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EFFECT OF PEPTIDOGLYCAN-POLYSACCHARIDE COMPLEX ON REPRODUCTIVE EFFICIENCY AND MASTITIS IN SHEEP

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Thesis submitted to the
Davis College of Agriculture, Forestry and Consumer Sciences
at West Virginia University
in Partial Fulfillment of the Requirements
for the Degree of

Master of Science
in
Reproductive Physiology

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ABSTRACT

Effect of Peptidoglycan-Polysaccharide Complex on Reproductive Efficiency and Mastitis in Sheep

Ida Holásková

Bacterial infections associated with mastitis reduce pregnancies in cattle. Effects on pregnancy and incidence of mastitis in sheep were investigated after immunization with peptidoglycan-polysaccharide (PG-PS) and killed cells from *Strep pyogenes*. Rambouillet (n=100) and mixed breed (n=18) ewe lambs were immunized (~d42 and 22 before breeding) with PG-PS (30µgPG/kg/bw) or killed cells or were not immunized (Control, n=117). IgG antibodies were detected by ELISA. Ewes were bred at synchronized estrus. All immunized and half of non-immunized ewes were challenged with PG-PS (60µgPG/kg/bw) d5 after breeding. Although proportion of ewes pregnant at d42 did not differ, probability of pregnancy decreased with total dose of PG-PS ($p<.05$). Incidence of mastitis in mixed breed ewes in the middle and at the end of lactation did not differ. In conclusion, immunization of ewe lambs with PG-PS or killed cells of *Strep pyogenes* did not improve pregnancy rate or incidence of mastitis after PG-PS challenge.

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LITERATURE REVIEW

I. Introduction

Breeding efficiency of domestic animals is a composite of several factors, including success of ovulation, manifestation of estrus, viability of gametes, fertilization rate, embryo survival and fetal development, and perinatal survival rate (Stott and Williams, 1962; Dailey et al. 2002). In cows, fertilization rates of 85 to 90 % have been reported (Kidder et al., 1954; Bearen et al., 1956; Hill et al., 1970), but up to 40 % of fertilized eggs did not survive through 40 days of gestation, so pregnancy rate was only 60 % (Tanabe and Casida, 1949; Tanabe and Almquist, 1953). Many studies were designed to estimate the embryonic and fetal loss in bovine. Great variation exists in the data collected due to different end-points of experiments, differences in breeds, locations, and farm management. Dailey et al. (2002) recently reviewed the literature and concluded that the majority of the loss occurred at the early embryonic stage, corresponding to early blastocyst stage. In both nonparous and parous beef cows, a large proportion of reproductive failure occurred by day 8 of gestation without noticeable influence on the estrous cycle length (Maurer and Chenault, 1983).

In the dairy industry, the goal is to have cows pregnant again within 90 days of calving (Louca and Legates, 1968), which means they must be bred during lactation. Lactation itself imposes a high metabolic demand on females. In addition, mastitis is a common problem in dairy cows. The financial losses due to the inflammation are not only in the form of unusable milk, but also due to the negative effect of disease on reproductive functions.

Cows show greater sensitivity to bacterial pathogens during pregnancy (Apitz et al. 1935). Barker et al. (1998) found that clinical mastitis during early lactation negatively influenced reproductive performance of Jersey cows. Specifically, number of artificial inseminations (AI) per conception was greater in cows with clinical mastitis after AI than for cows with mastitis before AI or after confirmed pregnancy.

Many species of bacteria cause mastitis in cows, sheep and goats. The main component of the cell wall of Gram-positive bacteria, peptidoglycan, induces the inflammatory response in the host. Peptidoglycan has very similar biological actions as endotoxin, the main cell wall component of Gram-negative bacteria (Stetson, 1956; Rotta, 1975).

It is of interest to find out how molecules involved in the inflammatory reaction caused by peptidoglycan affect early embryonic survival in ruminants. Moreover, immunizing against the bacterial pathogens, using isolated peptidoglycan-polysaccharide complex or the whole, dead, Gram-positive bacteria might be possible. If immunization is effective, the negative effect of bacteria on reproductive efficiency and/or mastitis could be lessened or abolished.

II. Early Embryonic Development

A. Morphological Events

A mammalian oocyte is a large haploid cell surrounded by a protective extracellular layer, the zona pellucida, and by granulosal cells organized into corona radiata. Fertilization in mammals triggers the completion of meiotic division of the oocyte. After fusion of sperm and oocyte, the diploid chromosome number is restored and a complex program of gene activity directs the embryogenesis. The embryo begins

to develop as a cell of about 100 to 150 μm in diameter (Blandau, 1961; Hartman et al., 1931). The early embryonic processes include cell divisions, cell migrations and differentiation, which result in two main cell lineages. One cell lineage will give rise solely to extraembryonic support structures, and the other lineage will form the embryo proper.

After fertilization, several mitotic divisions follow, spaced at approximately 12-hr intervals. This phase is called cleavage. There is no cell growth during this time, therefore, as cleavage continues, the large volume of the zygote's cytoplasm is divided into numerous and smaller cells-blastomeres. During this time, the embryo is still in the oviduct and enclosed by the thick, translucent mucoprotein, zona pellucida. A crucial event, compaction, happens at the eight-cell stage of the mammalian embryo. The blastomeres dramatically increase the cell-to-cell adhesions and flatten upon each other. Blastomeres forming the outside layer develop tight junctions between one another and initiate the process of epithelial differentiation. Cell divisions continue. When the number of cells reaches sixteen, the embryo is called a morula. At that time, two distinct populations of cells can be recognized, the inner cells connected by gap junctions and the outer layer of cells forming an epithelium. After the next series of mitotic divisions, the outer layer of cells, called the trophoblast, facilitates the transport of fluid from outside of the embryo to inside and the fluid-filled cavity, blastocoel, is formed. The embryo at this stage is called the blastocyst.

Very early embryonic development is controlled by the maternal mRNA and proteins in most of the domestic species. The onset of zygotic transcription in mice

begins at two cell stage, but in most of the organisms is delayed until the embryo is composed of hundreds to thousands of cells (Burdsal, 1999).

Implantation happens when the embryo becomes actually embedded in uterine tissue, or even enclosed by it, as in mice and rats (Enders and Schlafke, 1967). In all species, implantation involves a direct interaction of the trophoblast with the luminal epithelium of the uterus (Weitlauf, 1988). This process is preceded by attachment, which begins with apposition of the embryo to the site of implantation (Schlafke and Enders, 1975). There is specie-to specie variation with respect to the time, degree, and type of attachment at the maternal-fetal interface, as well as the orientation of the blastocyst, summarized by Wimsat (1975). Placenta was defined by Mossman in 1937 as an apposition or fusion of tissues from parent and offspring for physiological exchange and develops from the trophoblast. The discrete regions of placenta, which serve as zones of metabolic exchange, have the shape of finger-like projections, called chorionic villi. Different distribution of chorionic villi on placentas gives basis for the anatomical classification of placentas of the various species. In ruminants, villi are grouped in a variable number of structures called cotyledons, therefore the placenta is cotyledonary. The actual attachment in horses, pigs and ruminants is of epitheliochorial type, which means that maternal endothelial cells of blood vessels, uterine stroma and epithelium, then fetal trophoblast epithelium, interstitial cells and endothelial cells are layers that physically separate the maternal and embryonal circulations. This is the least intimate type of microscopic placental classifications (Senger, 1999).

Histological examination of cow eggs and embryos by Hamilton and Laing in 1946 revealed that the fertilized egg in a cow stays in the 1-cell stage for about 27 hours.

The transport through the oviduct takes about 96 hours. When it enters the uterine lumen, it is about 8 to 16 cell stage morula, and corresponds to the fifth day post ovulation. The blastocyst is formed about 8 to 9 days after ovulation (Chang, 1952). Then the blastocyst floats in the uterus until apposition of the trophoblast to the uterus and actual adhesion (Wimsatt, 1975), which take places about 40 days after ovulation in the cow (Hamilton and Laing, 1946). The blastocysts recovered 12 and 13 days after ovulation have started to elongate and were described by Chang (1952) as fragile, wrinkled and very sticky when pushed by a needle. In the sheep, the time corresponding to the various changes in the morphology and location of the embryo are as follows: one cell stage, 38 hours; morula stage, about 96 hours; morula entering the uterus 2-4 days post ovulation, than the blastocyst formation takes place at 5-7 days and implantation at 17-18 days (Chang and Rowson, 1965; Brinster, 1974). According to Brinster (1974), even though the overall pregnancy length largely differs among mammals (mouse 21 days, cow 284 days), the time from ovulation till blastocyst stage is quite similar, 3-9 days.

B. Endocrinology of early embryo development

One of the factors involved in establishment of successful pregnancy is an appropriate communication between the fetus and the maternal body. The embryo needs to signal its presence in the uterus, which, in turn, initiates the protective mechanism for the developing embryo. This phenomenon is referred to as maternal recognition of pregnancy. In many species, especially ungulates, a well-known component of the maternal protective mechanism is the prolonged functioning of the corpus luteum (CL), an endocrine gland formed in the ovary after ovulation of the follicle. The CL secretes

progesterone, which, during pregnancy, prevents cyclicity and ensures quiescence of the uterus. The weights of the uterine endometrium of cycling intact ewes were increased in response to early progesterone treatment (Wilson et al., 1972). Uterus and ovaries thus function as endocrine-circuit, as it was demonstrated by increasing the life span of corpus luteum after removal of the uterus (Wiltbank and Casida, 1956; Rowson and Moore, 1964; Inskeep and Butcher, 1966; Thacher et al., 1986; Bazer, 1992), and termination of pregnancy soon after removal of ovaries prior to 180-200 days of gestation in bovine (Estergreen et al., 1967).

The signal from embryo to mother is endocrine in nature. Moore (1968) proposed that in the sheep, the stimulus from the embryo has an antiluteolytic rather than luteotropic effect on CL. This corpus luteum life-extending function of the embryo is assumed on day 12 to 13 after estrus, two to three days before the CL of non-pregnant cycling sheep regress (Henricks et al., 1971). Regression of the CL during the cycle has been found to be associated with uterine prostaglandins (suggested by Babcock in 1966; Bland et al., 1971; Wilson et al. 1972; Inskeep and Murdoch, 1980) and from the ovary (Rexroad and Guthrie, 1979; Milvae and Hansel, 1983). This prostaglandin secretion is decreased and overcome by the presence of the embryo in the uterus (Kindahl et al., 1976; Pratt et al., 1977; Betteridge et al., 1984). Prostaglandins are synthesized from arachidonic acid (Granstrőm, 1981) and mediate hemodynamic derangements (Sorrels et al., 1971), changes in platelet function (van der Wolf et al., 1978), alterations in vascular permeability (Arrora et al., 1970), stimulatory effects on contractility of uterus (Cseply and Csapo, 1972; Wiqvist et al., 1972), and influences on the course of inflammatory processes (Crunkhorn and Willis, 1969). Cultured endometrium from uteri at day 17 of

gestation produced less prostaglandin $F_2\alpha$ ($PGF_2\alpha$) than endometrium from cyclic cows on day 17 (Gross et al., 1988). Day-13 ovine corpus luteum during pregnancy was found to have greater enzymatic activity of prostaglandin dehydrogenase (PGDH), which is an enzyme metabolizing $PGF_2\alpha$ into its inactive form, 15-keto-13,14-dihydro-prostaglandin $F_2\alpha$ (PGFM), compared to day 13 cyclic ovine corpus luteum (Silva et al., 2000).

C. Immunology of early maternal-fetal interface

The morphological and endocrine changes during early embryonic development are accompanied by the local and /or systemic presence of numerous bioactive compounds besides hormones that regulate or modify the uterine environment to favor the embryo. Many of these compounds are proteins, including growth factors, cytokines and adhesion molecules. Those proteins mediate many processes required for successful implantation, yet little is known about mutual interactions and sequence of events involving these molecules (Findlay at al., 1979; Sunder and Lenton; 2000). Immunological mechanisms associated with early pregnancy involve regulation of the invasion of the fetal cells into maternal tissue such that damage to the mother is prevented. Simultaneously, maternal recognition of pregnancy must be orchestrated so that allorejection of the fetus does not occur (reviewed by Entrican, 2002). For successful pregnancy, low concentrations of *inflammatory* cytokines, such as tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ), but predominance of *regulatory* cytokines as interleukin (IL) -10 (IL-10) and transforming factor-beta (TGF-β), are necessary (Entrican, 2002). In pathological situation for instance, infection, inflammatory cytokines may increase in concentration and endanger the pregnancy. A mice model of spontaneous embryo resorption (CBA mated to DBA/2 mice) had

provided valuable information on role of cytokines in the materno-fetal interface. Tangri and Raghupathy (1993) demonstrated that placentas of the resorption-prone mating combination (CBA x DBA/2) had enhanced expression of TNF-α, IFN-τ, and interleukin-2 (IL-2, inflammatory cytokine), when compared to placentas obtained from non-resorption-prone combination (CBA x BALB/c). TNF-α is involved in trombosis and smooth muscle contraction. IFN-τ may have both beneficial (reviewed by Hansen 1995; Bazer et al., 1997; Martal et al., 1997) or detrimental effects (Tangri and Raghupathy, 1993), which may depend on the species and stage of pregnancy (Entrican, 2002). Pro-inflammatory cytokine IL-12 is mediating immune response leading to the generation of T cells with a T helper 1 (Th-1)-type cytokine profile. On the other hand, T cells which express Th2-type cytokine profile are activated under the influence of IL-4 and are effective in antibody response. A balance of inflammatory and regulatory cytokines is necessary for protection against infectious disease as well as for not causing the damage to the host and embryo (Entrican, 2002).

