ABSTRACT

LOCAL IMMUNE RESPONSES FOLLOWING *IN UTERO* VACCINATION OF THE BOVINE FETUS WITH *ESCHERICHIA COLI*

By

Joseph Pius Otieno Wamukoya

Bovine fetuses were vaccinated during the last 6 weeks of gestation by the deposition of either killed *Escherichia coli* (*E. coli*) or sterile physiological saline into the amniotic fluid. At birth, the calves were divided into 4 groups. The first group comprised the control calves which received sterile physiological saline *in utero*. This group of calves was subdivided into 2 subgroups--one subgroup being necropsied at birth and the other given an oral challenge dose of live *E. coli* at birth and necropsied 5 days later. The fetuses in the following 3 groups were vaccinated with *E. coli in utero*. The second group of calves was necropsied at birth. The third group was given an oral challenge dose of live *E. coli* and necropsied 5 days later. The fourth group was given an oral booster dose of killed *E. coli* at birth and then an oral challenge dose of live *E. coli* 7 days later and necropsied 5 days thereafter.

Clinically, the signs exhibited by control calves, in response to challenge with *E. coli*, were not different from those exhibited

by calves in the vaccinated groups. However, a difference was observed in tests using fluorescein conjugated monospecific antisera to bovine IgM and IgG and to *E. coli*, to detect and enumerate antibody forming plasma cells. Whereas both IgM- and IgG-producing plasma cells were observed in the vaccinated groups, no plasma cells were detected in the 2 subgroups of the control calves and in the calf born less than 10 days after vaccination. Plasma cells producing IgG antibodies were more numerous, comprising about 70% of the total number of plasma cells counted. Only one-third of the total number of plasma cells counted were producing specific antibodies to *E. coli*.

The highest numbers of plasma cells were found in the jejunum, its draining lymph node, and the ileum. The highest numbers were also observed in calves which were born 18 to 20 days after vaccination. The group receiving a booster injection was found to have significantly more plasma cells than control calves.

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INTRODUCTION

Although colibacillosis has long been known to be responsible for many losses of newborn calves, no adequate preventive methods have been developed against the disease. Some of the preventive methods advocated, such as colostrum-feeding, actively immunizing the calf several days after birth with formalin-killed bacterins, and modifications of management have not proved to be adequate. In a recent survey, involving 77 Michigan dairy herds, Oxender *et al.* (1971) recorded high mortality rate in groups of calves fed colostrum. They also reported that overcrowding of calves, poor sanitation and ventilation, labor shortages and stress contributed to susceptibility of calves to *E. coli*. They found that when antibiotics were used extensively in a calf raising program, drug resistant bacteria may become a problem. Gay (1971) observed that although formalin-killed bacterins have been used for vaccination of calves against colibacillosis, the mortality rate did not differ between the vaccinated, colostrum-fed and untreated calves.

A reasonable approach to improving prevention of this disease is to explore the possibility of actively immunizing the calf *in utero* against *E. coli*. Some progress has been made in this area in recent years, and several attempts have been made to establish immunity in calves before birth. Gay (1971) and Conner

(1973) were the pioneers in attempting prenatal immunization. Their experiments were only partially successful in that some, but not all, of the prenatally immunized calves had humoral *E. coli* agglutinins in their sera. However, immunized calves were protected against oral challenge with live *E. coli*, although protection was independent of humoral agglutinins detectable by micromethods. Further basic work in this system is clearly necessary if it is to lead to practical applications.

The objective of this research was, therefore, to determine whether or not local antibody was produced in the gastrointestinal mucosa, mesenteric lymph nodes, and spleen following exposure of the fetus to *E. coli* antigen. To determine this, tests using fluorescein conjugated monospecific antisera to bovine IgM and IgG and to *E. coli* to detect and enumerate antibody forming plasma cells were applied. Immunoglobulin-G and IgM were used because it has been found that these are the main immunoglobulin components of bovine colostrum (Gay, 1971).

LITERATURE REVIEW

The purpose of this review is to provide a background appropriate to interactions of *E. coli* and host responses and some comments on the role of these reactions in preventive procedures. The topics discussed include general and antigenic properties of *E. coli*, factors determining host susceptibility, epidemiology, metabolic and physiological factors, and problems of passive and active immunization in prevention of neonatal enteric disease. Characteristics of bovine immunoglobulins are also reviewed.

Escherichia coli

Morphology

General properties. *Escherichia spp.* are referred to as colon bacilli because they are the predominant bacteria in the large bowel (Davis *et al.*, 1969). They are also referred to as coliforms. In culture broth, they produce a dispersed turbidity but under suboptimal conditions they form long, filamentous chains causing the broth to have a granular appearance (Davis *et al.*, 1969). Most *E. coli* species possess flagella and are motile.

The colonies of smooth (S) strains are shiny, convex and colorless on agar. When cultured on artificial media, they become rough (R) and form lusterless, granular colonies. The encapsulated (K

or Vi) strains produce mucoid colonies particularly when grown in low levels of nitrogen and phosphorus and high levels of carbohydrates (Davis *et al.*, 1969). All *E. coli* species have a characteristic fetid odor. They are more sensitive to inhibition by high concentrations of citrate than *Salmonella* and *Shigella spp.* (Davis , 1969).

Antigenic properties. More than 145 different somatic (O) antigens of *E. coli* have been identified by specific antisera and there are also at least 50 flagellar (H) antigens. In addition, 80 capsular (K) antigens have been demonstrated in the encapsulated strains. The presence of K antigens on the capsule masks activities of deeper lying O-antigens in immunological reactions (Davis *et al.*, 1969).

If motile species are treated *in vitro* with formalin, the labile H-antigens are preserved so that these bacteria can only agglutinate specific anti-H antibodies resulting in a fluffy precipitate (Davis *et al.*, 1969). Reactions with homologous anti-O antiserum are inhibited by the numerous peritrichous flagellae. On the other hand, if the H-antigens are denatured by heat, acid, or alcohol, the bacteria agglutinate with homologous anti-O antisera resulting in closely packed granular clumps. The surface antigens designated K can also partially prevent agglutination with homologous antisera to O-antigens but permit agglutination with antisera to H-antigens. The R-strains of *E. coli* have none of the above three specific surface antigenic properties and, since they have a tendency to agglutinate spontaneously, they are referred

to as "R-variants." They also possess O-antigens in the smooth form. Although the O and K antigens may be completely covered, the R-variants may still possess labile, H-antigens (Davis *et al.*, 1969). Formalin-killed, motile strains stimulate antibodies *in vivo* to all antigenic components of the bacteria (Davis *et al.*, 1969).

Escherichia spp. possess lipopolysaccharides on their lipoid-rich cell walls which are gram negative and which are essentially nonserotypic, somatic antigens, called endotoxins (Davis *et al.*, 1969). The endotoxins are responsible for toxic effects during colibacillosis. Taylor *et al.* (1971) have shown that dilatation of the rabbit gut loop can be achieved by some antigenic factors other than the classical O, H and K antigens. Whereas chloroform-killed suspensions of strains of *E. coli* derived from infants with diarrhea produced positive reactions in the ligated rabbit gut loop, similar attempts with antigenically identical organism preparations from healthy infants failed to do so. In the same studies, they also used strains of *E. coli* of non-human origin and tested their enteropathogenicity on both homologous and heterologous animal species. Their findings led them to conclude that there are several enterotoxic factors. It is of particular interest that a strain of 026:K60:H11 which they isolated from the calf produced a precipitin line with antiserum to culture extracts which were nonserotypic.

More studies have been done by Smith *et al.* (1967) to determine enterotoxicity of sterile cell-free preparations of pathogenic *E. coli* strains from pigs. They observed the presence of a heat stable (ST) enterotoxin in the fluid expressed from soft agar

cultures of porcine enteropathogenic *E. coli*. Later, the same workers showed that the production of ST was determined by a plasmid that could be transmitted to other strains of *E. coli* and *Salmonella spp.* This is similar to the genetic interrelationship which Davis *et al.* (1969) thought was achieved by transduction, lysogenization, and by conjugation whenever the conditions were unfavorable. In addition, Davis *et al.* (1969) have described the presence of another factor called "*colicin*" which is toxic to heterologous organisms and inhibits their growth. The colicins are thought to block oxidative phosphorylation, to retard protein synthesis, and to degrade DNA of other organisms. They are not bacteriolytic. They are thought to attach directly to certain receptors of the susceptible organisms.

Gyles *et al.* (1971) have reported the presence of heat labile (LT) enterotoxin in lysates of *E. coli* implicated in diarrhea of pigs.

