophils express Fc receptors for IgG_2 (reviewed by Burton and Erskine, 2003). Therefore, if the increasingly mature IgG_2 antibodies in J5 vaccinated steers recognized the same 8-10.5 kDa proteins on *E. coli* during opsonization for enhanced neutrophil phagocytosis as they did on the Western blots, these proteins should make ideal candidate antigens for improvements to the J5 vaccine. While our Protein A purified IgG_1 antibodies did not affect neutrophil phagocytosis of E. coli, these antibodies did recognize LPS on Western blots. If the same occurs in vivo, the current J5 vaccine may be effective in eliciting protection against the

systemic shock that accompanies severe coliform mastitis, as long as enough doses (at least 6) of the vaccine are administered. Because IgG_1 is produced as a result of T_H2-dominated immune responses, the exposed LPS in the J5 bacterin may have been recognized by surface IgM on naïve B cells. Subsequent processing and presentation in CD1 molecules to cognate T_H2 cells, and ensuing cytokine responses may have triggered IgM to IgG_1 isotype switching in the original B cells. If so, it may be beneficial to improve the current J5 vaccine by incorporating additional, detoxified LPS to stimulate strong anti-LPS IgG_1 responses. The modest IgG_1 responses elicited by three doses of the current J5 vaccine may be responsible for the reduced severity of coliform mastitis observed in some immunized cows. However, as for the IgG_2 response, the vaccine requires improvement to elicit strong serum IgG_1 responses in fewer administered doses.

In conclusion, we caused an increasingly mature serum antibody response in Holstein steers by repeated exposure of their immune systems to antigens in the J5 bacterin. Resulting antibodies increasingly recognized a variety of whole Gram-negative bacteria in ELISA assays, 8-10.5 kDa protein antigens (IgG_2) and LPS (IgG_1) from these heterologous Gram-negative bacteria in Western blot analyses, and effectively opsonized J5 *E. coli* for enhanced clearance of the bacteria by bovine neutrophils (IgG_2 only). However, at least 6 doses of the currently available J5 bacterin were required to mature these IgG_1 and IgG_2 antibodies and increase their blood levels above pre-immunization levels. Therefore, if the benefits of these serum antibodies are to be realized in the field (protection against coliform mastitis for example), the current J5 vaccine needs to be improved such that one to two doses elicits the same IgG_1 and IgG_2 responses we observed after six to eight doses of the current vaccine. We have identified two classes

of antigens worthy of pursuit in vaccine improvement, LPS (for IgG_1) and a cluster of small (8 – 10.5 kDa) proteins present in lysates of all Gram-negative bacteria we tested (for IgG_2). Isolation, identification, and immunogenicity testing of these antigens in lactating dairy cows will be the next major steps of our continuing research on vaccines to prevent coliform mastitis.

CHAPTER 5

APPENDIX

DATA FOR FIGURES IN CHAPTERS 2 AND 3

Table 3 Mean anti-J5 *E. coli* antibody response elicited by J5 *E. coli* bacterin from sera collected from five Holstein steers

Serum samples	Mean optical density$_{\lambda=405\text{-}450\ nm}$ ± S.E.M.		
	IgM	IgG_1	IgG_2
Pre-immunized	0.137 ± 0.021	0.337 ± 0.100	0.045 ± 0.051
1st	0.157 ± 0.022	0.588 ± 0.113	0.083 ± 0.056
2nd	0.229 ± 0.022	0.612 ± 0.111	0.092 ± 0.053
3rd	0.220 ± 0.015	0.573 ± 0.110	0.100 ± 0.051
4th	0.225 ± 0.019	0.552 ± 0.109	0.108 ± 0.051
5th	0.235 ± 0.027	0.727 ± 0.108	0.151 ± 0.051
6th	0.259 ± 0.029	0.840 ± 0.106	0.177 ± 0.050
7th	0.289 ± 0.024	0.882 ± 0.103	0.239 ± 0.052
8th	0.315 ± 0.023	0.975 ± 0.102	0.270 ± 0.051
9th	0.340 ± 0.035	0.947 ± 0.101	0.262 ± 0.051
10th	0.399 ± 0.041	0.982 ± 0.100	0.294 ± 0.051
11th	0.389 ± 0.040	0.964 ± 0.101	0.335 ± 0.053
12th	0.457 ± 0.046	0.811 ± 0.113	0.282 ± 0.073

Table 4 Mean anti-J5 *E. coli* antibody endpoint titers of selected serum samples collected from five Holstein steers

	Log_2 anti-J5 antibody endpoint titers ± S.E.M.		
Serum samples	**IgM**	**IgG_1**	**IgG_2**
Pre-immunized	5.4 ± 0.458	11 ± 0.513	7.6 ± 0.696
3^{rd}	6.4 ± 0.458	12 ± 0.513	9.4 ± 0.696
6^{th}	8.2 ± 0.458	14 ± 0.513	11.6 ± 0.696
9^{th}	9.2 ± 0.458	15 ± 0.513	12.2 ± 0.696
12^{th}	10.0 ± 0.458	15 ± 0.513	12.8 ± 0.696

Table 5 Mean percentage of phagocytosing bovine neutrophil of opsonized GFP-transformed J5 *E. coli*

Incubation (min)	**Opsonin-Control**	**% Phagocytosing neutrophil Mean ± S.E.M.**
60	BSA	10.48 ± 0.907
60	IgG_1	9.11 ± 0.907
60	IgG_2	18.61 ± 0.907
60	None	10.64 ± 0.907
90	BSA	8.20 ± 0.907
90	IgG_1	12.58 ± 0.907
90	IgG_2	23.01 ± 0.907
90	None	7.86 ± 0.907
120	BSA	10.20 ± 0.907
120	IgG_1	10.16 ± 0.907
120	IgG_2	21.98 ± 0.907
120	None	10.39 ± 0.907
150	BSA	11.60 ± 0.907
150	IgG_1	10.66 ± 0.907
150	IgG_2	25.46 ± 0.907
150	None	11.72 ± 0.907

CHAPTER 6

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