III. Early Embryonic Loss

Loss of pregnancy is a serious problem resulting in financial losses for dairy producers. Robinson (1977) pointed out that only 50 to 55% of healthy breeder cows in which eggs were fertilized using artificial insemination, calved. Diskin and Sreenan (1980) examined 256 Aberdeen Angus and Hereford cross breds and found that in genitally normal heifers, fertilization failure accounted for 10% of overall reproductive failure, while embryo death accounted for more then 30%. Most of the embryonic mortality occurred between days 8 and 16 of gestation. A study of variability in fertility of bulls affecting the fertilization rates and embryonic mortality of Holstein and Guernsey

heifers by Kidder et al. (1954) showed that high fertility bulls, based on nonreturn rate 70% and above, had a100% fertilization rate and estimated embryonic death rate of 24 %. Several factors cause early embryonic death. Besides genetic and endocrine bases, disease at the time of early embryonic development can adversely affect survival of the embryo.

IV. Mastitis

A. Prevalence and Economical Losses

Mastitis, inflammation of the mammary gland, is a significant cause of financial losses in the dairy industry, and it has a negative impact on reproductive efficiency. The USDA report in 1954 showed the estimated loss of $225,804,000 (USDA, 1954) and at least $2 billion in 1993 (DeGraves and Fetrow, 1993) to mastitis in the United States. In other words, 10-11 % of total productive capacity was lost per year, when producers took in to consideration decreased milk production, increased replacement cost, discarded milk, cost of drugs, veterinary fees, and labor costs. Erb et al. (1985) estimated in 33 dairy herds that bovine mastitis together with failure to conceive at first service increased risk of culling 5.2 to 10 times. Similarly, ovine mastitis is causing significant problems on milking sheep farms and in the sheep milk cheese industry worldwide, especially in Mediterranean countries. A study of 17 sheep flocks of the Manchega breed and 5 flocks of the Assaf breed in central Spain revealed that subclinical mastitis oscillated from 9 to 83 % of animals with an average of 35 %, and between 4.5 and 67% of mammary glands with the a mean of 21 % (Las Heras et al., 2001). These values were similar to reports from northern Greece (Stefanakis et al, 1995) and Portugal (cited in Las Heras et al., 2001), but slightly higher than those from Italy (cited in Las Heras et al., 2001) and

England (Watkins et al., 1991). In the United States, with a relatively smaller socioeconomic importance of the sheep-cheese industry, the primary concern due to mastitis is the negative effect on lamb growth. Keisler at al. (1992) examined subclinical mastitis of Hamphire and Finn x Dorset x Rambouillet crossbred sheep and found that incidence of inflammation was from 17 to 50 %. The same group also found that the subclinical mastitis had no effect on lamb growth. However, this was at day 40 postpartum, and lambs had access to supplemental feed.

B. Clinical Classifications

The primary cause of mastitis is bacterial infection, which results from passage of the microorganism through the teat canal, either by growth or by suction during milking (Plastridge, 1958). After infection, inflammation results in changes in udder secretions and glandular tissue that may or may not be associated with clinical signs of mastitis. *Subclinical mastitis* means that neither swelling of the udder is detected nor is there observable abnormalities in the milk, but via special screening tests, number of somatic cells in the milk would be increased (Swartz, 2001). Milk somatic cells consist of neutrophils, macrophages, lymphocytes, and epithelial cells (Sordillo et al., 1997) that are released into the milk in exceedingly high concentration when the mammary gland is inflamed. Microscopic evaluation of milk or using the on-site screening tests such as California Mastitis Test (CMT), Wisconsin Mastitis Test (WMT) or the catalase test, detect the number of such immune (somatic) cells in the milk. This provides valuable health and market-related information about the cow and milk, and is called a somatic cell count (SCC). Blosser (1979) concluded that most of the losses due to mastitis are attributed to subclinical mastitis. *Clinical mastitis* is the presence of white blood cells in

the milk and can be mild or acute (severe). In the case of *mild clinical mastitis,* cells clumped as flakes and clots, give an unusual appearance of milk. *Acute clinical mastitis* is painful to the animal; the udder is hot and very sensitive, fever and loss of appetite may occur. Somatic cell counts and clinical mastitis have positive genetic correlation (Kadarmideen and Pryce, 2001). *Chronic mastitis* is a persistent udder infection.

C. Mastitis Pathogens

The organisms most commonly involved in mastitis include both gram-positive and gram-negative bacterial species, like *Escherichia coli, Micrococcus aureus, Micrococcus pyogenes, Staphylococcus aureus, Staphylococcus pyogenes, Nocardia, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus equines, Streptococcus pyogenes, Streptococcus hemoliticus, Streptococcus uberis, Streptococcus zooepidemicus, Arcanobacterium pyogenes, Corynebacterium bovis, Klebsiella pneumonidae*, and also yeast (Law, 1923; Brown et al., 1926; Schalm, 1963; Gonzales et al.,1990; Moore et al., 1991; Barker et al., 1998; Waage et al., 1998). *Streptococcal* (*Strep*) species of different origin were found frequently in raw and pasteurized milk (Brown et al., 1926). It was of interest, whether the milk-borne *Strep* pathogens could cause septic sore throat epidemics. Smith with Brown (1915) advanced the theory that the human sore throat epidemics were not caused by the *Strep* which usually cause bovine mastitis, but by *Strep* of human origin that occasionally enters the udder of a cow. Davis and Capps (1914) infected cow's udder with human streptococci by rubbing the culture on abraded teat. Brown et al. (1926) found Beta hemolytic streptococci in considerable numbers in certified milk from five dairy herds representing about 900 dairy cows in Wisconsin. However, there was no evidence that any of them were pathogenic to

humans. Plastridge (1958) pointed out that there are three main principal organisms associated with bovine mastitis, Gram-positive *Strep agalactiae, Strep uberis and Micrococcus Pyogenes*, which usually cause chronic mastitis. DeGraves and Fetrow (1993) summarized the organisms involved in subclinical mastitis, caused by contagious bacteria as *Strep agalactiae* and *Staphylococcus (S.) aureus*, or by environmental organisms such as nonagalactiae *Strep* species, usually *Strep uberis* or *Strep dysgalactiae*. Barker et al. (1998) found environmental Streptococcal species more prevalent than E. Coli or other Gram-negative pathogens in a herd of Jersey dairy cows. In the 2001 newsletter of National Mastitis Council, producers were informed that the most frequently isolated *Strep* from bovine mammary gland is *Strep uberis*. Coliform mastitis is caused by Gram-negative bacteria that include *E. coli*, *Klebsiella spp*., and *Enterobacter spp*. (Harmon, 1994). The main microorganisms producing subclinical mastitis in milking sheep are *S. epidermis*, then *Strep agalactiae*, *Strep uberis*, and *Strep dysgalactiae* (Fernández-Garayzábal et al, 1998).

D. Effect of Mastitis on Physiology and Reproduction

Paape et al. (1974) induced acute coliform mastitis using *E. coli* endotoxin and noticed dynamic changes within a short time after infusion. A rapid decrease in concentration of circulating neutrophils was detected within 90 minutes. The extra vascular mobilization of neutrophils into mammary tissue was noticed in 3 hours by increased somatic cell counts in milk, which peaked in 18-24 hours at 1000-fold. Swelling of the udder in two hours suggested over-population and lysis of blood neutrophils. Increase in body temperature was critical between four and six hours post infusion. An increase in plasma corticosteroid concentration was detected at two hours,

peaked at four hours and returned to normal by two hours. Giri et al. (1984) also recognized a rapid increase of somatic cells in milk, as well as a significant increase of bovine serum albumin in whey after instillation of 50 μg of endotoxin into teats of cows.

Fever, as a component of inflammatory reaction to acute mastitis, also may have an effect on the oocyte and on the embryo. Lavický et al. (1988) recorded fever response when components of bacterial cell wall were given intravenously to 12-40 days old calves at a dose of 50 μg/kg body weight. Moor and Crosby (1985) hypothesized and found that temperature fluctuation in mammalian oocytes during maturation adversely affected their subsequent developmental potential, as the cooling of ovine oocytes slowed meiosis. Fever and hyperthermia may affect embryo survival. Ealy et al. (1994) found increases in pregnancy rates of heat-stressed cows in Florida when cows were cooled from two to three days before until five to six days after breeding. A fever response was observed in lactating dairy cows four and half and six hours after intravenous and intramammary endotoxin infusion (Jackson et al, 1990). However, Barker (1999) did not find reduction in pregnancy rate to be associated with fever, when sheep were challenged with peptidoglycan-polysaccharide intravenous injection day 5 after breeding.

Sordilo et al. (1997) pointed out that cytokines, which are released during pathological conditions associated with mastitis, are extremely potent molecules, which, at elevated levels, may be detrimental to the host. The physiological alterations of homeostasis associated with mastitis have been related to decreased reproductive efficiency, including early embryonic loss (Barker et al, 1998, Loeflerr et al. 1999; Oliver et al., 2001). Loeffler et al. (1999) studied effects of diseases on fertility in dairy cows in the Netherlands and found that the impact of the disease depended strongly on time since

the disease last occurred. In the case of clinical mastitis, its impact on conception was very weak if occurring prior to artificial insemination (AI), but caused greater than 50 % reduction in pregnancy risk if occurred 3 weeks after AI.

V. Bacterial Cell Wall, Lipopolysacharide and Peptidoglycan

A. Chemical Structure

Most bacterial cells have three main regions: cytoplasm, cell envelope and cell appendages (proteins in the form of fimbriae and flagella), reviewed by Todar (2002). The cell envelope consists of plasma membrane, cell wall and capsule. Capsule is the outside layer made of polysaccharide, known to mediate adherence of bacteria to particular surfaces. The cell wall is an essential structure protecting bacteria from osmotic lysis. The main constituent of the cell wall is peptidoglycan (PG). Gram-positive bacteria have a thicker cell wall, 15-18 nanometers (nm), composed of many layers of PG, as opposed to Gram-negative bacteria, 10 nm with one layer of PG. Gram-negative bacteria have the outer membrane, which often contains lipopolysaccharide (LPS or endotoxin), and is generally toxic to the host. Peptidoglycan is the main and strongly immunogenic component of a gram-positive bacteria's cell wall (Krause and McCarty, 1961; Heymer et al, 1985; Rosenthal and Dziarski, 1994), just as is LPS on the Gram-negative bacteria.

Peptidoglycan (synonyms: glycopeptide, mucopeptide, or murein) is a heteropolymer consisting of a polysaccharide backbone forming glycan strand, composed of β(1-4)-glycosidically linked N-acetylglucosamine (NAG) residues. Each alternate N-acetylglucosamine residue is substituted by D-lactic acid ether in cis C-3 hydroxyl group. This derivative of glucosamine is called muramic acid (NAM, Heyman et al, 1964). The

carboxyl group of NAM is substituted by an oligopeptide which contains alternating L- and D- amino acids. Adjacent peptide subunits are cross-linked either directly or via an inter peptide bridge. This gives rise to a large macromolecule encompassing the bacterial cell. The glycan strand of various peptidoglycans is rather uniform, but different amino acid sequences can be found in PG of various bacteria. In most cases, L-alanine is bound to muramic acid, followed by D-glutamic acid, a diamino acid L-Lysine and D-alanine. However, in some species of *Staphylocci (i.e. S. aureus)* and some *Strep*, an additional D-alanine is found at the C-terminus (Schleifer and Seidl, 1974). Other important cell-wall components besides the PG include the group-specific carbohydrate hapten, which contains rhamnose and NAG, and are referred to as polysaccharide (PS). Often, complexes of peptidoglycan (PG) and polysaccharides (PS) are isolated from gram-positive bacteria and used as antigen in different animal models. These complexes are referred to as PG-PS.

B. Clinical Manifestations of Bacterial Infection

Infection of a host with Gram-positive or Gram-negative bacteria results in similar clinical manifestations, such as inflammation, fever, leukocytosis, hypotension, decreased peripheral perfusion, malaise, decreased appetite, sleepiness, and arthritis (Wang et al, 2000). These symptoms are caused by mediators released from host cells that were exposed to the bacterial cells and their components (Wang et al., 2000). The main parts of bacteria responsible for these clinical manifestations are endotoxins and peptidoglycans (Dziarski, 1980).

C. Immunochemistry of Peptidoglycan

From the experiments of Abdula and Schwab (1965), and Karakawa et al. (1966, 1967, 1968) it was determined that it was possible to obtain anti-PG sera from rabbits that were immunized intradermally or intravenously with PG. However, the antibody response was never as marked as when the vaccine was prepared from the heat-killed whole cells of gram-positive cocci. There have been at least five different antigenic epitopes of PG described: epitope **a** - the glycan strand, epitope **b** – N-terminal, epitope **c** –c-terminal of interpeptide bridge, epitope **d** – the uncrossed peptide subunit tetrapeptide, and epitope **e** - peptide subunit pentapeptide (Karakawa et al. 1967; Rolička and Park, 1969).