Factors Determining Host Susceptibility

Intrinsic factors

Nonspecific. Although adaptation of the gastrointestinal tract to extrauterine life has not been studied in bovine fetuses and calves, the findings of Sunshine *et al.* (1971) in the rat may be representative of the changes that take place in all mammalian fetal and newborn animals. Sunshine *et al.* (1971) observed that the intestine of rat fetuses is lined with a flattened, stratified epithelium. Epithelial ridges and well defined villi appear in the

duodenum and formation of villi proceeds caudally until at the time of birth villi are found lining the entire length of the small intestine. Goblet cells and Paneth cells are not present until after the first two weeks of life. The microvilli of the duodenum and jejunum are poorly developed in the newborn animals and suckling animals and do not demonstrate the normal adult structures until just prior to weaning. They found that increased activity of the enzymes in the late fetal life correlates closely with the formation of villi in crypts three to five days prior to birth. The rate of turnover decreases rapidly and remains depressed during the suckling period, only to increase at least fourfold at the time of weaning. All these changes make it possible for neonatal and suckling animals to ingest and absorb whole protein molecules intact, including colostrum immunoglobulins, by pinocytosis and then transfer the proteins via the lymphatic ducts to the circulation.

Specific factors

Protective antibodies. Lack of protective antibodies has been attributed to the susceptibility of the newborn to enteropathogenic organisms. Studies in man have shown that IgA is responsible for protecting infants from enteric infections. Hanson (1961) described a form of IgA occurring in human milk which was different from serum IgA. Tomasi *et al.* (1962, 1963, 1955) found that this form of IgA occurs in other secretions and is the predominant immunoglobulin in these fluids. The molecule is usually heavier (11S) than the serum IgA (7S) because it bears an additional polypeptide chain or secretory piece.

Much evidence for a local antibody system has accumulated through the study of the appearance of antibody in the intestinal, lacrimal, and mammary secretions following infections or immunization (South, 1971). Her studies have shown that all species phylogenically more advanced than amphibians have lamina propria plasma cells.

Genetic factors. Although genetic studies in relation to *E. coli* in the calf have not been made, it is reasonable to propose that some breeds of cattle are more genetically resistant to colibacillosis than others.

Extrinsic factors. Other factors contributing to host susceptibility are simple management failures like improper planning, overcrowding of animals, lack of colostrum-feeding, poor sanitation and ventilation, and labor shortages (Oxender *et al.*, 1971). Stress of any kind in calves will lead to infections with *E. coli*. Oxender's investigations (1971) also showed that when antibiotics were used extensively in a calf raising program, drug resistant bacteria may become a problem. They (Oxender *et al.*, 1971) concluded that the use of antibiotics does not replace good management practice in raising calves.

Epidemiological and Microbiological Aspects

Serotypes of *E. coli* associated with colibacillosis in neonatal animals have been studied by Glantz (1971), who reported that serotypes pathogenic for one animal may not be pathogenic for another. Some *E. coli* serotypes are species specific but others are not and

the reasons for this are not clear. Glantz (1971) has reported that there are about 50 serogroups of *E. coli* occurring in calves. He reported the isolation of 0138, 0139, and 0141 from the intestinal tracts of pigs diagnosed as having edema disease or enteritis. The 08 and 012 serotypes were isolated from a variety of disease conditions such as septicemias, toxemias, enteritis and, less frequently, edema disease. Diarrhea was most commonly associated with 026 and septicemias with the serotypes of OX36.

Escherichia coli serotypes isolated from newborn calves belonged to 54 different O groups (Glantz, 1971). Serotypes 02a, 015, 019, 0101, 0115, 088 and OX28 predominated. The 02a was unusual in calves as it is commonly isolated from poultry. The 02a and 09 serotypes were isolated from calves with severe enteritis, while the serotypes 08, 026, 055, 078, 0101, 0115, 0117 and 0119 were noted for producing septicemia. The pathogenicity of OX28 was not verified. The K80 antigen, usually associated with 078, was identified with 021, 025, and 0124. Four serotypes of 021 had the K81 antigen usually associated with 0138.

All these enteropathogenic *E. coli* serotypes are also found in healthy animals constituting the normal flora of the gut. Smith (1971) has reported that the normal flora of the alimentary tract of domestic animals usually consists of *E. coli*, *Clostridium welchii*, streptococci, lactobacilli, *Bacteroides*, veillonellae, and yeasts. The contents of the alimentary tract of a diarrheic, dead calf consist of *E. coli* in increased numbers in the abomasum and large intestine with a moderate increase in the small intestine.

Clostridium welchii diminished in numbers, numbers of lactobacilli increased in the abomasum, small intestine, rectum and large intestine, and streptococci increased in number in a manner similar to *E. coli*. The *Bacteroides* and veillonellae and yeasts were almost nondetectable.

Metabolic and Physiological Factors in the Pathogenesis of Neonatal Diarrhea

Fisher (1971) observed that diarrheic calves expelled volumes of watery feces in excess of their milk intake. Some calves died in a few days as a result of the diarrheic syndrome (acidosis and dehydration). Packed cell volume (PCV) changed remarkably. In other calves which suffered from diarrhea for a much longer period before death, the changes in packed cell volume were less noticeable. This is because in prolonged cases, dehydration is not marked and therefore the electrolyte/fluid balance remains almost normal. In dehydrated cases, rapid loss of fluids leads to hemoconcentration resulting in high packed cell volume.

The effect of severe diarrhea on the plasma of the calf is to decrease the volume, usually to decrease the sodium and chloride concentration, to increase the urea concentration, and sometimes to increase the potassium concentration. The level of plasma proteins goes down because of lowered intake of food and due to losses through extravasated tissues. This results in a further loss of body fluids. The hydrogen ion concentration in plasma rises and the bicarbonate concentration decreases so that metabolic acidosis ensues. Spontaneous recovery can occur, whereupon the plasma

parameters return to normal. Recovered cases remain emaciated for a long period of time.

Perspectives in the Control of Neonatal Enteric Disease

Problems of passive and active immunization in control of neonatal enteric disease. Generally, formalin-killed bacterins have been used for vaccination of calves against colibacillosis, but still the mortality rates did not differ between vaccinated, colostrum-fed and untreated calves (Gay, 1971). Moreover, Fey (1960) made a startling observation that calves that had died of colisepticemia and which presumably had been fed colostrum were, in fact, still markedly deficient in circulating gammaglobulins. Further study on healthy calves revealed that a deficiency in gammaglobulins was not uncommon and it was postulated that failure of some colostrum-fed calves to acquire globulins from the colostrum was the main predisposing factor which rendered the calves in the field susceptible to invasion by *E. coli*. The fact that calves are resistant to colisepticemia once they have acquired a certain level of immunoglobulins is an all-or-none phenomenon which would more logically be associated with a nonserotype-specific and more universal antibody rather than a serotype-specific antibody (Gay, 1971). Gay (1971) observed that if colostrum-deprived calves were raised in isolation, they became resistant to colisepticemia by approximately 7 to 14 days of age. Then he argued that if serotype-specific antibody were the protective antibody then such resistance would require that all such calves had been exposed to all

pathogenic serotypes of *E. coli* within a few days after birth.

When Gay (1971) vaccinated fetuses by laparotomy using a single serotype, calves were protected when challenged with other strains of *E. coli*. This points out the value of stimulation of antibodies against *E. coli* before the calf is born, but also indicates that this antibody was not necessarily serotype specific. Gay (1971) also observed that calves exposed for the longest time produced heterogeneic antibody to many strains of *E. coli* and also to *S. typhimurium*, whereas calves vaccinated for 16 days showed only a partial response in this respect and the calf vaccinated for only 14 days showed a serotype-specific response.

Bovine Immunoglobulins

Three antigenically distinct classes of bovine immunoglobulins have been described (Butler, 1969). All these immunoglobulins occur both in serum and in the mammary gland and lacrimal secretions and are designated IgM, IgG and IgA. The IgG class is subdivided into 2 subclasses, IgG1 and IgG2.

Bovine IgM

This is a macroglobulin comprising less than 10% of the serum and colostral immunoglobulins (Butler, 1969) and possessing physicochemical and biological properties similar to IgM of other species. Pike *et al.* (1964) observed that the antibodies to lipopolysaccharide O-antigen of gram-negative enteric bacteria were predominantly IgM in nature. Robbins *et al.* (1965) confirmed this finding using *S. typhimurium* and added that the IgM antibody was more efficient in

complement fixing and as an opsonization agent than the IgG class. In addition to these findings, Davis *et al.* (1969) have reported that gram-negative bacteria coated with specific antibody are lysed by complement, apparently by the same reaction sequence as in red cell lysis. The gram-positive bacteria and myobacteria, however, are not susceptible to the action of complement and the basis for their resistance is not understood. Hill *et al.* (1966) reported that the mouse protective activity of IgM antipneumococcal antibodies was some 100,000 times greater than that of IgG.

This protein is eluted in the first peak of gel filtration with Sephadex G200 or in the third major peak of a continuous gradient DEAE cellulose of DEAE sephadex fractionation, when a 33% saturated $(NH_4)_2SO_4$ preparation of serum, milk or colostrum is used as a starting material (Butler, 1969). Butler also separated the contaminating alpha-2 macroglobulin (transferrin, Murphy *et al.*, 1965) from IgM by Pevikon block electrophoresis. The isolated macroglobulins have a sedimentation coefficient of 19S.

Gammaglobulin-M is easily reduced by 2-mercaptoethanol which also abolishes its antibody activities. Butler (1969) found that the antigenic distinctiveness of bovine IgM demonstrated by immunodiffusion appeared to reside in the FC fragment. Gammaglobulin-M has μ-heavy chains and kappa or lambda light chains similar to other species. On electrophoresis IgM precipitates close to the sample-well and the rate of formation of the precipitin arc is slow, most likely due to its molecular weight. It is a better agglutinating and complement fixing antibody as compared with other immunoglobulins.

Bovine IgG

This is the most abundant immunoglobulin, comprising 85-90% of the serum and whey immunoglobulins. The bovine IgG shares antigenic determinants with human, caprine or ovine IgG as far as γ-chains are concerned (Butler, 1969). Bovine IgG resembles that of sheep and is close to the horse in κ/λ chain ratio. The molecules of IgG have a sedimentation coefficient of 7S. The class can be subdivided antigenically into 2 subclasses by anion exchange chromatography, immunoelectrophoresis, immunodiffusion, electrophoresis, and ethanol-fractionation (Butler, 1969). Immunoglobulin-G2 is plentiful in serum but its concentration is less in colostrum, milk and saliva. Immunoglobulin-G1 is the principal immunoglobulin of mammary and salivary secretions. The IgG2 subclass elutes in the first peak of anion exchange chromatography DEAE cellulose DE52 at a continuous gradient while IgG1 elutes in the second peak.

In an earlier study Kickhofen *et al.* (1968) subdivided the gammaglobulins of the IgG class of cattle into 3 subclasses on the basis of their behavior on DEAE sephadex and immunoelectrophoresis. They referred to IgG1 as IgGS (secretory), IgG2 as γ2 and an intermediate IgG-2-like subgroup as γ1. Earlier than this, Pierce *et al.* (1965) had reported 3 subgroups of IgG but have since reclassified the intermediate subclass as IgG1. Groves *et al.* (1967) showed an extra band in colostrum which they called IgG2. Kickhofen *et al.* (1968) found the IgGS to be 163,000 MW, and γ1 and γ2 both to have a MW of 150,000 each. Butler (1969) concluded that the division of the bovine IgG immunoglobulins into only 2 subclasses may be an oversimplification.

Bovine IgA

This immunoglobulin can be separated from IgM and IgG by sephadex G200 gel filtration and anion exchange chromatography and acrylamide gel electrophoresis. It appears more abundantly in whey than in serum (Butler, 1969). Serum IgA contains no bound glycoproteins, a fact which explains the difference between serum and mammary IgA (Butler, 1969). The principal immunoglobulin in the mammary gland, lacrimal and salivary secretions of man and colostrum of the rabbit and mouse is a secretory IgA (Tomasi *et al.*, 1963; Hanson *et al.*, 1961; Hurlimann *et al.*, 1968; Tomasi *et al.*, 1965; Asofsky, 1968; Cebra *et al.*, 1967), but in the cow it is the IgG1 (Gulger, 1959p Hanson *et al.*, 1959; Larson *et al.*, 1957; Steck, 1962) and no secretory piece has been isolated for bovine mammary IgG1. Nansen *et al.* (1972), in their investigation of the immunoglobulins in the bovine lacrimal fluid, have shown that IgA is the main immunoglobulin constituting approximately 40% of the total protein content. But this is in disagreement with Butler (1969), who suggested that IgG1 was the predominant immunoglobulin in the lacrimal fluid of cattle. Nansen *et al.* (1972) further showed that while IgA levels varied, mainly independently, from the serum IgA level, there was a significant positive correlation between the lacrimal and serum levels of both IgM and IgG1 indicating that the bulk of these immunoglobulins may be derived from the plasma. The concentration of bovine IgA is higher in colostrum and milk than in serum. The immunoglobulin-A has α-heavy chains and kappa or lambda light chains (Butler, 1969).

Bovine Colostrum

It is apparent that the calf is not totally agammaglobulinemic at birth (Klaus *et al.*, 1969), although Brambell (1958) and Gerwutz *et al.* (1968) observed that the calf is agammaglobulinemic at birth. The bovine mammary gland selectively secretes serum-gammaglobulins during all phases of lactation, but especially during the period of colostral synthesis when the gammaglobulin levels may exceed those of serum by five times. Dixon *et al.* (1961) observed that colostral IgG levels were considerably higher than those of the maternal serum and that there were no differences between colostral and serum IgM levels. Klaus *et al.* (1969) and Pierce *et al.* (1963) reported that the absorption of colostral immunoglobulins was a nonselective process in the gut of a newborn calf and inferred that effective passive immunity to enteric bacteria in the newborn calf is intimately related to the efficient transfer of specific IgM antibodies from the maternal circulation into colostrum. Blakemore *et al.* (1956) and Garner *et al.* (1958) had shown that homologous gammaglobulins, given intravenously to pregnant cows, were transferred to mammary gland secretions during the weeks preceding calving. The antibody level was found to be 13 times higher in colostrum than in maternal serum. The findings showed that immunoglobulins of similar electrophoretic behavior and antigenic relationship were present in bovine serum and colostrum.

Logan *et al.* (1974) reported that calves fed colostrum were resistant to oral challenge infection with *E. coli* whereas the calves deprived of colostrum exhibited typical signs of colibacillosis

They observed that purified colostral immunoglobulin fractions (IgA, IgG and IgM) were less effective in preventing colibacillosis than colostrum--with IgA being least effective. The presence of IgA inhibited the effectiveness of IgM and both IgM and IgG diminished the activity of IgA against *E. coli*.

Bovine Fetal Immunoglobulins and Bovine Intestinal Secretory Immune System

After antigenic stimulation, serum gammaglobulins are first detected in the bovine fetus during the fifth month of pregnancy (Horner *et al.*, 1973), although Schultz *et al.* (1971) had reported an earlier detection of IgM in fetal serum at 4 months of pregnancy. Silverstein *et al.* (1963), however, had noted that the age at which the fetus was first capable of an immune response depended on the nature of the antigen. Horner *et al.* (1973) reported that serum gammaglobulins occur in greater percentages and in higher concentrations in fetuses derived from dams in a tropical environment. The presence of gammaglobulins in fetal sera is indicative of an *in utero* immune response in the fetus because it is generally accepted that transplacental transfer of maternal gammaglobulins does not occur in cattle (Brambell, 1958).

The bovine secretory immune system in the intestinal mucosa has been studied by many workers. Studies by Curtain (1971) suggested that IgG1 predominates in bovine mucous secretions and also in plasma cells locally situated in the external mucosa. Vaerman (1970) failed to detect IgA cells in lamina propria of bovine intestinal tissues, but Porter (1973) observed levels of IgA in

the lamina propria of the calf comparable with those found in the pig in which an intestinal secretory IgA system has been adequately defined. Furthermore, Mach *et al.* (1971) had also found IgA to be a major immunoglobulin in the washings from the epithelium of the bovine intestinal tract. But in all this work, IgM was consistently found in higher levels than IgA. This led Porter (1973) to propose that IgM may be involved in the primary response of the secretory immune system switching to IgG and then to IgA.

MATERIALS AND METHODS

Antigen

Cultures[a] of *E. coli* serotype 026:K60:NM were stored (tightly sealed in the dark at room temperature--22 C). Subcultures on trypticase soy agar,[b] with no dextrose, were prepared monthly, incubated overnight at 37 C, tightly sealed and stored.

Animals

Cows

Thirty date-bred pregnant Hereford heifers were obtained from the Premier Corporation, Fowlerville, Michigan. Two date-bred pregnant Holstein cows were obtained from the Dairy Department of Michigan State University, East Lansing, Michigan. The cows were fed hay and water *ad libitum* in a confined yard.

Guinea Pigs

Fifteen albino guinea pigs were obtained from Michigan Health Laboratories, Lansing, Michigan. The guinea pigs were fed guinea pig pellets and supplied with water *ad libitum*.

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[a]Obtained from Dr. Paul Glantz, Pennsylvania State University, University Park, Pennsylvania.

[b]BBL 11043, Division of BioQuest, Cockeysville, Maryland.

Rabbits

New Zealand white rabbits were obtained from the local suppliers. They were fed prepared rabbit feed and supplied with water *ad libitum*.

Immunoelectrophoresis and Double Immunodiffusion

Immunoelectrophoresis (IEP)

Barbital sodium[a] buffer 0.1 M (pH 8.0) was prepared by the method described by Leid and Williams (1974). The buffer was allowed to stand at room temperature for 24 hours to allow maximum electrolytic dissociation before it was dispensed into the electrophoresis chamber.[b]

Two percent agar gel was prepared by boiling 10 g of agar noble[c] in 250 ml distilled water and then made into 0.05 M (pH 8.0) by adding 250 ml of the 0.1 M barbital sodium buffer (pH 8.0). The mixture was boiled until there were no more air bubbles and then dispensed into test tubes in volumes of 13 ml. Methiolate, diluted 1/10,000, was added to the gel which was then stored at 4 C.

Immunoelectrophoresis was performed following the method described by Grabar *et al.* (1964). Antigens were placed in wells in a layer of 2% agar gel on microscope slides placed on electrophoresis frames. Strips of moist filter paper connected the agar with the reservoir of buffer in the chamber. A current of 9 amperes

[a]Obtained from Scientific Products, 17150 Southfield Road, Allen Park, Michigan.