The main target cells in a host responding to endotoxin and peptidoglycan are the monocytes and macrophages, activated through two pattern recognition receptors CD14 and Toll-like receptors (TLRs) (Dziarski et al 1998, Poltorak et al 1998; Yoshimura et a, 1999; Schwandner et al 1999). The main proinflammatory mediators induced by immune cells of host by endotoxins and peptidoglycans are the cytokines TNF-α, IL-1, and IL-6. Wang *et al.* (2000) challenged this hypothesis and found that over 120 genes were activated in human monocytes by *S. aureus*, peptidoglycan, and endotoxin. All three bacterial stimulants induced gene transcription predominantly for chemokine IL-8, and macrophage inflammatory protein (MIP-1α), while cytokine genes (TNF-α, IL-1, and IL-6) were induced to a lower extent. The gene expression was evaluated using cDNA arrays, confirmed by quantitative RNase protection assay and high secretion of chemokines was confirmed by enzyme-linked immunosorbent assays. Peptidoglycan also induced other chemokines like MIP-2α, MIP-1β, and monocyte chemoatractant

protein-1 (MCP-1) in higher amounts than cytokines. Gold et al. (1985) showed that when β-lactam antibiotics are administered to humans with *Strep* infection, soluble non-cross-linked PG polymers are released by cocci, which results in IL-1-mediated inflammatory reaction.

VI. Pregnancy Loss Due to Endotoxin and Peptidoglycan

It has been known that animals show greater susceptibility to bacterial pathogens during pregnancy (Apitz et al., 1935).

A. Endotoxins and Pregnancy Loss

Experimentally induced endotoxin infection can lead to abortion and death of the dam (Zahl, 1943; Rieder, 1960; Chedid, 1962). According to Giri et al. (1991) endotoxin from Gram negative bacteria have two main physiological effects: First, endotoxin causes regression of the CL which is often linked to abortion in many species like mice, goats, pigs, mares and cows (Skarnes and Harper,1972; Rious-Darrieulat, 1978; Wrathall et al., 1978; Fredricksson et al., 1985; Fredricksson et al., 1986; Giri et al.1990). Second, endotoxin produces metabolic, circulatory, hematological and reproductive disturbances partly via release of prostaglandins, thromboxane, leukotriens, and cytokines (Anderson et al., 1975a, b; Fletcher and Ramwell, 1977; Morrison and Kleine 1977; Jesmok and Borgia, 1985; Lang et al. 1985, Cort and Kindahl, 1990; Trebichavský et al., 2002). Chaoaut at al. (1990) showed that LPS led to release of TNF-α, leading to dose-dependent enhancement of embryo resorptions up to 100 %. The same group (Chaoaut at al., 1990) also examined the effect of injecting various cytokines directly to pregnant mice. They found that 1000 units of TNF-α enhanced resorptions from 43 to 79% in CBA x DBA/2 – abortion-prone mice, and from 7% to 89% in non-abortion prone mice.

Gamma interferon and recombinant interleukin 2 (IL-2) also enhanced resorptions from 38 to 68% and to 76% and from 6 to 44% and 55 % in four different mating combinations, in abortion –prone and non-abortion-prone mice, respectively. On the other hand, cytokines such as IL-3 and GM-CSF, belonging to CSF family, increased the chances of fetal survival and reduced resorptions in the abortion-prone CBA/J x DBA/2 mating combination from 55% to 22 % (IL-3) and 47 to 8 % (GM-CSF). These results indicate that balance of inflammatory and anti- inflammatory cytokines is involved in mediation of embryonic resorption and survival, and that the deleterious effect of inflammatory cytokines on pregnancy can be prevented.

Skarnes and Harper (1972) provided direct evidence for synthesis of prostaglandin F in mouse uterine endometrium as a result of parenteral administration of bacterial endotoxin and concluded that abortifacient action of endotoxin is mediated by $PGF_{2\alpha}$. A single injection of 5 μg of endotoxin on day 16 of gestation caused maternal diarrhea and intrauterine fetal death. Higher doses (10, 20 μg) caused abortion. Interestingly, the same group found high amounts of PGF or its metabolites in the urine of males as well as pregnant female mice. Hence, they suggested that endotoxemia evokes a generalized, not local synthesis of F-prostaglandins. Skarnes and Harper (1972) proposed that prostaglandins were working indirectly through other substances, like serotonin. Serotonin is a lipid insoluble amine that functions as both a neurotransmitter and an endocrine hormone regulating gastric secretion and smooth muscle contraction in blood vessels (Randal et al, 1997). Administration of serotonin antagonist helped to lessen the abortion-causing action of endotoxin (Skarnes and Harper, 1972). Cseply and Csapo, as well as Wiqvist et al. (1972) proposed that $PGF_{2\alpha}$ acted as a strong

vasoconstrictor and as smooth muscle constrictor, which contributes to its abortifacient action by decreasing the blood flow to uterus and by premature contractions of uterus.

B. Peptidoglycan and Pregnancy Loss

Peptidoglycan-polysaccharide complex (PG-PS) makes the structural support of the cell wall of Gram-positive bacteria. Bacteria lysed in the host release this compound and thus elicit an immune response, which has been found to be detrimental to pregnancy maintenance in sheep (Barker 1999), and humans (Gibbs and Blanco, 1981). Women infected with aerobic *Strep* of Group A, B, D, *Strep pneumonidae* or viridans streptococci had either endometriosis (inflammation of uterus) or chorioamnionitis (inflammation of chorioamnion) with early onset of fever as the main clinical presentation (Gibbs and Blanco 1981). Fortunato et al. (1998) stimulated cultured human amniochorion from uncomplicated gestations with PG-PS from Group A lytic *Strep* and found significant increases of IL-6 and IL-8, both mRNA expression and protein. However, IL-6 and IL-8 release response was found much greater with LPS challenge (Fortunato et al. 1998). Some suggested that abnormal colonization of bacteria in the genital tract increases prostaglandin synthesis, which initiates labor in humans (Bennet et al, 1987; Lamont et al, 1990; Reisenberger et al., 1997), however, others suggest that the early onset of labor is not caused by mere presence of bacteria, but by the endogeneous host mediated mechanism involving inflammatory cytokines (Romero et al., 1989; Mitchell at al., 1990, Fortunato et al., 1998).

VII. Vaccination with Peptidoglycan-Polysaccharide Complex: Possible Prevention of Early Embryonic Loss and Mastitis

A. Principles of Vaccination

Vaccination by definition is the deliberate induction of adaptive immunity to a pathogen by injecting a vaccine, which is a dead or attenuated form of the pathogen (Janeway et al., 2001, p.706). Edward Jenner, in 18th century, was the first to use vaccinia. He injected a cowpox virus in a young boy, which lead to immunity to the human smallpox virus (Janeway et al., 2001, p.1). Vaccination causes naïve lymphocytes to proliferate and differentiate into effector cells and memory cells (Sprent and Tough, 1994). The cellular and molecular events that occur after successful primary vaccination depend on the type of organism in question, bacterial, viral, parasitic, or fungi, and its "favorite" environment in the host – extracellular (interstitial spaces, blood, lymph, or epithelial surfaces) versus intracellular (cytoplasmic or vesicular, Janeway et al., 2001, p.386). The type of organism and location of infection determine the type of the immune response. Immune responses can be humoral by the generation of protective antibodies (immunoglobulin (Ig) G, IgA, IgE) or cellular by the activation of CD4 T cells (Th1, Th2, supressor, or CD8 –CTL). The cascade of events initiated after inoculation with an antigen includes inflammatory response and activation of innate immunity; activation of specialized antigen presenting cells; antigen presentation to lymphocytes in peripheral lymphoid organs; activation and signaling through the lymphocyte receptors; clonal expansion of lymphocytes and generation of effector cells; destruction of antigen; and generation of immunological memory.

It is the immunological memory that enables successful vaccination and prevents reinfection with pathogens that have been repelled successfully by an adaptive immune response. Immunological memory is the most important biological consequence of the development of adaptive immunity, although its cellular and molecular basis is still not fully understood. At the lack of antigen, B cell proliferation and hypermutation stops. As well, the B-T cell interaction will be terminated, and the effector cells die out. Only a relatively small subset forming the memory cells will remain to serve quickly in future antigen exposure. Recent suggestion of Callan and coworkers (2000) is that memory cells have different clonotype than the primary effector cells, which are predestined to die by apoptosis after depletion of the antigen.

B. Gram-Negative Vaccines

Rioux-Darrieulat (1978) prevented abortion induced by endotoxin, using antisera to endotoxin, raised in different animals. This form of passive immunization protected pregnant mice even more effectively, when both antiserum and antiserotonin were used. A summary of investigation of immunization and immunotherapy for mastitis during the eighties and early nineties (Tyler et al. 1993) led to development of vaccines for gram-negative mastitis, but development of vaccines for other mastitic pathogens has been slower. Vaccine to coliform mastitis J5 has been developed and tested. Hogan et al. (1999) reported that heifers immunized with *E. coli* J5 bacterin subcutaneously 60 and 32 days before calving, and then again within 48 hours after calving, had reduced severity and local signs of clinical mastitis, after being challenged with *E. coli* via intramammary infusion one month after calving. (This report seems to be misleading, because the data do not appear to support the conclusion that the vaccine was effective. Perhaps the choice

of statistical procedure to analyze the results was not appropriate.) Profitability of using J5 vaccine was evaluated by DeGraves and Fetrow (1991). They enumerated that it was profitable to use J5 vaccine if 1% or more of cow lactations resulted in clinical coliform mastitis.

C. Gram-Positive Vaccines

Preventive herd management practices that include dry cow therapy, segregation of sick and healthy cows, and selective culling have helped to decrease mastitis prevalence. However, *S. aureus* is still a major problem on many farms (Sears, 2002). *S. uberis,* as one of the major environmental pathogens, is also problematic since it is ubiquitous in the dairy environment and is associated with sub-clinical mastitis. Development of effective Gram-possitive bacterial vaccines could greatly enhance milk production and reproductive performance as well.

Schleifer and Seidl (1974) synthesized a C-terminal D-alanyl-D-alanine pentapeptide (Glycine-L-Alanine-L-Alanine-D-Alanyl-L-Alanine) component of bacterial PG and rendered it immunogenic by coupling it to a carrier protein –human serum albumin. Immunization of rabbits with this conjugate lead to generation of antisera and, these antibodies showed cross-reaction with solubilized peptidoglycan carrying the same pentapeptide and with another conjugate with similar, but different, pentapeptide. This indicated that these three antigens (whole and two different PG and pentapeptide) had identical determinant groups with respect to the antisera employed. Daily pretreatment of rabbits with small doses of peptidoglycan induced tolerance to the pyrogenic effect (Lavický et al., 1988).

Reports of vaccination of dairy cows against environmental pathogens such as *Strep uberis* and *Strep dysgalactiae* are limited (Potter, 2002). Current research is oriented towards identification of conserved potential virulent factors on the bacterial cell surface as targets for vaccines (Song et al., 2001; Potter, 2002). Vaccination with live *Strep uberis*, and soluble surface extract derived from the same bacteria did result in protection against experimental challenge (Hill et al., 1994), but it was less effective against heterologous strains of *Strep uberis* (Finch et al., 1997). Killed *Strep uberis* administered repeatedly also provided some protection against subsequent challenge, however, that treatment did not reduce inflammation of the mammary gland (Finch, 1994). Recently, vaccination with a concentrated *Strep uberis* culture supernatant, containing PauA, a plasminogen activating protein produced by this pathogen has been used by Leigh et al., (1999, 2000). PauA was proposed to be one of the determinants of *Strep uberis* aiding in dissemination of the bacteria into tissues at the site of infection (Leigh, 1994; Rosey et al., 1999). Subcutaneous vaccination resulted in 37.5 to 62.5 % protection from clinical mastitis after challenge with heterogous strain (Leigh et al, 1999).

Potter (2002) focused on development of vaccines to *Strep uberis* and *Strep agalactiae* using recombinant GapC (6xHis), Mig, and CAMP factors. GapC is the bacterial surface-associated glyceraldehydes-3-phospate dehydrogenase capable of providing broad spectrum protection (Potter, 2002). The mig gene of *Strep dysgalactiae* encodes two Mig protein-binding receptors, α_2-macroglobulin (α_2-M) and immunoglobulin G (IgG), and it was speculated that binding of bacterial Mig proteins to plasma protein α_2-M or IgG in a host prevents phagocytosis by bovine neutrophils (Song

et al., 2001). Song et al. (2002) also found that Mig protein of *Strep dysgalactiae* bound bovine immunoglobulin A (IgA). IgA binding proteins seem to represent bacterial protection against phagocytosis, because the binding of human IgA to receptors in *Strep pyogenes* has been found to interfere with bacterial adhesion to the host cells (Fluckiger et al., 1998). Vaccination with recombinant *Strep uberis* GapC or CAMP-3 resulted in significant reduction in inflammation of cows' mammary glands several days post-challenge, especially for *Strep uberis*. Inflammation was not reduced in *Strep dysgalactiae* vaccinates, suggesting lack of cross-species protection (Fontaine et al. 2002).

VIII. Summary

Mastitis is an unresolved problem in the dairy industry. Main components, PG-PS and LPS, of the bacteria involved in mastitis cause an array of biological disturbances in the host, including negative effect on embryo /fetus survival. A partial solution to this problem could be to administer agents that prevent the action of inflammatory mediators or drugs that kill bacteria. Prevention of mastitis via management practices and immunization against mastitis would be more economical and effective and should result in protection of early pregnancy. The aim of this study was to find out if immunization with bacterial peptidoglycan can lessen the negative impact of bacterial challenge soon after breeding and during lactation.