[b]Gelman Deluxe Electrophoresis Chamber.

[c]Obtained from Difco Laboratories, Detroit, Michigan.

per frame was passed through the system for 70 minutes. After this time, the current was discontinued and long, horizontal troughs cut at the center of each slide. An appropriate antiserum was placed in each trough and then incubated in a moist chamber at room temperature for at least 12 hours to allow diffusion to take place. After incubation, the slides were examined for precipitin arcs using indirect illumination.

Double Immunodiffusion (DID)

One percent agar gel was prepared in 0.05 M barbital sodium buffer (pH 8.0) by boiling 5 g of agar noble in 250 ml distilled water and then adding 250 ml of 0.1 M barbital sodium buffer (pH 8.0). This mixture was boiled until there were no more air bubbles. Methiolate, diluted 1/10,000, was added to the gel which was then stored at 4 C.

Double immunodiffusion was performed by placing antigens in the peripheral wells in a layer of 1% agar gel on a microscope slide. An appropriate antiserum was placed in the central well and then incubated in a moist chamber at room temperature for at least 12 hours and then examined for precipitin lines.

Preservation of the Precipitin Arcs and Lines

The slides with the desired precipitin arcs and lines were washed in 2% sodium chloride (NaCl) solution for at least 2 days with 2 changes to remove excess nonprecipitated proteins and in distilled water for 1 day to remove excess salt (Grabar *et al.*, 1964). The slides were then dried and stained with amido Schwarz

blue-black prepared according to the method described by Grabar *et al.* (1964). The stained slides were destained with 2% acetic acid with at least 2 changes. Finally, the slides were preserved by submerging them into acetic acid-glycerol-water mixture for 15 minutes to apply a thin coating over the gel. The slides were then dried and stored in a dry place.

Determination of Protein Concentrations

Protein concentrations were measured following the method described by Lowry *et al.* (1951). In the case of immunoglobulin solutions, concentrations were calculated from the optical density (OD)[a] at 280 nm, multiplied by a factor derived from the extinction coefficient (Oriol, Binaghi and Boussac-Aron, 1968; Binaghi and Oriol, 1968).

Tube Agglutination Tests

Twenty, 12 x 75 test tubes were placed in a rack. To tube 1, 0.8 ml of phosphate buffer saline (PBS) was added and 0.5 ml to the remaining tubes. A volume of 0.2 ml of the rabbit antiserum was added to tube 1. Doubling dilutions of this mixture was performed until tube 19 upon which 0.5 ml of the mixture was discarded. A volume of 0.5 ml of the *E. coli* antigen, diluted 1:50, was added to each tube and thoroughly mixed. The final mixtures were incubated at 37 C for 48 hours. After 48 hours, without shaking, the tubes were observed for clearing of the supernatant and agglutination (a modification from Davis *et al.*, 1969).

[a]Read on a Beckman DB Spectrophotometer, manufactured by Beckman Instruments, Inc., Fullerton, California.

Preparation of Antisera

Bovine Serum and Immunoglobulins

Antiserum to whole bovine serum. Whole blood was obtained from a normal cow by jugular venipuncture. The blood was allowed to clot for one hour at room temperature and then allowed to further clot overnight at 4 C. Serum was separated from the clot and a portion of it was diluted 1:5 with 0.1 M phosphate buffer saline (pH 7.3). Six milliliters of the diluted serum were mixed with 6 ml of complete Freund's adjuvant[a] (CFA) and thoroughly emulsified. Rabbits were immunized by inoculating 1 ml of the mixture into each thigh muscle and 0.25 ml subcutaneously over each shoulder. Two weeks later, the rabbits were boosted with an equivalent amount and bled out 7 days after the booster inoculation. The antisera were stored at -70 C without preservative.

For immunization of guinea pigs, the protein concentration of bovine serum had to be determined (Oriol *et al.*, 1968) following the method of Lowry *et al.* (1951). Each guinea pig was inoculated with 100 µg of protein mixed with CFA in a similar manner as described above. Two weeks later, the guinea pigs were boosted and killed 10 days after boosting. Blood was collected by severing the posterior vena cava and allowing blood to flow into the thoracic cavity, from where it was withdrawn with a syringe. The antisera were stored at -70 C without preservative.

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[a]Obtained from Difco Laboratories, Detroit, Michigan.

Bovine immunoglobulins. The globulin fraction of the bovine serum was removed by a 50% saturated $(NH_4)_2SO_4$ precipitation (X3) while gently stirring at room temperature. The precipitate was dialyzed against PBS until free of sulphate ions and then filtered through a 0.45 μ millipore filter.[a]

Sephadex G200[b] gel was prepared following the procedure described by the manufacturers. Four milliliter volumes of the globulins, dialyzed against 0.1 M Tris-HCl buffer (pH 8.0) and filtered through 0.45 μ millipore filter, were applied on a 2 x 100 cm column equilibrated with the same buffer. The eluates were collected in 3 ml volumes and the OD at 280 nm determined. The elution pattern is shown in Figure 1.

The ascending and descending portions of F1 and F2 were all pooled separately, concentrated in carbowax and dialyzed in PBS. Each pool was then analyzed for the type of immunoglobulin it contained using IEP. The ascending portion of F1 was found to contain IgM and transferrin as indicated in Figure 2, while the descending portion contained IgM, IgG1 and IgG2. Subclasses of IgG were contained in F2. The descending portions of F1 and F2 were further pooled, concentrated and dialyzed in PBS.

Antisera to bovine IgM. The eluates of the ascending portion of F1 from gel filtration were run on IEP and precipitin arcs were

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[a]Obtained from Millipore Filter Corporation, Bedford, Massachusetts.

[b]Obtained from Whatman Biochemicals, Ltd., Springfield Mill, Piscataway, New Jersey.

Figure 1. Elution pattern of bovine globulins from Sephadex G200.

a - the portion that contained predominantly IgM contaminated with alpha-2 macroglobulin (transferrin).

F1 - fraction containing predominantly IgM immunoglobulins.

F2 - fraction containing predominantly IgG class of immunoglobulins.

F3 - fraction containing alpha and beta globulins and some albumin.

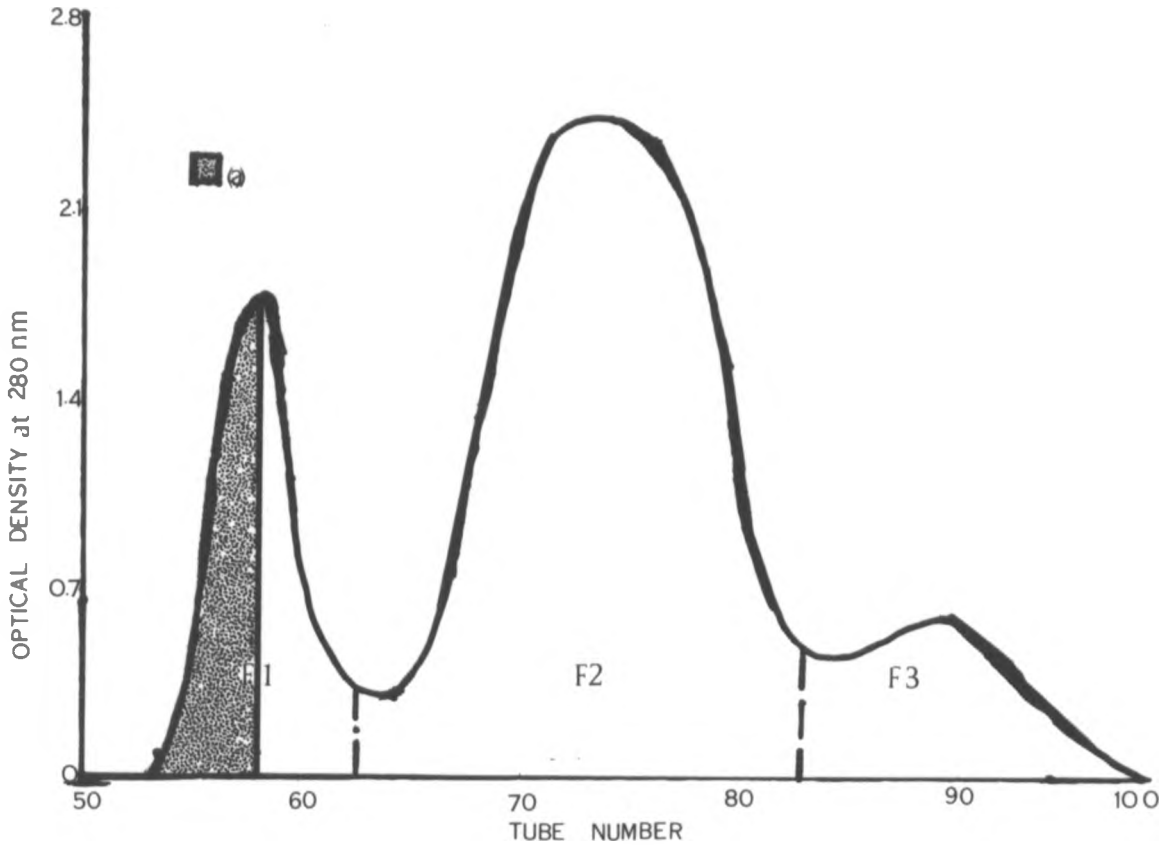
2.8
2.1
1.4
0.7
0
OPTICAL DENSITY at 280 nm
F1
F2
F3
50
60
70
80
90
100
TUBE NUMBER

Figure 1

Figure 2. Immunoelectrophoretic pattern of (a) in Figure 1.