OBJECTIVES

The primary goal of this study was to induce the protective immune mechanism by repeated inoculation of ewe lambs with PG-PS or the whole killed bacteria of *Strep pyogenes*. The experimental hypothesis was that the immunization will enhance early pregnancy maintenance in ewe lambs which are exposed to pathogen shortly after breeding. Similarly it was hypothesized that immunization with PG-PS and/or with killed cells will decrease occurrence of mastitis during the first lactation in ewe lambs.

The secondary goal was to compare effects of whole heat killed cells of *Strep pyogenes* and PG-PS isolated from *Strep pyogenes* on two characteristics, pregnancy and mastitis. The associated experimental hypothesis was that the PG-PS and whole killed cells elicit different response in terms of pregnancy maintenance and mastitis. If the effect will not be different and both preparations are beneficial for animals used, the whole cells will be easier to obtain.

Ewe lambs have the advantage of lower cost and shorter gestation than dairy heifers, as well as the fact that they have not previously experienced mastitis.

MATERIALS AND METHODS

I. Bacteria Growth and Peptidoglycan-Polysaccharide Isolation

Peptidoglycan-polysaccharide-complex was isolated from *Strep pyogenes* as summarized by Stetson and Dziarski (1994), except for modification of Wells et al. (1986) which did not include removal of the teichoic acid and carbohydrates. Therefore, the name of the product was peptidoglycan-polysaccharide complex (PG-PS). This product closely resembled the commercially available PG-PS 100P (Lee Laboratories, Grayson, GA, product # 210868) as assessed by Barker (1999).

Aseptic techniques were used following the Biosafety Level 2 standard and special practices and regulations (HHS, Center for Disease Control 93-8395). All the manipulations with cultures were performed in a laminar flow hood with UV light decontamination. The solutions were sterilized before use. The chlorine solution was poured into the waste media after filtration, and all waste and utensils were sterilized by autoclaving.

(i) Growing and harvesting the bacterial cells: Freeze-dried specimen of *Strep pyogenes* group A, type 3 (American Type Culture Collection, (ATCC), 10389) was rehydrated with 5 - 6 ml of Brain Heart Infusion (gift from Dr. M. Elliott, Department of Immunology and Microbiology, West Virginia University) according to ATCC propagation procedure and used to inoculate Trypticase Soy Agar (TCA) with 5% defibrinated sheep blood (Fisher, B21239X). Plates were incubated at 37˚C for 48 hours; the rest of the revived culture was divided into aliquots of 2 mL volume (1.7 mL of *Strep pyogenes* culture plus 0.3mL of sterile glycerol) and frozen at - 80˚C. The colonies of *Strep pyogenes* were identified on the agar plates as entire, glistening, circular, smooth,

small, and by almost translucent agar in immediate vicinity of growth due to lysis of red blood cells (Girard, 2000). The isolated colony was used to inoculate 10 ml of sterilized Todd Hewit Broth (THB) (Fisher, DF0492-17-6), and incubated in loosely capped glass tubes over-night at 37˚C. After 24 hours, the content were transferred into 100 mL bottles with screw caps containing sterilized THB using 1:10 dilutions, and left in incubator at 37˚C overnight. The glass bottles with sterilized, 900 mL of THB were inoculated after 24 hours with the bacterial content of 100 mL tubes and left in incubator at 37˚C for 24 hours again.

Bacteria were captured by filtration using sterile disposable 500 ml bottle-top filters with PES membrane (Nalgene, HX8199H, pore size 0.45 μm) using vacuum pumps. One filter was used for one liter of media with bacteria. Bacteria were washed on filters once with 50 mL of sterile phosphate buffered saline (PBS, 0.1M NaH_2PO_4 and 0.15 M NaCl, pH 5.3) and harvested by scrapping with a sterile metal spatula, put in tubes, weighed and washed two more times with 50 mL PBS, followed by centrifugation at 2,500-x g for 20 min at 4˚C and removal of supernatant. Tubes with cells and supernatant from last wash were stored in refrigerator until sonication. The average yield from 1 L of media was 1.18 g wet bacterial cells. Bacterial growth was carried out in batches of about 10-12 liters. After 13 batches, 134 g of wet cells were produced.

(ii) Rupturing the cells by sonic treatment: The supernatant from the last centrifugation was gently removed. Cells were pooled and resuspended in PBS (approximately 50 g wet weight in 180-200 mL PBS). Suspensions were sampled for Gram-staining (Gram Stain Set, Fisher, 2300583). Weight of wet cells in pooled suspension prepared for sonication was recorded. The suspension was sonicated in 70 ml batches for 90 minutes

(Heat System Ultrasonics, Inc.: Long Island, NY, used the middle probe and amplitude 5). The sonication was done in the hood, and the vessel with cell suspension was partially submerged in the ice water bath, adding the ice frequently. During sonication, the suspension was covered by aluminum foil and gauze soaked in 70 % alcohol to ensure sterility.

The ultrasonic liquid processor converts electrical energy to mechanical vibration. This causes cavitations in the liquid sample such that microscopic vapor bubbles are formed momentarily and implode, breaking cells or tissue by powerful shock waves.

After sonication, suspension was transferred to sterile tubes and centrifuged for 30 min at 10,000 x g, at 4˚C. The supernatant, containing the cell wall fragments, was aspirated with a sterile Pasteur pipette, and stored in sterile containers. Pellets were weighed, resuspended, pooled, sonicated, and centrifuged again, until the pellet was less than 20% of the original wet weight. Total sonication time for 134 g of cells was 54 hours. Success of the sonication treatment was checked by Gram-staining of samples from the supernatants. Gram-stain principle is differential retention of dye in the two main classes of bacteria. To perform the procedure, cells were stained with crystal violet and added iodine. The slide was decolorized with alcohol/acetone and counterstained with safranin or basic fuchsin. Crystal violet forms a complex with iodine in the protoplast of organism/cell, recognized microscopically as dark purple. Organisms that retain the dye after decolorization with alcohol are classified as Gram-positive, and those that are decolorized when counterstained are Gram-negative. Bacteria containing lipopolysacharides have higher lipid content in their cell envelopes compared to other bacteria, which makes them more permeable to alcohol, thus the dye is washed away.

When the Gram-positive cell walls are fractionated after sonication, the cells are unable to retain the dye, thus they behave as Gram-negative. This is what was observed. Sterility of the cell wall fragments was confirmed by culturing 0.1 mL on sheep blood agar (Sartor et al. 1985).

(iii) Purification of the PG-PS: To remove the cell constituents, pooled supernatants were treated sequentially by different enzymes (Sigma, St. Louis, MO) in dialysis tubing.

a) hyaluronidase (H6254 Type V) in 0.1 M phosphate/0.15 M NaCl (pH 5.3);
b) deoxyribonuclease (D5025; 375,000 units) and ribonuclease (R5000, Type II-A) in 0.1 M phosphate buffer (pH 7.2);
c) papain (P4762) in 0.1 M phosphate buffer (pH 7.2) containing 0.001 M cysteine (C8882), 0.001 M EDTA and 0.05 % sodium azide, and
d) pepsin (P6887)in 0.012 N HCl (pH 2);

The amount of 1.24 mg of deoxyribonuclease and 0.0025 mg of all other enzymes per mg wet weight of cells was used. Dialysis tubing (110 mm wide) was cut to 10 inches length and softened by boiling in 2 % sodium bicarbonate and 1 mM EDTA solution for 10 minutes, rinsed in deionized water, boiled in deionized water for 10 minutes, and then rinsed and stored at 4˚C while submerged in deionized water until used. The dialysis bags were filled with the cell wall fragments (CWF) suspension, clamped with closures (Fisher, Spectra/Por Nylon, 0867154) and placed in a large beaker containing appropriate buffer with a few drops of chloroform (Sigma, C5312) for 12 hours 4˚C. The next day, hyluronidase was added to CWF, while dialyzing for six hours on a hot plate (37˚C) using a stir bar and changing the buffer every two hours. After six hours, the appropriate buffer for the next enzymatic digestion was poured in to the beaker for overnight dialysis

with few drops of chloroform, and the process was repeated. Enzyme digestions were during the day and buffers were changed overnight at 4˚C. For each enzyme, quality control needed to be carried out, using hyaluronic acid, DNA, RNA, hemoglobin, and homogenized animal tissue (chicken meat was used) as substrates for enzymes hyaluronidase, deoxyribonuclease, ribonuclease, papain, and pepsin, respectively. The CWF was collected and washed by repeated centrifugation at 100,000 g (ultracentrifugation), keeping the pellet. Thus the technical name of the final product is PG-PS 100P (Lee Laboratories, 1997). The pellets were resusupended in 0.1 M phosphate buffer and ultracentrifuged, the process was repeated two more times. The final pellets were resuspended in 200 mL of sterile water, put in dialysis bags and dialyzed against water at 4˚C for 12 hours.

Chloroform:methanol lipid extraction took place at room temperature. Dialysis bags were opened in 1000 ml beakers, and volume of CWF in water was measured in a graduated cylinder. Chloroform : methanol (CM) (Sigma, C5312, M1775, respectively) mixture (2:1) was added to the CWF water suspension such that the final ratio of CM to CWF was 5:1 and stirred for two hours. After letting the CM : CWF in water suspension sit for awhile, the phases visibly separated. The chloroform phase with the lipid, the bottom phase, was aspirated with a sterile pipette connected to a vacuum pump. The volume of methanol with sample was measured. After subtracting the initial volume of the sample, the volume of methanol was determined. Chloroform was added to methanol with sample to keep the 2 to 1 chloroform : methanol ratio and then more CM was added to equal the initial extraction volume. The process was repeated twice. The water phase with CWF was then dialyzed against water, changing the water three times during twelve

hours. The product was then poured from dialysis bags into a pan, shell frozen with dry ice and 2-propanol, covered with sterile gauze and lyophilized for about 20 hours to ensure adequate drying. Dried PG-PS was white, thin aggregates. The original 134 g of wet cells of *Strep pyogenes* produced 0.88 g of PG-PS, which means that 1 gram of wet cells yielded 0.0065 g of PG-PS.

(iv) PG-PS product analysis: The amount of methylpentose molecules in PG-PS was determined by the rhamnose assay described by Dische and Shettles (1948). Rhamnose (R3875) and cysteine hydrochloride (C6852) were purchased (Sigma, St. Louis, MO), and commercial PG-PS was purchased (Lee Laboratories). Prepared solutions were: 2 mL of rhamnose standard solution with concentration 0.005 g/mL of water; 250 µL of PG-PS solution (0.00008 g /µl); 117 mL of sulfuric acid solution (16.71 mL of water and 100.28 mL of sulfuric acid); and 2.6 mL of 3% cysteine hydrochloride. Water was added to twelve pairs of tubes with 200, 150, 100, 50, 20, 10, and 5 µL of rhamnose solution and 50, 25, 12, 8, and 5 µL of PG-PS sample solution, and to two empty (blank) tubes, to bring the volume to 1 mL in each. Then, to all tubes in cooling ice, 4.5 mL of water/sulfuric acid was added. The mixture was then warmed to 20 to 22˚C for few minutes, held in an actively boiling water bath for 10 minutes, and then cooled in tap water. When cool, 0.1 mL of 3% aqueous cysteine hydrochloride solution was added to each tube and shaked. Reaction of this mixture gave a greenish yellow color. The absorbance was measured via a spectrophotometer (Beckman DU-600). A standard curve was developed from known rhamnose concentrations, using linear fit (Absorbance = 0.15414 + 0.00421 x Concentration) of version 3.0 of JMP (SAS Institute, Cary, NC) statistical software. The concentration of rhamnose in the PG-PS product was estimated,

adjusted for volume of PG-PS solution in each tube and averaged. Two readings that exceeded the linearity of the spectrophotometer (> 3) were excluded. The average rhamnose concentration was 0.51 mg in 1mg of PG-PS (51 %).

(v) Determining the dosages of PG-PS: Concentration of 51 % of rhamnose in isolated PG-PS corresponds to the PG content (Lichtman et al., 1993). One g of the wet cells of *Strep pyogenes* had 0.0033g of PG. Barker (1999) showed marked pregnancy reduction with 60 µg PG/kg body weight (bw). Therefore, that dose was chosen to be the challenge injection and 30 µg PG/kg bw to be an initial and booster injection. We had 0.51g PG in 1 g of PG-PS product, (1÷0.51=1.96). To attain 30 µg PG/kg bw for first two injections, 58.82µg of PG-PS (30 x 1.96) was required per kg. Considering average weight of ewes to be 50 kg, approximately 3 mg of PG-PS per ewe and 150 mg for 50 ewes was suspended in 150 mL of sterilized 0.9 % sodium chloride solution. Prior to injection, product was sonicated for 90 min to ensure the solubility and prevent the aggregation of PG-PS molecules. The dose for one ewe was 3 mL of suspension. Dose for the challenge injection (60µg PG/kg bw) was calculated the same way.

II. Preparation of Whole Killed Cells

Strep pyogenes were grown in three batches as described earlier, producing 27 g of wet cells. Prior to harvesting the cells, the flasks with bacteria in media were submerged into 60 ºC water bath for one hour to kill bacteria by pasteurization. Then, the cells were harvested by filtration as before and washed and spun 4 times using centrifugation (Sorvall ®Super T21, 5000 x g, 30 min, 4ºC) and 0.9 % sterile sodium chloride (saline). The supernatant was discarded. After the last wash, cells were

suspended in sterile saline (14 mL for each g of wet cells). The suspension was divided into aliquots and frozen until required.

The dose of killed cells was determined via the approximate PG-PS content in whole cells based on rhamnose assay. Approximately 0.0065 g of PG-PS was in 1 g of wet cells. To ensure 30 μg PG/kg/bw, 0.00003 g divided by 0.0065 is 0.00461 g of cells per kg, which means 0.23 g of wet cells per 50 kg ewe, suspended in 3 mL of sterile saline. The dose of whole killed cells preparation for immunization injection was based on the PG-PS instead on PG (30 μg PG-PS/kg/bw, instead of 30 μg PG/kg/bw).

III. Assignment of Treatments

A total of 200 Rambouillet ewe lambs were assigned at random into 4 treatment groups (n = 50 per group).

- Group 1 (**G1, Immunized with PG-PS**) sheep were immunized by subcutaneous administration of a preparation of PG-PS (30 μg PG /kg body weight (bw)) on day 0 early in October 2000. A second (booster) injection of PG (30 μg/kg bw) was given on day 22. At day 36, estrus was synchronized, and ewes were bred to rams at estrus around day 42. On day 5 after breeding (~ day 47 from first inoculation), ewes were injected with a higher dosage of PG-PS (60 μg PG/kg bw) via jugular vein.

The final injection on day 5 after breeding is served to mimic the real bacterial challenge, imitating infection and inflammation such as mastitis. Therefore, for the rest of this document, the last injection is referred to as a challenge.

- Group 2 (**G2, Immunized with killed cells**) ewes were immunized subcutaneously at day 0 and day 22 with heat killed bacterial cells, then had estrus

synchronized on day 36, and were observed for estrus and bred. On day 5 after breeding (~ day 47), ewes were challenged with intra-venous injection of PG-PS (60 μg PG/kg bw).

- Group 3 (**G3, Not immunized, challenged**) ewes received the physiological solution-saline at the time of first two injections as G1 and G2, then were synchronized, observed for estrus, bred and challenged with the same dose of PG-PS (60 μg PG/kg bw) as G1 and G2 ewes on day 5 after breeding.

- Group 4 (**G4, Saline**), as a control group, received only saline at day 0, 22 and 47.

Blood samples were collected from the jugular vein every two weeks, from day 0 until day 84.

An additional 35 mixed breed ewes were randomly divided into 4 groups and were treated as above. In this group, blood samples were taken once every week from day 0 and daily after challenge (day 5 after breeding) until day 42 of pregnancy (day 84 from initial inoculation). For the first 6 hours after challenge, blood samples were taken every 15 minutes and then every day until pregnancy check (d 42). The body temperature was monitored in the group of 35 ewes for 12 hours after each injection (day 0, 22 and 47) and then 24 hours post injection.

IV. Animals

Experimental animals were ewe lambs born in Fall 1999 to March 2000. At the USDA Sheep Experiment Station in Idaho 200 Rambouillet sheep were on the ranch from early spring to late summer and then housed in pens of about 50 ewes outside. Of 200 Rambouillet ewes initially treated, 160 responded to estrous synchronization, thus

were bred and challenged with PG-PS or killed cells or treated with saline. The second group was 35 mixed breed Dorset and Suffolk ewes at the West Virginia Agriculture and Forestry Experiment Station Animal Science farm in Morgantown, housed in a three-sided barn with expanded mesh metal flooring. Of 35 mixed breed ewes 32 responded to estrous synchronization and were bred and challenged. All procedures were approved by West Virginia University Animal Care and Use Committee (ACUC #9801-06). Estrous behavior of ewes in the presence of vasectomized rams was observed prior to beginning the experiment in the Fall 2000. Vaginal progesterone pessaries (MAP, Canada) were inserted into the ewes, and the vasectomized rams were removed from the group. Five days later, ewes received one intramuscular injection of prostaglandin $F_2\alpha$ (3 mL, 5 mg/mL; Lutalyse; Pharmacia and Upjohn, Inc. Kalamazoo, MI). After another 5 days, pessaries were removed, and ewes received an injection (5 mL im) of commercial gonadotropins (PG-720-1, Intrervet, Charlotte, NC). The majority of ewes were in estrus within 48 hours. Fertile rams with chest markers were put in pens with ewes at the ratio of one ram per ten ewes.

V. Collection of Data on Pregnancy and Mastitis

Pregnancy status was examined in all ewes on day 42-post breeding by transrectal ultrasonography (Schrick et al. 1993), using an Aloka 500 console with a 7.5 MHz linear transducer designed for examination of the human prostate (Corometrics Medical Systems, Wallingford, CT). The age of an embryo was based upon size. Pregnancy data were confirmed by another pregnancy diagnostics at day 60 of gestation using the sector scanner.

Milk samples were collected from mixed breed ewes (N=35) in the middle and at the end of lactation (39 and 98 days post partum, respectively) by a described procedures (Keisler et al. 1992, Fernández-Garayzábal, 1998). The first 5 mL of milk from each half of each ewe's udder was discarded. Teat ends were cleansed with gauze moistened in 70% ethanol, and about 5 mL was collected directly on to the California Mastitis Test plates and evaluated according to manufacturer's instructions. Additionally, 5 mL of milk was collected directly into a sterile tube without touching it and placed on ice. Within 2 hours of collection, 0.01 ml of milk was transferred using disposable bacteriological eye loops onto Petri plates containing Trypticase Soy Agar (TCA) with 5% defibrinated sheep blood (Fisher, B21239X). Plates were incubated at 37 ºC for 48 hours before examination for microbial growth. Determinations were made on the basis of colony morphology and Gram-staining (Schneierson, 1965).

Mastitis could not be evaluated in Rambouillet ewes due to an unexpected disease. A *Camplylobacter* infection that resulted in 65 % of late abortions or early death after birth affected most of the animals at the sheep experiment station.

VI. Assays of Antibody

Activation of humoral immunity by vaccination was determined by measuring the IgG antibodies to PG-PS in serum. Enzyme-linked immuno-sorbent assay (ELISA) protocol was adapted from Barker et al. (1998) and Yamamoto et al. (1996).

Flat bottom ELISA plates (96WL Easywash, HB, 07-200-642, Fisher Scientific) were coated with 100 µL/well of sonicated PG-PS solution (0.002 µg PG/µL phosphate buffered saline (PBS) = 2µg/mL in PBS with 0.04 % sodium azide) and were covered and left overnight at 4 °C. Plates were washed four times with PBST (PBS +0.5 % Tween

20, no sodium azide in a wash buffer), using an automated washer and then 200 µL/well of blocking agent PBSTG (50 µl Tween 20 and 0.5 g porcine gelatin in 100 mL of PBS, stirred and heated at 37°C for 10 min) was added and left overnight at 4 °C. Fresh PBSTG was prepared every time before each use, as it gelatinizes in the refrigerator. Therefore, before the next wash, plates were left at room temperature for an hour for gelatin to soften. Plates were washed four times with PBST, and 100 µg/well of primary antibody –sheep plasma samples gradually diluted in PBSTG were added and incubated overnight at 4 °C. Plates again were washed four times with PBST, and 100 µl/well of secondary antibody - alkaline phosphatase conjugated rabbit antisheep IgG (H+I) diluted 1: 3000 in PBSTG (RBT anti-sheep IgG AP OB6159-04 Fisher Scientific) was added and incubated at 37 °C for 1 hour. Plates were washed six times with PBST. The enzyme substrate P-nitrophenyl phosphate (1 mol/L diethanolamine was added with 0.5 mmol $MgCl_2$ pH 9.8, OB201-01, Fisher Scientific) was disolved in substrate buffer (24.5 mg $MgCl_2.6H_2O$ and 48 ml diethanolamine add to 400 mL double distilled H_2O, adjusted to pH 9.8 with 12N HCl, brought to 500 mL with distilled water, stored at room temperature) immediately before use, and 100 µL/well was added. Plates were covered with aluminum foil to ensure darkness for 10 minutes thus allowing development of color. Plates were read at a wave length of 405 nm (Universal Microplate Spectrophotometer; µQuant; BIO-TECH Instruments, Inc., Winooski, VT).

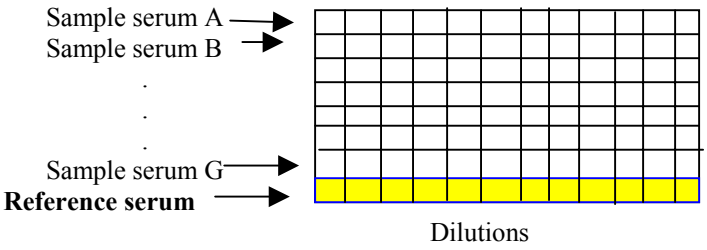
Serum samples analyzed by ELISA were from prebleed (d 0), day 13, 35, 47, and 84 from 10 randomly selected Rambouillet ewes from each treatment group (200 samples); and prebleed, day 47, 68, and 289 of all mixed breed ewes (134 samples). Each sheep serum sample was assigned to a row on the ELISA plate and was diluted with

PBSTG using 12 dilutions, beginning with 1:40 in the first well and continuing at doubled dilution rate to 1:81,920 for samples from days 0, 13, 68, 84, and 289. For samples of days 35 and 47, first well dilution was 1:80 and continued to 1:163,840 in the 12th well. One ewe's serum (Group 1, Day 13, ewe number R1580) with representative antibody response was designated as the *"reference serum"* and was repeatedly used on each plate to measure variability between plates and titer. Two wells on each plate had everything but primary antibody (blank). Dilutions of the sera were prepared in less expensive, U shaped round bottom well plates (Cooke Engeneering Co., Alexandria, VA; "U" Plate Disposable 220-24 Microtiter) and transferred to the ELISA plates using a multi-channel pipette.

The highest dilution of serum when the antibody is still detectable is referred to as titer. The optical density (OD) data from spectrophotometer were transformed into titers utilizing the OD measurements of reference serum, as described in Figure 1. Statistical analysis was done using the log of titers.

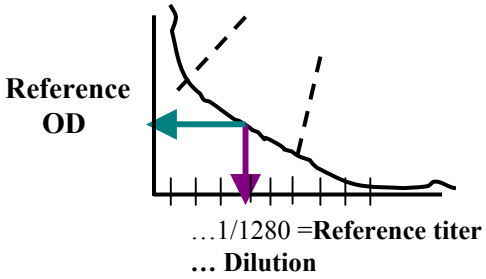
Figure 1. Algorithm of IgG titer determination.

1. ELISA results have Optical Density readings (OD).

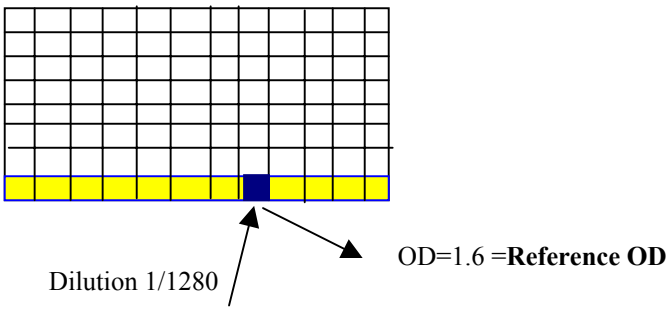


2. For the **Reference serum** on each plate:

- Plot the reference serum ODs against its dilutions.
- Determine the linear part of the curve.
- Determine dilution associated with the1/2 maximum point on the linear portion of the curve and corresponding dilution (**Reference titer**, in this case, 1/1280).



- On the ELISA plate, read what **Reference OD** of **Reference serum** is associated with the dilution 1/1280 (ex. 1.6).



- For each sample on the plate, find the reading closest to Reference OD of 1.6.
- Read what dilution is associated with that well and record it.

3. Move to another ELISA plate and repeat the process again.

VII. Statistics

The primary experimental hypotheses were:

1. Immunization positively affects the pregnancy outcome.

2. Immunization positively affects the mastitis outcome.

The secondary experimental hypotheses:

3. Effects of peptidoglycan and killed cells on pregnancy do not differ.

4. Effects of peptidoglycan and killed cells on mastitis do not differ.

Variables measured were proportion of ewes pregnant on day 42 of gestation, and incidence of mastitis in the middle and at the end of lactation. Immune response was verified by monitoring fever and IgG antibodies.

To test specified hypotheses, preplanned comparisons 2 and 3 indicated in Table 1 were evaluated using Pearson Chi-square test of homogeneity and Fisher's exact test for proportion of ewes pregnant and occurrence of mastitis

Table 1. Layout of treatments and planned contrasts for statistical analysis

Treatment/ Comparison	**G1** Immunized w. PG-PS	**G2** Immunized w. killed cells	**G3** Not immunized challenged	**G4** Saline
Comparison 1	-1	-1	-1	3
Comparison 2	-1	-1	2	
Comparison 3	-1	1		

In comparison 2, homogeneity of pregnancy counts or mastitis counts is tested for all immunized (G1 and G2) and Not immunized, challenged (G3) ewes. In comparison 3, homogeneity of pregnancy counts or mastitis counts is tested for immunized with PG-PS (G1) and immunized with whole killed cells (G2).