1. IgM precipitin arc

2. Transferrin precipitin arc

Figure 3. Immunoelectrophoretic profile of the monospecific guinea pig antibovine IgM.

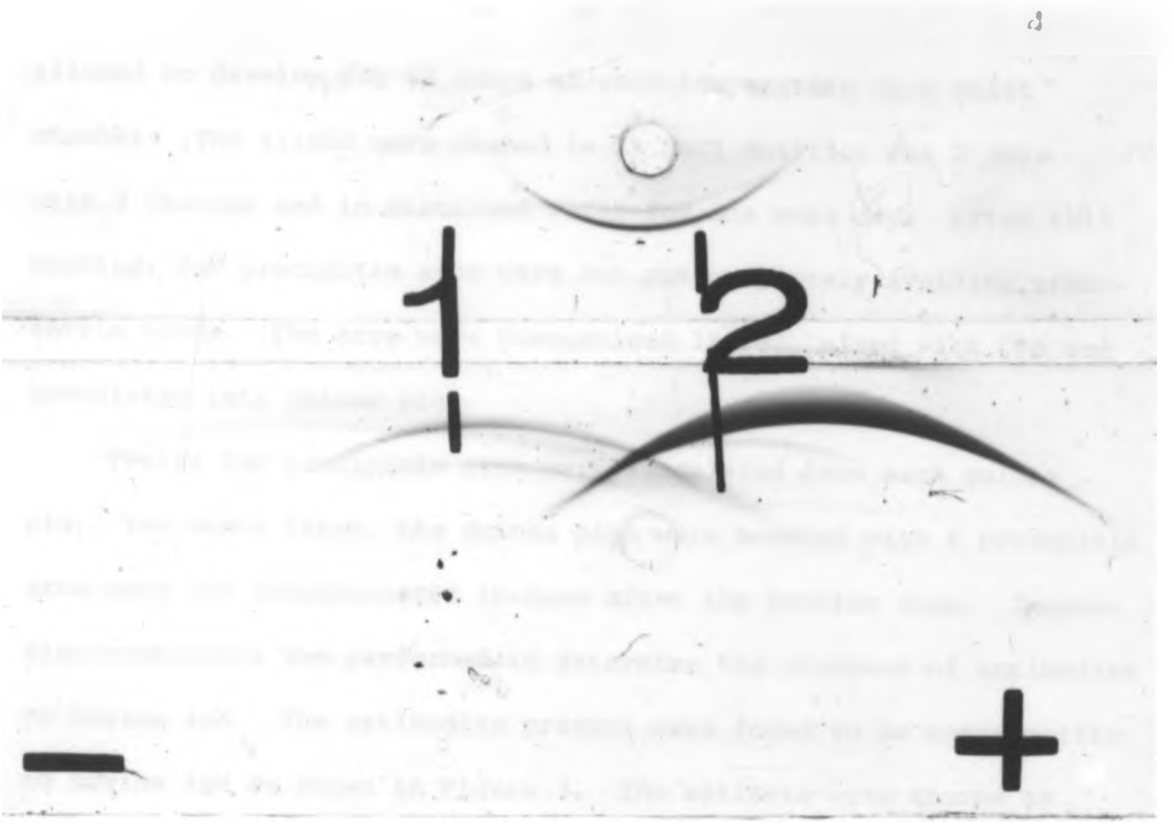

Figure 2

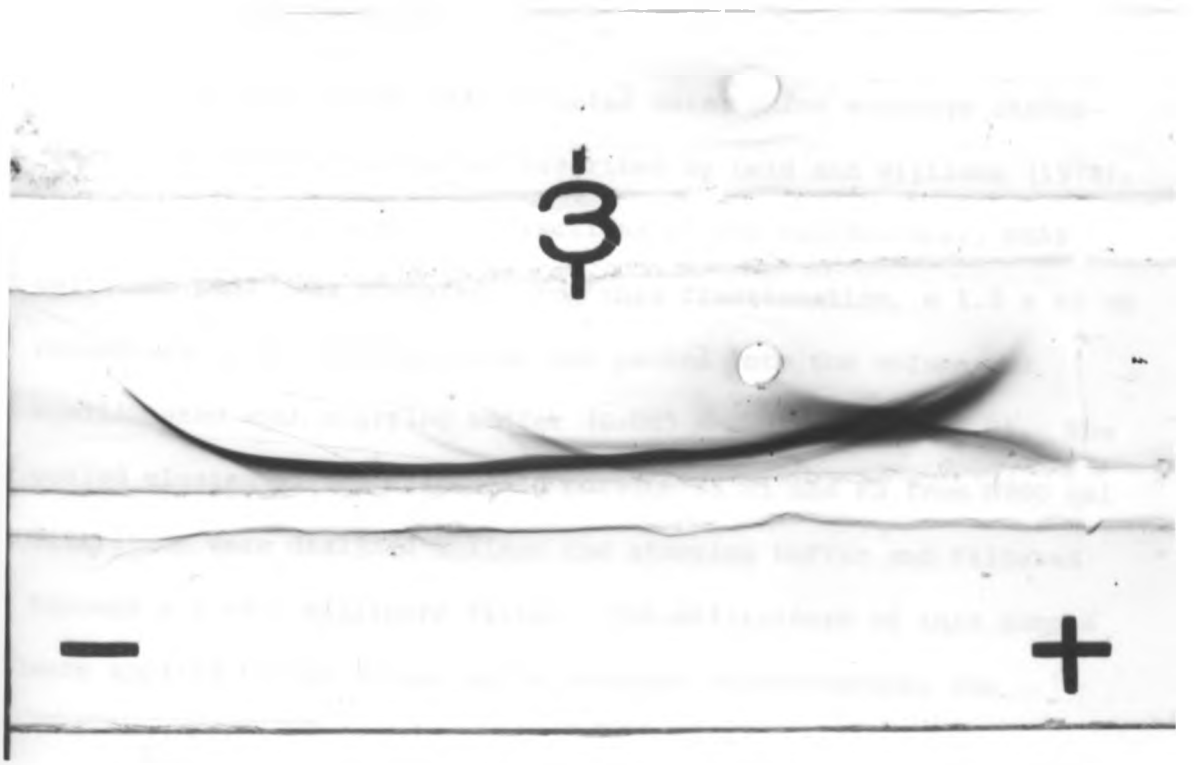

Figure 3

allowed to develop for 48 hours at room temperature in a moist chamber. The slides were washed in 2% NaCl solution for 2 days with 3 changes and in distilled water for one more day. After this washing, IgM precipitin arcs were cut out completely avoiding transferrin bands. The arcs were homogenized in PBS, mixed with CFA and inoculated into guinea pigs.

Twelve IgM precipitin arcs were inoculated into each guinea pig. Two weeks later, the guinea pigs were boosted with 6 precipitin arcs each and exsanguinated 10 days after the booster dose. Immunoelectrophoresis was performed to determine the presence of antibodies to bovine IgM. The antibodies present were found to be monospecific to bovine IgM as shown in Figure 3. The antisera were stored at -70 C without preservative.

Antisera to Bovine IgG

The IgG subclasses were isolated using anion exchange chromatography following the method described by Leid and Williams (1974).

In accordance with the directions of the manufacturer, DEAE cellulose DE52[a] was prepared. For this fractionation, a 1.5 x 40 cm column was used. The cellulose was packed into the column and equilibrated with starting buffer (0.005 M phosphate, pH 8.0). The pooled eluates of the descending portion of F1 and F2 from G200 gel filtration were dialyzed against the starting buffer and filtered through a 0.45 μ millipore filter. Ten milliliters of this sample were applied to the column and a stepwise chromatography for

[a]Obtained from Whatman Biochemicals, Ltd., Springfield Mill, Piscataway, New Jersey.

fractionation of IgG subclasses achieved by 4 buffer changes. Proteins were eluted in a stepwise manner, using 0.005 M phosphate buffer (pH 8.0), followed by 0.01 M, pH 8.0, 0.05 M, pH 5.8, and finally 2 M NaCl solution. All the phosphate buffer solutions were made 0.015 M in NaCl solution. The OD at 280 nm of the eluates was determined. The elution profile is shown in Figure 4. Fractions 1, 2, and 3 (F1, F2, F3) were pooled separately, concentrated in carbowax and dialyzed in PBS. Immunoelectrophoresis was performed to determine the types of immunoglobulins in each fraction. Fraction 1 was found to contain only IgG2 and F2 contained predominantly IgG1 with IgG2 as a contaminant. Before dialysis F1 and F2 were further pooled and concentrated. The total protein concentration was determined.

The pooled sample was mixed with CFA and 100 µg of the protein inoculated into each guinea pig. Two weeks later, the guinea pigs were boosted with an equivalent quantity of protein and exsanguinated 10 days later. Immunoelectrophoresis was performed and the profile is shown in Figure 5. The antisera were stored at -70 C without preservative.

Antisera to *Escherichia coli*

Two rabbits were inoculated with 10^9 bacterial cells/ml suspension diluted from the immunizing inoculum. Each rabbit was injected intravenously, 4 times, at weekly intervals using doses of 0.10 ml, 0.25 ml, 0.50 ml, and 1.0 ml, respectively. A week after the final inoculation, the rabbits were euthanatized with ether and exsanguinated. The titers of the antisera were

Figure 4. DEAE anion exchange chromatography of bovine serum.

F1 - eluates of 0.005 M phosphate pH 8.0

F2 - eluates of 0.05 M phosphate pH 5.8

F3 - eluates of 2 M NaCl solution

(c) and (d) were pooled

Figure 5. Immunoelectrophoretic pattern of the guinea pig antibovine IgG.

1. IgG_2 precipitin arc

2. IgG_1 precipitin arc

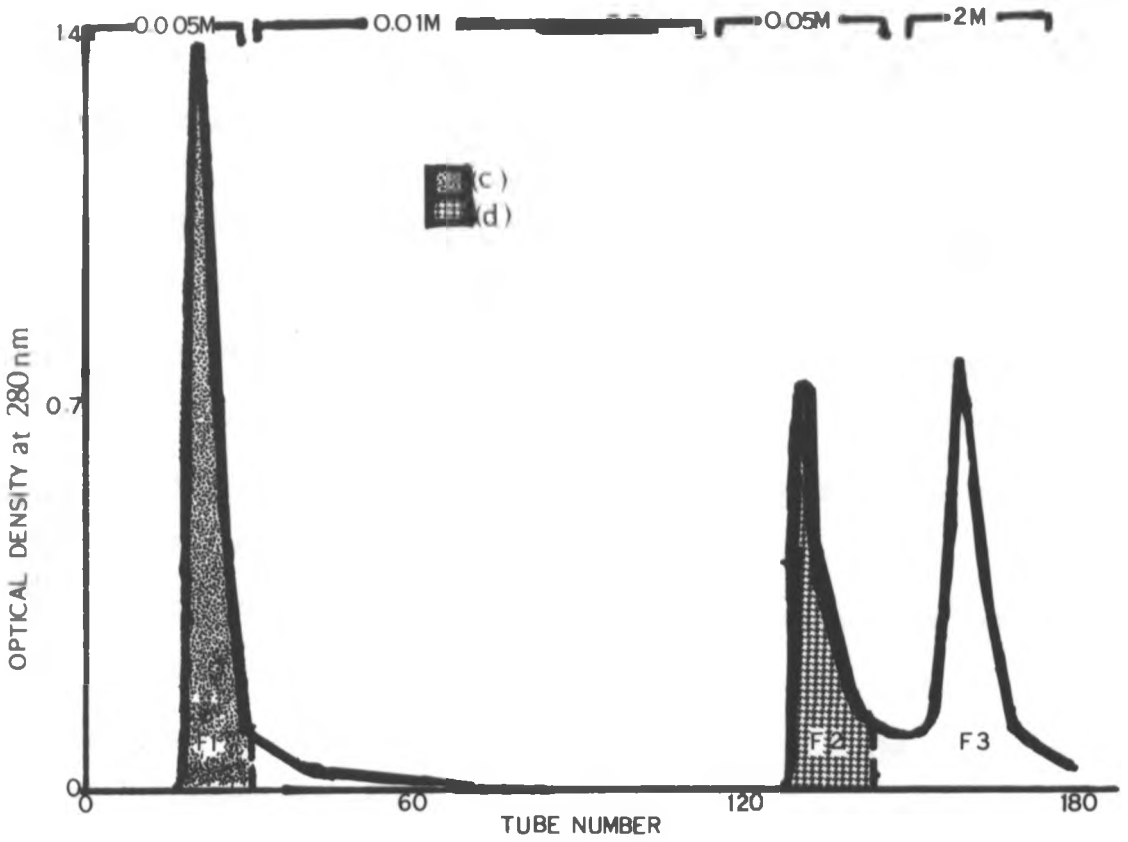

Figure 4

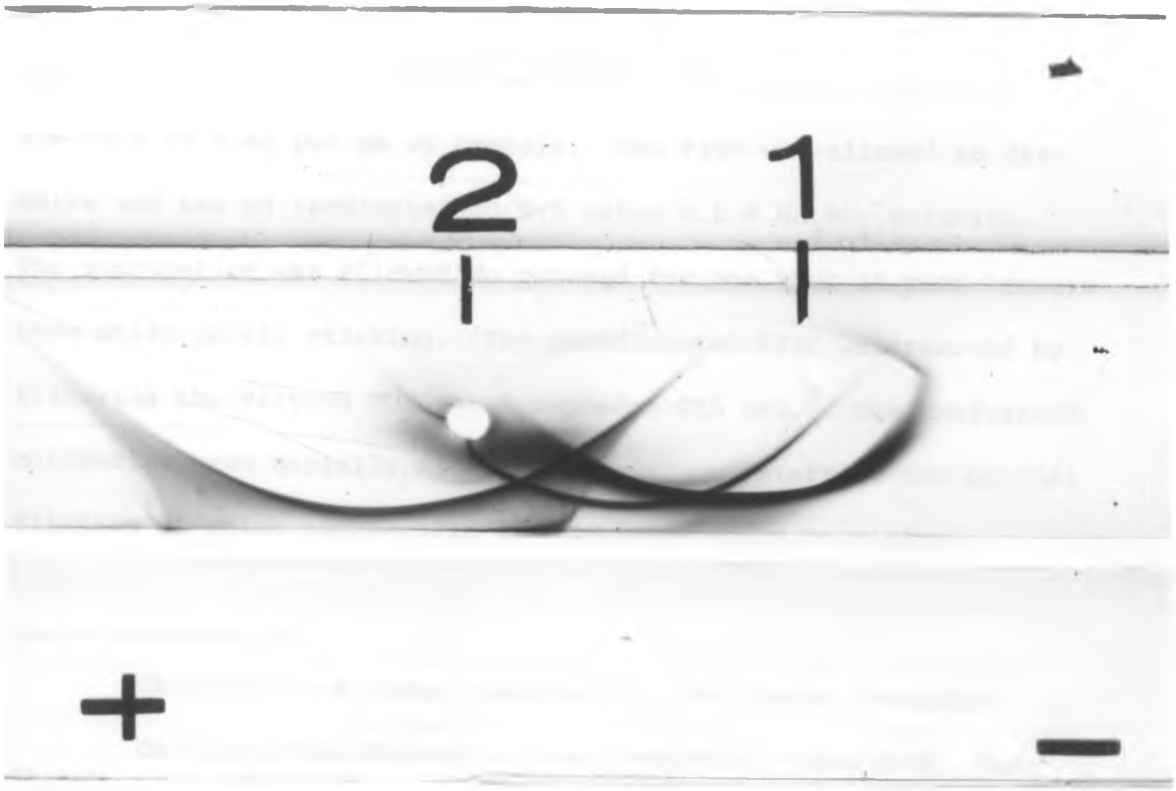

Figure 5

determined using tube bacterial agglutination test. The antisera were stored at -70 without preservative.

Conjugation of Antisera to Fluorescein Isothiocyanate (FITC)

Anion exchange chromatography was performed on the various antisera as described earlier and fractions showing antibody activity to bovine IgM, IgG or *E. coli* determined by IEP or bacterial agglutination test. The total protein concentrations in the various active fractions were determined.

Conjugation of the various antisera fractions to FITC[a] was achieved following the method described by The *et al.* (1970). Solutions of 0.15 M Na_2HPO_4 (pH 9.0) were prepared. The 0.15 M Na_2HPO_4 solution was adjusted to pH 9.5 using 0.1 M Na_3PO_4 (pH 12) solution. The filtered antibody sample was dialyzed in the conjugate buffer for 3 hours at room temperature. After dialysis, the sample was placed in a 25 ml beaker and FITC isomer 1 added at the rate of 4 mg per gm of protein. The FITC was allowed to dissolve and the pH readjusted to 9.5 using 0.1 M Na_3PO_4 solution. The conjugation was allowed to proceed for one hour at room temperature while gently stirring. The unconjugated FITC was removed by filtering the mixture through a Sephadex G25 gel.[b] The conjugated antibodies were serially diluted in order to determine the optimal dilution at which nonspecific fluorescence would be minimal.

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[a]Obtained from Sigma Chemical Co., St. Louis, Missouri.

[b]Obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey.

Fetal Immunization

Vaccination Process

To prepare the antigen (immunizing inoculum), a culture was grown overnight on trypticase soy agar slants, washed off with 0.85% NaCl solution, seeded on bottles of the same agar medium, and incubated for 20 to 24 hours at 37 C (Conner *et al.*, 1973). The growth was suspended in 0.85% NaCl solution and pooled. The pooled suspension was examined by bacteriologic cultural technique to determine purity and a viable count was obtained. Formalin, 0.4%, was then added and the suspension incubated in a shaking water bath at 37 C for 20 to 24 hours. The cell suspension was standardized to 5×10^{10} cells/ml in saline containing 0.02% of methiolate. This was stored at 4 C and used within 5 to 20 days.