Logistic Regression analysis of JMP statistical software (Version 5.0, 2002, SAS Institute, Cary NC) was used for prediction of pregnancy from cumulative dose of PG-PS (day 0 + day 22 + day 47 doses in G1, G3, and G4 ewes). Fever and antibody response were analyzed using repeated measures analysis of variance of SAS GLM Procedure (1999, SAS Institute, Carry, NC) and Chi-square test of association of JMP (2002). Significance level was 0.05.

VIII. Responses of Body Temperature and Antibodies

Body temperature (BT) was measured in rectum of ewes using digital thermometers every hour for 12 hours after the inoculations (day 0 and 22) and then at 24 hours and after the challenge injection (day 47) in mixed breed ewes. Repeated measures analysis of variance enabled to verify that treatment influenced BT, to determine whether the time after inoculation influenced BT, and whether there was a treatment by time interaction. Since G3 and G4 ewes all received saline only at first and second inoculations, they were grouped together (G3,4 or Saline). Body temperature changes after first inoculation are represented in Figure 2. There was a significant interaction of treatment and time ($p<0.0001$). Although BT increased within first 3 hours in all groups, BT continued to increase in G1 and G2. Temperature was highest in G2 with a peak 40.2°C at 10 hours after inoculation. Analysis of contrasts "Hour * Saline *versus* G1 and G2" and "Hour * G1 *versus* G2" were both significant ($p < 0.0001$ and $p < 0.01$, respectively). The BT was elevated after the second injection (Figure 3) in G1 and more so in G2 ewes, reflecting significant hour by treatment interaction ($p < 0.0001$). Again, the contrasts "Hour* Saline *versus* G1 and G2" was significant ($p < 0.0001$) as well as "Hour * G1 *versus* G2" ($p < 0.0001$). At the challenge injection (Figure 4), there was

also hour by treatment interactions, ($p < 0.05$). Only "Hour * Saline *versus* G1, G2, and G3" comparison was significant ($p < 0.0001$). Occurrence of fever, determined by increase in BT > 0.7 °C from the BT before injection, was also evaluated (Lavický et al, 1988). Summary of occurrence of fever is in Table 2. After first inoculation, 67 % of G1, 89 % of G2 and 47 % of Saline treated sheep had fever ($p > 0.1$). Distribution of occurrence of fever after second injection (D 22) was 44 % in G1, 100 %, in G2 and 12 % in Saline group ($p < 0.0001$). On the day of challenge injection (Day 47) all of the sheep in G1 and G2 had fever, 75 % of ewes challenged and not immunized (G3) had fever and 25 % of saline treated ewes ($p < .05$).

Figure 2. Body temperature after first inoculation (Day 0)

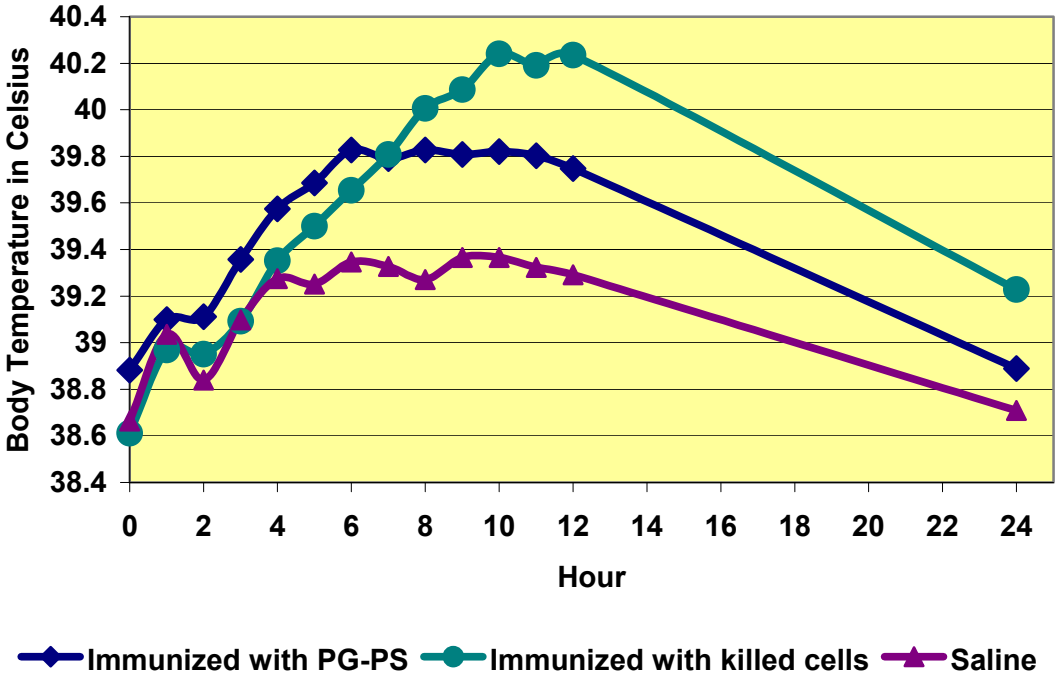


Figure 3. Body temperature after second inoculation (Day 22)

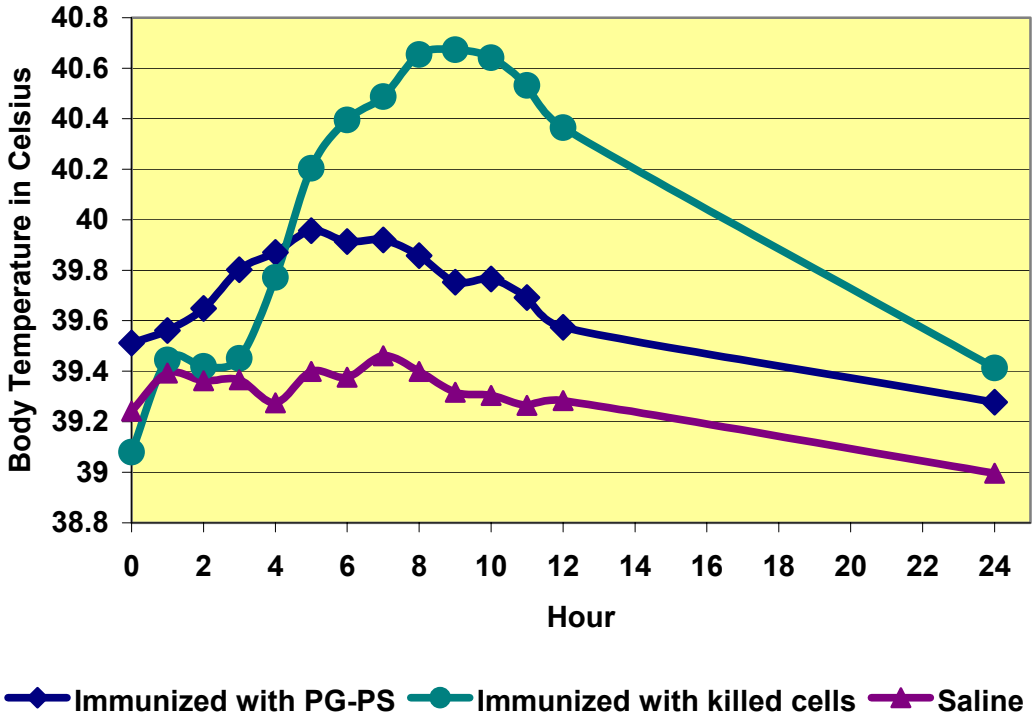


Figure 4. Body temperature after PG-PS challenge injection (Day 47)

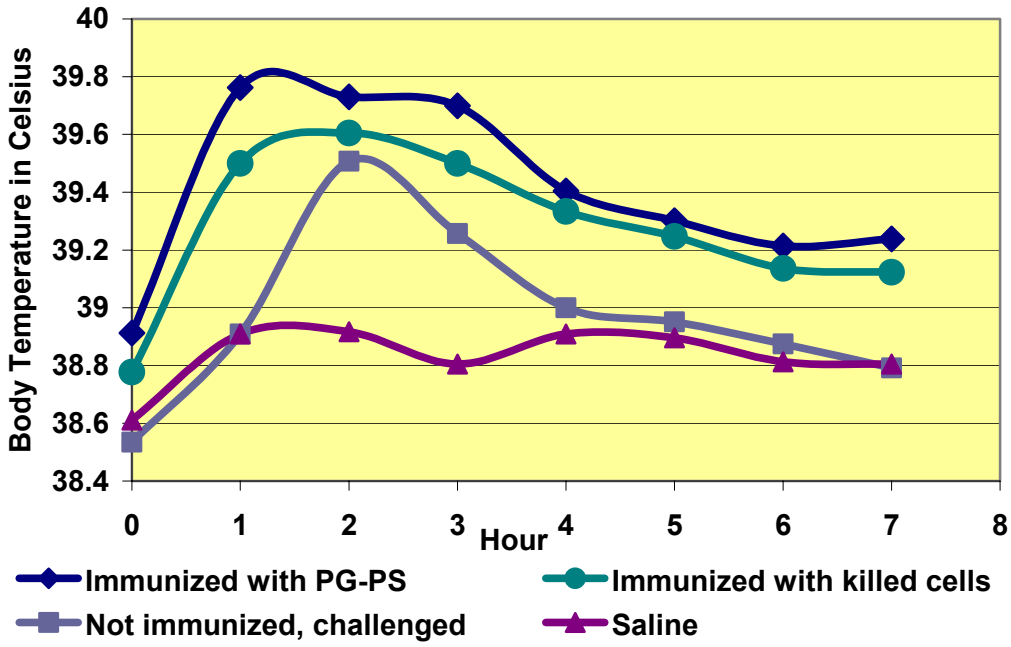


Table 2. Distribution of fever (increment of 0.7 ºC) occurrence in mixed breed ewes at the times of inoculations.

Treatment **Day / Fever**	**G1** Immunized **with PG-PS**	**G2** Immunized **with killed cells**	**G3** Not Immunized, **challenged**	**G4** Saline	**p-value**
D 0	6/9 (67%)	8/9 (89%)	8/17 (47%)		0.11
D 22	4/9 (44%)	9/9 (100%)	2/17 (12%)		<0.0001
D 47	7/7 (100%)	9/9 (100%)	6/8 (75%)	2/8 (25%)	0.0011

The PG-PS IgG antibody response was evaluated separately from Rambouillet and mixed breeds and is presented in Figures 4 and 5, respectively. Treatment and day interaction was detected for both breeds ($p < 0.0001$ in both). Also, titers in immunized animals (G1 and G2) compared to challenged not immunized ewes, differed depending on time in each group, ($p < 0.0001$ in each). Titers for PG-PS-treated ewes (G1) compared to killed cells-immunized ewes (G2) differed in Rambouillet ewes ($p < 0.05$), but not in mixed breed sheep ($p = 0.2$). In Rambouillet ewes, killed cells were more effective than PG-PS in eliciting IgG responses. It was observed by the magnitude of titers, that the mixed breed ewes appeared to have stronger IgG response than Rambouillet ewes.

Figure 5. IgG antibody titers in Rambouillet ewes measured on Day 0, 13, 35, 47, and 82

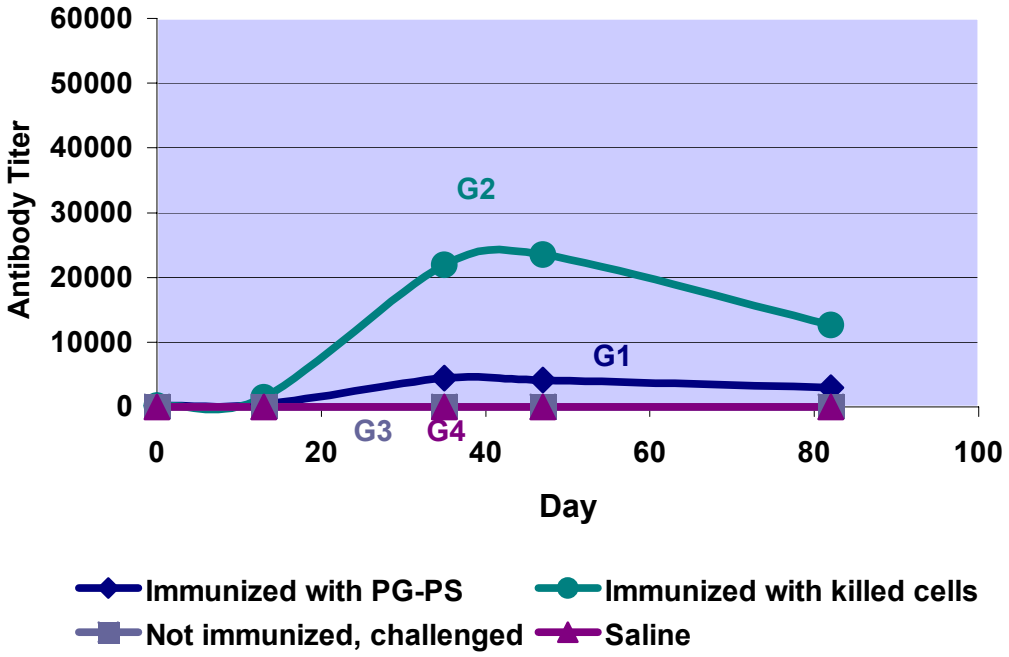
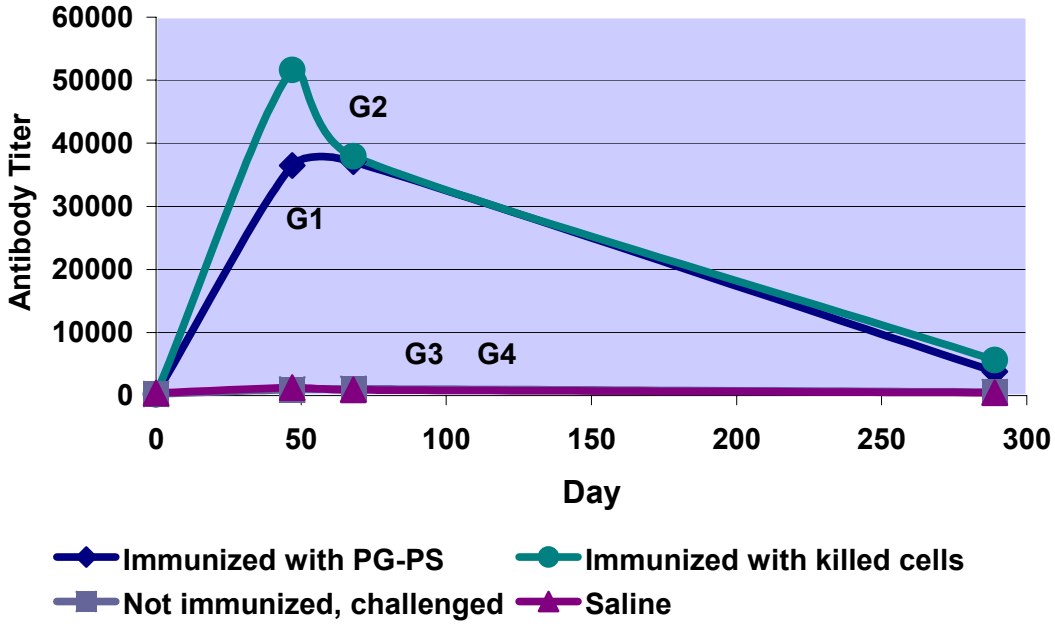


Figure 6. IgG antibody titers in mixed breed ewes measured on Day 0, 47, 68, and 289

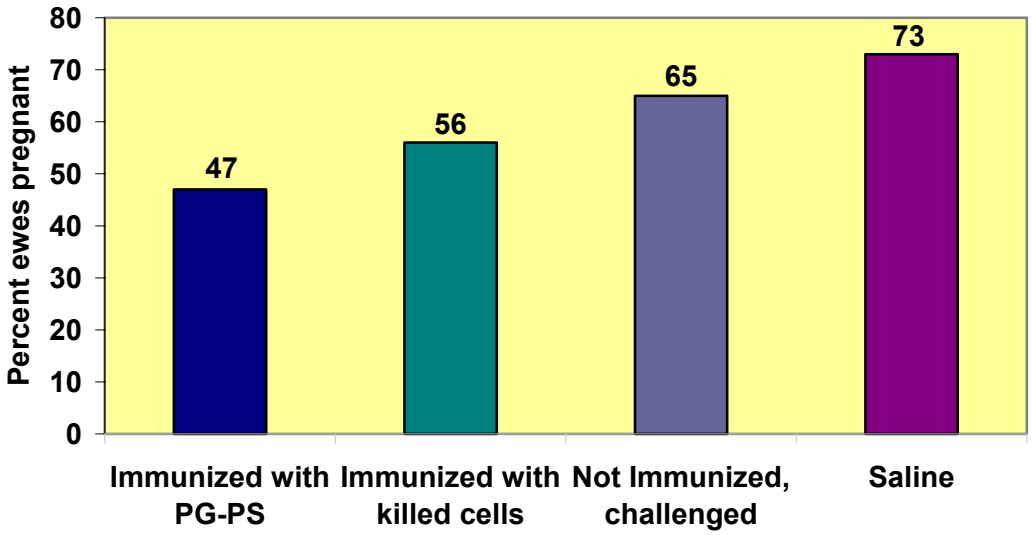


RESULTS

I. Pregnancy in Rambouillet Ewes

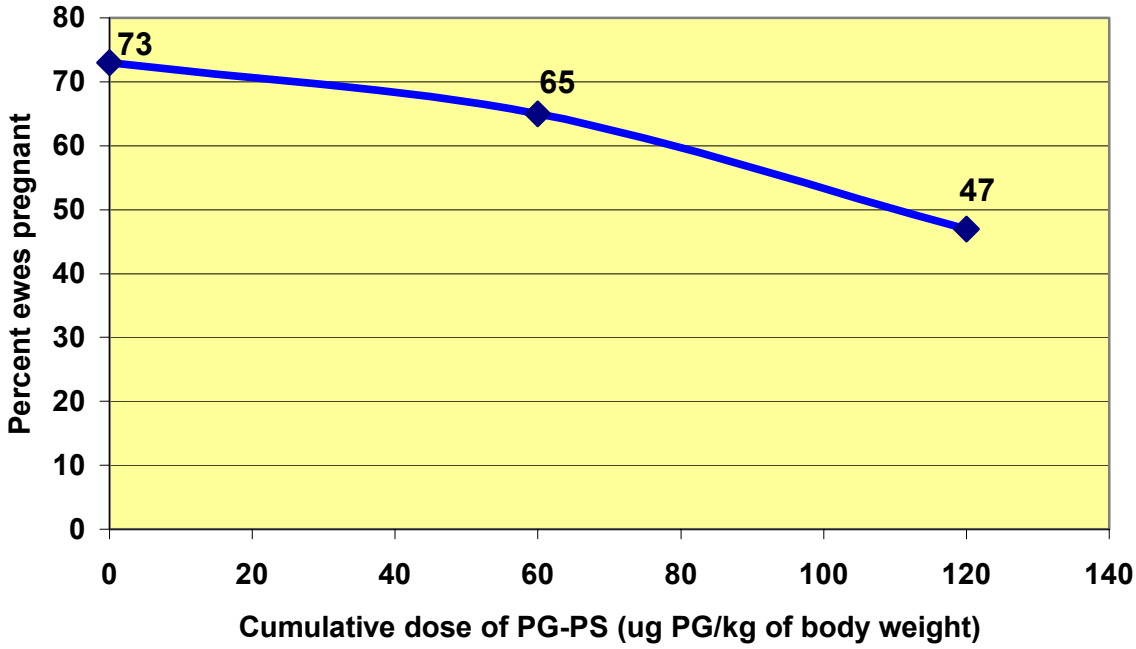
Pregnancy examination in Rambouillet ewes revealed that the proportion of ewes pregnant did not differ significantly due to treatments (Figure 7). Immunization was found ineffective on pregnancy maintenance when proportion of all immunized ewes (G1 and G2) pregnant, 52 %, was compared to 65% pregnant in not immunized, challenged group (G3). Effect of PG-PS did not differ from effect of killed cells on pregnancy maintenance (47% versus 56%).

Figure 7: Effect of treatment on percent pregnant in Rambouillet ewes. N = 160, p = 0.12



The cumulative PG-PS dose (D0 + D22 + D47) was a useful predictor of pregnancy ($p < 0.05$). With increased cumulative dose of PG-PS, proportion of ewes pregnant decreased as depicted in Figure 8.

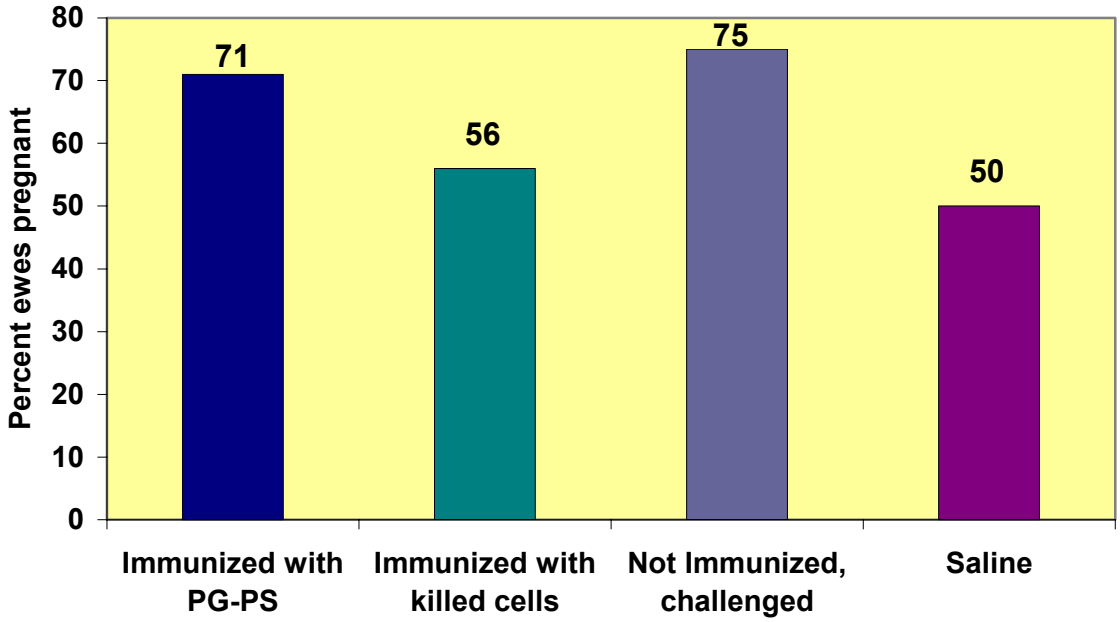
Figure 8: Model predicting the percent of Rambouillet ewes pregnant from cumulative dose of PG-PS
Model: P(y) = 1/[1+e^-(-1.05 + 0.009*x)]
N = 119, p < 0.05



II. Pregnancy in Mixed Breed Ewes

Proportion of pregnant ewes did not differ significantly in four treatment groups, Figure 9. The percentage immunized ewes detected pregnant (63%) was not statistically different from not-immunized ewes pregnant (75%, $p = .67$). The PG-PS immunized ewes seemed to have higher pregnancy proportion (71%) than killed cells-immunized ewes (56%), but the difference was not significant ($p = 0.63$).

Figure 9. Effect of treatment on percent pregnant in mixed breed ewes
N = 32, p = 0.68



III. Mastitis in Mixed Breed Ewes

Incidence of mastitis expressed in percent in Figures 10 and 11 was not statistically different in four treatment groups in the middle ($p = 0.12$) and at the end ($p = 0.32$) of lactation. Four of the fourteen immunized ewes had mastitis in the middle of lactation (29%), but four out of 7 (57%) ewes not immunized had mastitis, but this was not different when evaluated by two-tailed Fisher's exact test. At the end of lactation, immunized and not immunized ewes had similar incidences of mastitis, 62 and 57 %, respectively. The type of immunizing agent (PG-PS versus killed cells, G1 vs. G2) did not affect the occurrence of mastitis in the middle, nor at the end of lactation.

Bacteria obtained from mastitic milk samples of 22 ewes at the end of lactation consisted of 28 colonies, out of which, 15 were Gram-positive (54%). Distribution of bacteria-containing milk samples did not differ between treatments ($p > 0.05$).

Figure 10. Effect of treatment on occurrence of mastitis in mixed breed ewes, middle of lactation (39 days post partum) N = 28, p = 0.13

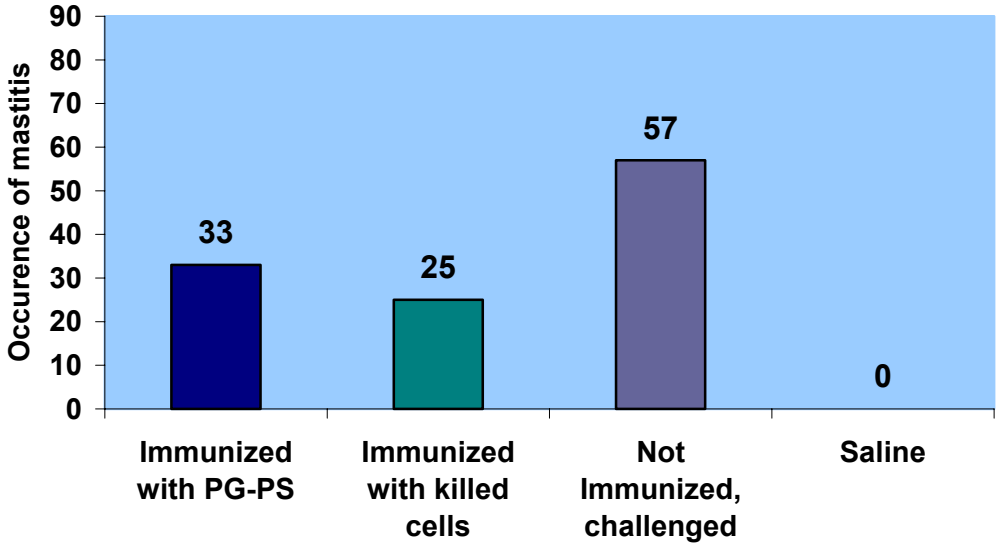
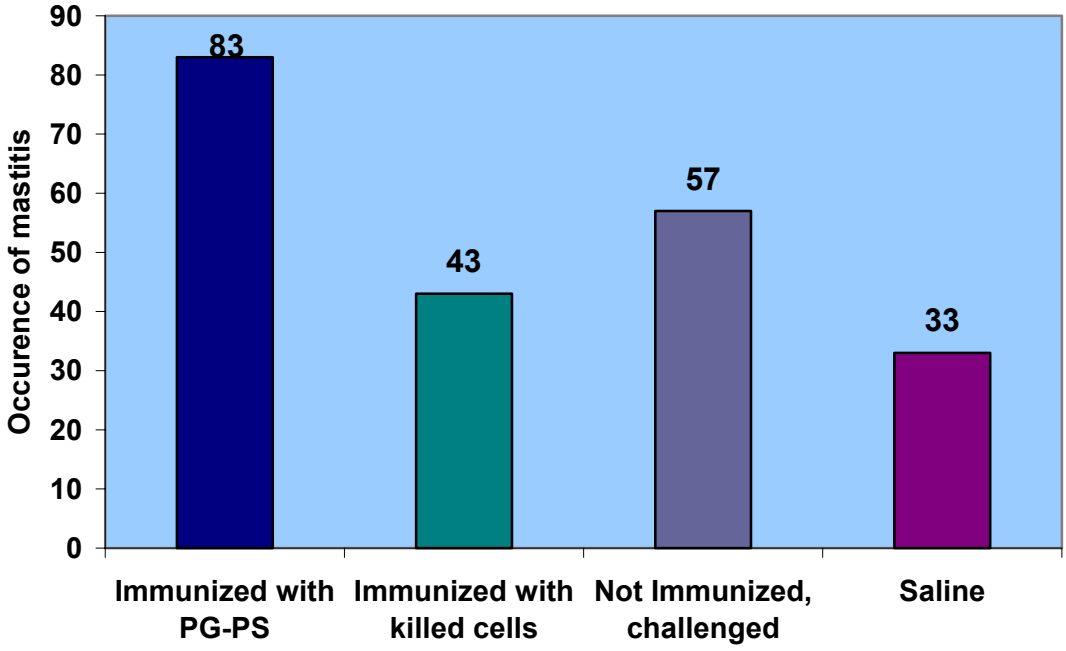