The vaccination was performed in the right flank of the cow (Conner, personal communication, 1975). The area of operation, shown in Figure 6a, was within a triangle drawn from the stifle joint to the distal aspect of the 10th rib and then downward to a point approximately 30 cm anteriorly from the latero-anterior aspect of the udder along the milk vein and back to the stifle joint. With the cow standing in the stocks, the position of the fetus was located by ballotement within the triangle described. The site for insertion of the needle (where fetus was bounced) was prepared by clipping the hair and sanitizing the skin. A small area of the skin, the abdominal muscles and the peritoneum was anesthetized with 2.5% procaine hydrochloride, then scrubbed and disinfected again. Wearing sterile surgeon's gloves, the operator

Figure 6a. Site of operation during prenatal fetal immunization.

Figure 6a

inserted a sterile 2 inch, 12-gauge needle through the abdominal wall in the anesthetized area to serve as a cannula. Through this cannula, a sterile 12 inch, 16-gauge needle with a 5 ml syringe attached was passed and directed towards the uterus as shown in Figures 6b(i) and 6b(ii). The needle was passed through the uterine wall and advanced until the fetus was touched. At this time the fetus usually struggled violently. After withdrawing the needle a fraction of an inch, suction was applied by drawing back on the plunger of the syringe. When amniotic fluid was aspirated into the syringe, the syringe was detached and replaced with one containing 2.25 ml of either killed *E. coli* antigen or sterile 0.85% NaCl solution and injected directly into the amniotic cavity. The needle and cannula were then withdrawn and an antibiotic powder applied to the skin at the injection site. Of the 30 cows, 21 received *E. coli* antigen and 9 received sterile physiological saline.

Postvaccination Procedures

To prepare the viable culture comprising the challenge inoculum given orally to newborn calves, trypticase soy agar broth was inoculated from the stock agar culture and incubated 20 to 24 hours at 37 C. The cell suspension was standardized to 10^{11} bacterial cells/ml. The broth culture was stored at room temperature in the dark and used within 48 hours.

Postvaccination monitoring included the recording of rectal temperatures (at 2 days) and close observation on a 24-hour basis. At birth, the calf was immediately separated from its mother before

Figure 6b(i). Surgical instruments involved in prenatal immunization.

Figure 6b(ii). Process of vaccinating the fetus *in utero*.

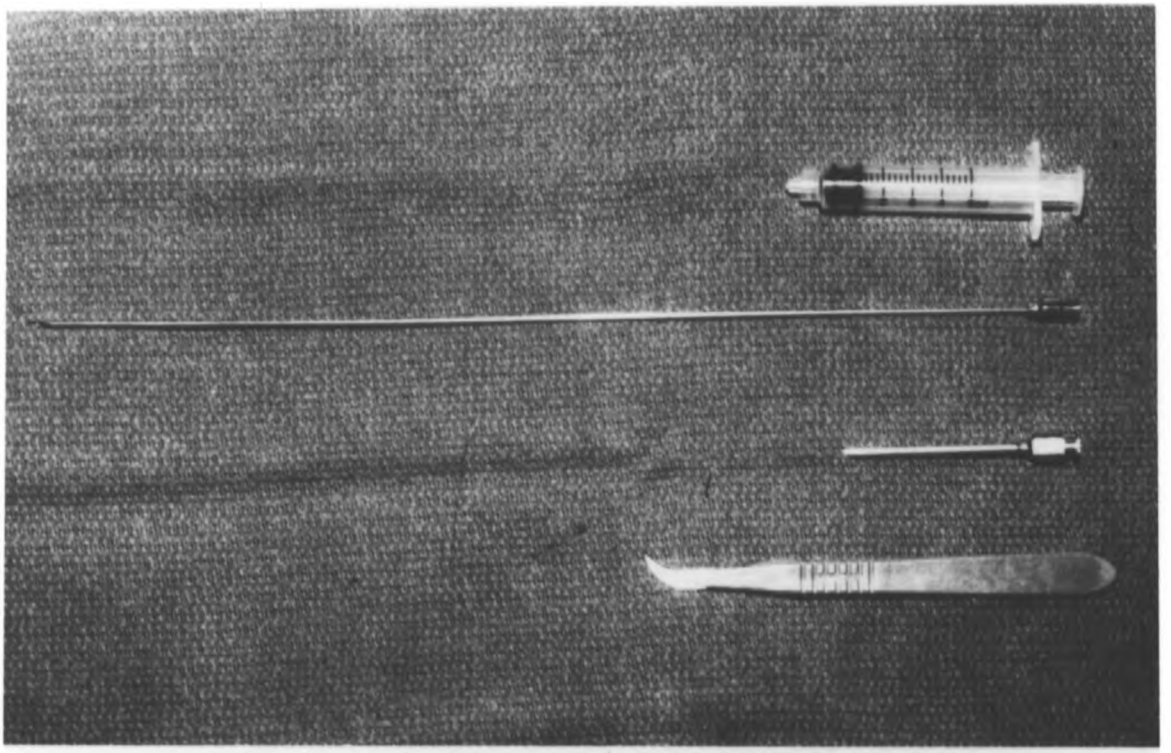

Figure 6b(i)

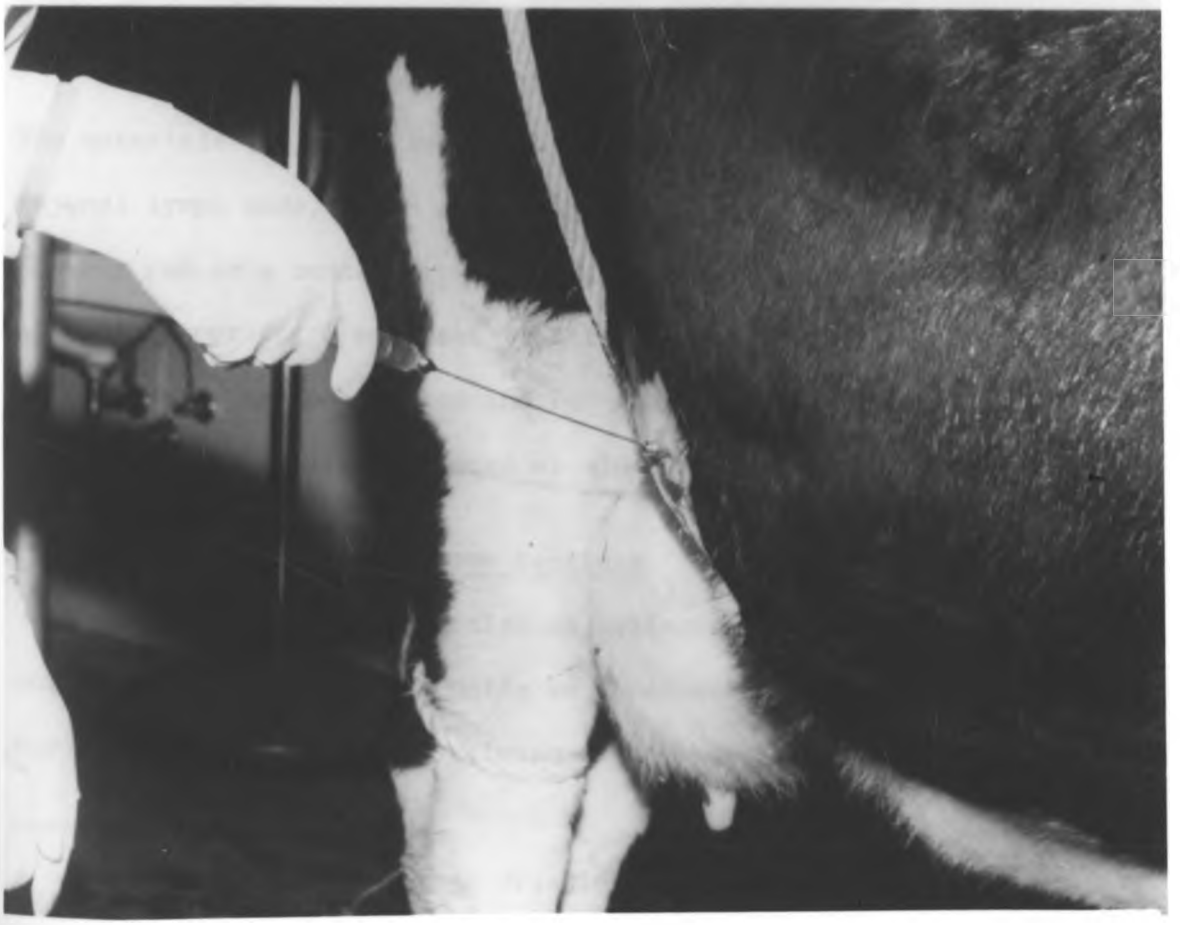

Figure 6b(ii)

there was any opportunity to suckle. Calves that were not necropsied at birth were colostrum-deprived and fed on milk replacers twice a day. Table 1 shows the various treatments to which calves were subjected.