Figure 11. Effect of treatment on occurrence of mastitis in mixed breed ewes, end of lactation (98 days post partum) N = 26, p = 0.32



IV. Additional Results

Association of fever (increased body temperature by 0.7 ºC) at the time of the challenge (day 47) and pregnancy maintenance also was evaluated in mixed breed ewes using Fisher's exact test. Fifty percent of ewes (4/8) without fever were pregnant on day 42 after breeding and 67 % of ewes that had fever after the challenge (16/24) were pregnant. There was no association found between fever on day 5 and maintenance of pregnancy on day 42 of gestation.

DISCUSSION

Pregnancy An immune response was successfully elicited as documented by the formation of IgG PG-PS-reactive antibody. Rotta (1975) proposed that PG could be used for the prevention of infectious diseases, because rabbits repeatedly injected with PG developed tolerance to fever, and mice developed resistance to infection with group A streptococci after PG injection. Rotta (1975) also stated that the non-specific resistance of mice to infection induced by the streptococcal cell wall was dependent on PG, not the PS or cell wall proteins. Baines et al. (1996) demonstrated that early embryo loss in mice induced by a bacterial infection (LPS) could be prevented by prior immunization with LPS, as long as the anti-LPS antibody titers remained above a threshold value of 1/500. However, the immunity was short-lived and a protective effect was lost after 5 weeks. Immunization with isolated PG-PS or with heat killed *Strep pyogenes* did not positively affect pregnancy outcome in Rambouillet nor in mixed breed ewes. Moreover, the total amount of PG-PS ewes received lowered the probability of successful pregnancy in Rambouillet ewes, as shown by logistic regression analysis. However, immunization with PG-PS or with killed cells did not seem to have any lasting negative carry-over effect on conception, because the pregnancy rate averaged across all groups on day 42 post breeding was 95%. This included the second service ewes, which did not conceive or lost their embryo at the time of the PG-PS challenge but were able to conceive at the next estrous. In the group that was not immunized and was challenged on day 5 after breeding, 65% of ewes were pregnant on day 42. This result is similar to 64% pregnant found by Barker (1999) in older ewes injected with PG-PS without immunization. Negative tendency of the effect of immunization may be attributed to sensitizing the

immune system of sheep to the PG-PS. If that was the case and ewes were sensitized to the PG-PS using the immunization injections, then their bodies reacted by mounting an even stronger immune response at the challenge, which could be manifested by more inflammatory mediators with negative effect on pregnancy in ewes. Schwab et al. (1967) reviewed experiments of many investigators that demonstrated certain form of chronic arthritis in animals using multiple or single injections of microbial agents. One hypothesis of the etiology of the rheumatoid arthritis is that poorly degradable bacterial PG are transported to the joint and are capable of inducing abnormal immune response on the part of the host (cited in Hamilton et al 1982). This results in release of inflammatory mediators leading to the destruction of the joint. It may be that the nonbiodegradability and of bacterial PG is pathogenic factor related to negative effect of PG-PS on pregnancy.

Baines et al. (1994) compared embryonic loss in mice kept in a murine pathogen free animal facility to that of normally housed mice and found that the number of implantations per pregnancy was significantly higher in mice grown in murine pathogen-free environment. In living systems constantly exposed to pathogens, the defense reactions of the host to pathogens are redundant and may cause unnecessary exaggerated reaction of immune system. Thomas (cited in Jesmok and Borgia, 1985), after challenging horseshoe crabs with very low dose of LPS and observing a violent response, pointed out that it was the defense mechanism that became itself a disease and the cause of death. In mammals, production of inflammatory reaction associated agents, such as cytokines, chemokines, prostaglandins, as well as neuropeptides and other molecules may directly or indirectly influence survival of a host and its embryo.

Results of this and other experiments related to the problem of embryonic loss due to exogenous pathogens lead to following postulates:

1. PG-PS-reactive antibodies do not guarantee protection against early loss of pregnancy in sheep. Immunization with PG-PS and killed cells of *Strep pyogenes* induced the humoral immune response demonstrated by IgG antibodies to PG-PS. Mastitis developed during the ensuing lactation in immunized ewes. Treated groups with high IgG titers did not show higher maintenance of pregnancy.

2. Prostaglandins, especially increased $PGF_2\alpha$, are involved in abortions (Skarnes and Harper, 1972, Giri, 1990) via regression of CL and/or via uterine smooth muscle contractions, and in early embryonic losses. Barker (1999) found that lower pregnancy maintenance was associated with high concentrations of PGFM (prostaglandin $F_2\alpha$ metabolite) in PG-PS treated ewes. Also, Ramadan et al. (1997) observed increased venous $PGF_2\alpha$ after bacterial challenge. Kastelic (1991) reported that luteal regression occurred before embryo death prior to day 25 of gestation. Starbuck (2002), based on ultrasonographic examination of dairy cows, suggested that when an embryo died between day 30 and 37, it happened before luteal regression. Mitchell et al. (1990) cultured human decidua with endotoxin and inflammatory mediators (IL-1α, IL-1β, and TNF- α). All of these induced significant concentration-dependent increases in PGE_2 and $PGF_2\alpha$ production.

3. Nonspecific maternal immune effector cells, macrophages and natural killer cells, when activated, are involved in embryonic resorption in mice (Gendron and Baines, 1988, Gendron et al., 1990, Duclos et al., 1995). Inflammatory mediators secreted by macrophages, IL-1, TNF-α and nitric oxide (NO), play an important role in embryonic

loss (Baines et al. 1997). Menom et al. (1995) observed inflammatory cytokines IL-1 and IL-6 as well as IL-8 and TNF-α (Fortunato et al., 1996) produced by amniochorionic membranes of human, after *E coli* challenge in vitro. A macrophage activation marker in the decidua of mice was expressed before early embryo loss (Haddad et al. 1997).

4. Nitric oxide (NO) is produced from arginine in high amounts after increases of certain cytokines and bacterial toxins (Nathan, 1992). It is speculated that NO inhibits lymphocyte apoptosis which may modulate the immune system to prolonged inflammatory response (reviewed by Dimmer and Zeiher, 1997). Ando et al. (1998) observed that NO was elevated both in the liver and abdominal cavity after intraperitoneal administration of LPS. Duclos et al. (1995) and Haddad et al. (1995) postulated that NO secretion by macrophages activated at the materno-fetal interface in a mouse could be one of many possible mechanisms by which macrophages affect placental development.

5. Fever may or may not be directly related to early embryonic loss. Fever was detected in the majority of inoculated animals after administration of PG-PS as well as killed cells at all three times of inoculation. This is contrary to Rotta's et al. (1975) observation of decreased fever after repeated inoculations of rabbits with PG. However PG-PS and PG differ in the presence or absence of highly immunogenic PS portion. Even though fever was monitored for the purpose of verification of the inflammatory reaction to PG-PS and killed bacteria, the possibility of fever directly affecting embryo survival could not be excluded. Results of this experiment as well as Barker (1999) showed no association of fever and pregnancy. Similarly, Giri et al (1991) found intravenous endotoxin infusion in the first trimester of gestation did not affect the body

temperature of cows that aborted, differently than as those non-aborting. However, induction of fever by partial submersion of pregnant rabbits in warm water for 20 minutes interrupted pregnancy Cameron (1943). Zahl and Bjerknes (1944) suggested that fever induced by bacterial endotoxin caused death of the blastocyst, thus accounting for pregnancy loss during the pre-implantation stage. Romero et al. (1989), based on reasoning that IL-1 is mediating the fever response related to preterm labor in humans, cultured amnion cells with IL-1 and observed concentration-dependent increases in PGE_2 production. This group concluded that IL-1 could serve as signal for initiation of labor in cases of intrauterine or systemic infection.

6. Reproductive steroid hormones, especially progesterone (P_4) are involved in the pathology of bacteria-induced embryonic losses. Barker (1999) recognized reduced concentration of P_4 in all PG treated ewes, but not all treated ewes lost their pregnancy. Kelly et al. (2001) reviewed the steroid actions and concluded that P_4, to which the human endometrium is exposed, is a major factor governing cytokine concentrations in the endometrium. Estriol was found to be regulatory on T-cell migration and cytokine profile in the human (Zang et al, 2002).

Mastitis. Immunization of ewes did not have a significant effect on occurrence of mastitis. Yancey (1999) stated that it is difficult to evaluate the success of vaccines against mastitis. Whether the definition of successful vaccine should be based on reduction of severity of clinical symptoms, prevention of new intramammary infections, or spontaneous cure rate, has not been defined. Ideally, a successful vaccine should do all of that. Yancey (1999) also pointed out that specific enhancement of immune

response via vaccination could even worsen the mastitis. This would explain why cows that had mastitis are more susceptible to infection. This sensitivity is likely an example of hypersensitivity. Hypersensitivity is a harmful immune response that produces tissue injury and may cause serious disease (Janeway et al., 2001, p.472). Little (1937) suggested that teats had to be sensitized to *Strep agalactiae* by exposing the teat to it repeatedly before establishment of infection. Spencer and Angevine (1950) concluded that cows may be indeed sensitized to *Strep agalactiae* and that hypersensitivity to *Strep agalactiae* appears to be involved in pathogenesis of mastitis. This was supported by the fact that intradermal injections of formalinized cultures produced larger and more persistent swelling in infected then in normal cows. In the later report, however, Spencer and Simon found that prior sensitization by subcutaneous injection of living organism did not increase susceptibility to experimental exposure by intramammary infusion of 1,100-24,000 organisms.

PG-PS versus whole killed cells of Strep pyogenes. No differences in between however, ewes inoculated with killed cells had slightly delayed onset and longer lasting fever and significantly higher IgG titers compared to PG-PS inoculated sheep. Higher fever in ewes treated with killed-cells-treated can be explained by the presence of other pyrogenic molecules. Higher IgG response of the G2 group of sheep related to differential, perhaps more effective, antigen presentation of pathogen in the case of whole cells, which in turn results in higher antibody response.

Unplanned Gram-negative challenge. During the gestation of Rambouillet sheep in Idaho, a *Campylobacter* infection outbreak occurred at the facility. *Camplylobacter,* Gram-negative bacteria, as opposed to Gram-positive bacteria that was used to isolate the

PG-PS, causes late abortions in ruminants. Only out of curiosity, possible association of PG-PS and gram-negative bacterial infection was numerically evaluated in terms of abortions. There was no statistical association found between immunization with PG-PS or killed cells and distribution of late abortions and early neonatal deaths due to *Campylobacter*.

Materials and methods effectiveness The protocol for estrous synchronization using progestagens, prostaglandin and gonadotropin was very successful. Out of 200 Rambouillet ewes synchronized, 76 % came into heat within 72 hours, 82 % within 7 days and 95 % were pregnant on day 45-post synchronization. The ultrasound procedure of evaluating pregnancy and estimating the age of the embryos had a high degree of accuracy. When comparing the ultrasound-estimated age of embryos to the breeding records, there was only a 5 days average under-estimation of the embryonic age.

In conclusion, immunization with isolated PG-PS or with heat killed *Strep pyogenes* did not positively affect the pregnancy in Rambouillet, nor in mixed breed ewes. Moreover, total amount of PG-PS ewes received, lowered probability of successful pregnancy in Rambouillet ewes. Incidence of mastitis was not lessened in immunized in mixed breed ewes during and after weaning. Effects of killed cells and PG-PS on pregnancy and mastitis did not differ.

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