Of the control (saline) calves, some were necropsied at birth and others were given an oral challenge dose of live *E. coli* and necropsied 5 days later. Of the calves vaccinated *in utero* with *E. coli* antigen, some were necropsied at birth, others were challenged with live *E. coli*, and still a third group was given an oral booster dose of *E. coli* antigen and 7 days later given an oral challenge dose of live *E. coli* and necropsied 5 days later.

Collection and Preservation of the Tissues

Biological specimens were collected from 14 calves at necropsy. The materials collected included the spleen, duodenum, jejunum, jejunal lymph node, ileum and ileal lymph node. Tissues were immediately put in a container with 2-methylbutane[a] and placed in liquid nitrogen vapor for 3 minutes. The tissue was then placed into plastic bags, sealed, marked and returned to liquid nitrogen vapor for 30 minutes, and then stored at -70 C.

Cutting and Staining of Frozen Sections

Frozen sections of the tissues collected from the calves were cut at a thickness of 10 μ using an International-Harris cryostat. For every section cut for staining with conjugated antibodies, one

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[a]Obtained from Eastman Organic Chemicals, Rochester, New York.

Table 1. Treatments to which calves were subjected at birth

Calf no.	*In utero* vaccination with	No. of days born after vaccination	Treatment after birth
1534	*E. coli*	38	oral challenge dose with live *E. coli*
1690	*E. coli*	20	necropsied at birth
2452	*E. coli*	28	necropsied at birth
2459	*E. coli*	34	oral challenge dose with live *E. coli*
1518	*E. coli*	20	necropsied at birth
2290	*E. coli*	10	necropsied at birth
1214	*E. coli*	18	boosted, and oral challenge with live *E. coli*
1377	*E. coli*	18	oral challenge dose with live *E. coli*
1567	saline	39	oral challenge dose with live *E. coli*
334	saline	33	necropsied at birth
2816	saline	36	necropsied at birth
299	saline	24	oral challenge dose with live *E. coli*
4657	saline	13	oral challenge dose with live *E. coli*
4717	saline	50	necropsied at birth

section was cut to be stained with hematoxylin and eosin (H&E) stain. Sections to be stained with conjugated antibodies were immediately fixed in chilled acetone for 15 minutes (Richardson *et al.*, 1972). The fixed sections were then stained with an appropriate conjugated antibody for 30 minutes at room temperature (Richardson *et al.*, 1972). The indirect staining was accomplished by flooding the sections with a suspension of *E. coli* antigen, diluted 1:50, for 30 minutes at room temperature, rinsed in PBS and flooded again with conjugated antibody against *E. coli* for another 30 minutes. The sections were then washed in PBS for 15 minutes and mounted in buffered glycerine (1 part PBS and 9 parts glycerine).

Fluorescence Microscopy and Photomicrography

A Wild M20 microscope[a] equipped with a Wild plan-Fluotar 40/0.65 objective was used with an HBO 200 mercury vapor illuminator and a UV-fluorescence exciting filter 33 mm. For photomicrography, a Wild attachable camera[a] fitted with a built-in photocell for connection to a light meter with an electronic control for the exposure time was used with a Tri-X Pan 135, high speed ASA 400 black and white film.

Staining, Microscopy and Photomicrography of Histologic Sections

The sections for histopathologic examination were stained with H&E stain following the procedure described by Luna *et al.* (1968). For examination of the stained sections, an A020 microscope was

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[a]Obtained from A Diagger and Co., 159 West Kinzie Street, Chicago, Illinois 60610.

used and for photomicrography an AO model 1035 35mm camera was used with a Kodachrome II color film.

RESULTS

Clinical Observations of the Experimental Calves

None of the calves that were given an oral challenge dose of live *E. coli* succumbed to the infection, although some of the calves exhibited some clinical signs. Calf 1534 had been vaccinated *in utero* with *E. coli* antigen and was born 38 days after the vaccination. Although this calf passed some bloody feces a few hours after challenge, it showed no signs of diarrhea or fever. Calf 2459, born 34 days after *in utero* vaccination, showed no clinical signs after receiving an oral challenge dose of live *E. coli*. Calves 299 and 4657, which had received sterile physiological saline *in utero*, had mild diarrhea after challenge but did not die. The only calf (1214) which had been vaccinated *in utero* with *E. coli* antigen and boosted at birth with the same antigen before being challenged with live *E. coli*, did not show any clinical signs after the challenge. Calf 1377 showed signs of weakness and inappetence but had no diarrhea. This calf had been vaccinated *in utero* with *E. coli*.

Because both vaccinated and control calves showed no signs of colibacillosis when challenged, it was decided to vaccinate, *in utero*, 2 dairy calves, one with *E. coli* antigen and the other with sterile physiological saline. At birth, both calves were given an

oral challenge dose of live *E. coli*. The calf which had received saline *in utero* died within 36 hours of challenge and exhibited typical signs of colibacillosis. The other calf, which had received *E. coli in utero*, survived the challenge until necropsied 5 days later.

Observations on Sections Stained with Conjugated Antibodies

General

Observations were made on sections stained with conjugated antibodies prepared by the method outlined. Two sections were made from each frozen tissue. One section was stained with conjugated, antibovine IgM and the other with antibovine IgG. In addition, a section from each frozen tissue collected from calf 1214 was cut and stained with conjugated antibody against *E. coli*. The optimal dilutions of the various conjugated antisera fractions were found to be as follows:

anti-IgM - 1:4

anti-IgG - 1:16

anti-*E. coli* - 1:8

Each stained section was observed under a fluorescent microscope at (X25) magnification. Plasma cells counted in 20 consecutive microscopic fields were recorded. There were no fluorescent cells observed in any of the control calves although some plasma cells had been detected by light microscopy as shown in Figure 7. Figure 8 shows plasma cells demonstrable in the H&E sections of the calves vaccinated with *E. coli in utero*.

Figure 7. Photomicrograph of histologic section stained with H&E. Plasma cell in a section of a control calf (arrow).

Figure 8. Photomicrograph of histologic section stained with H&E. Plasma cells in a section of a vaccinated calf (arrows).

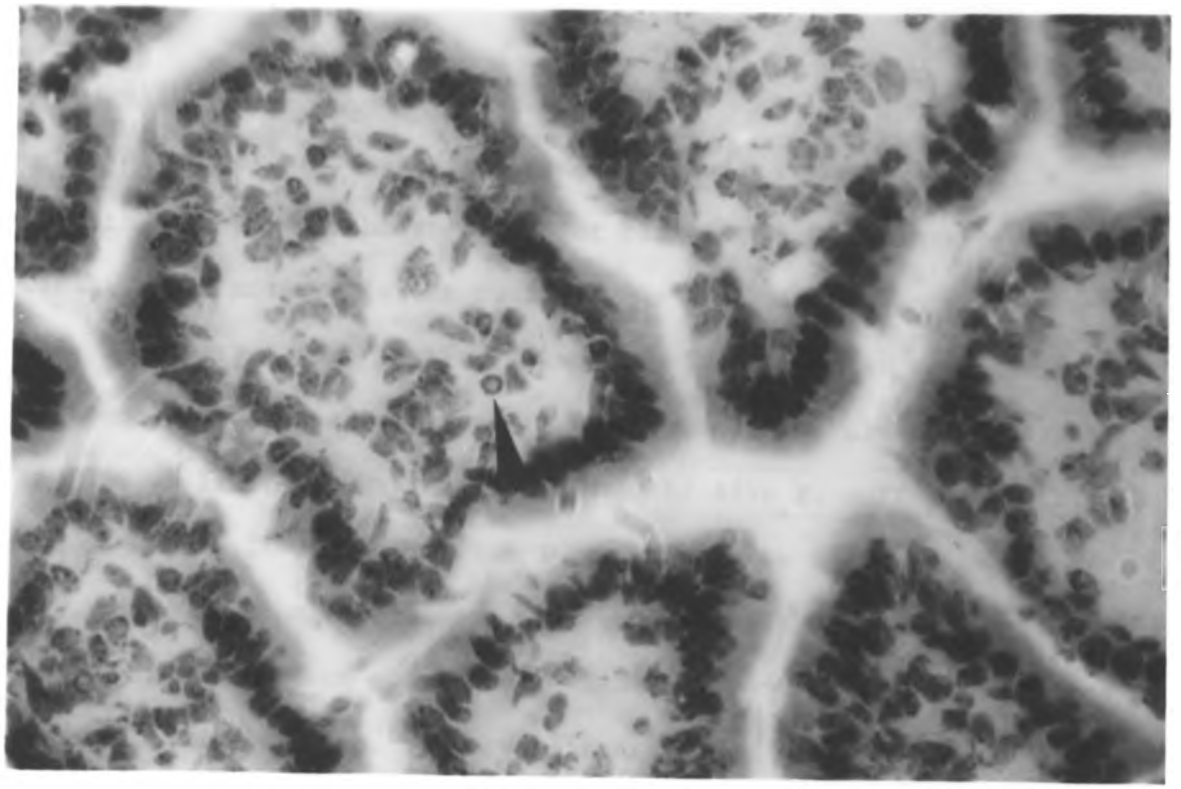

Figure 7

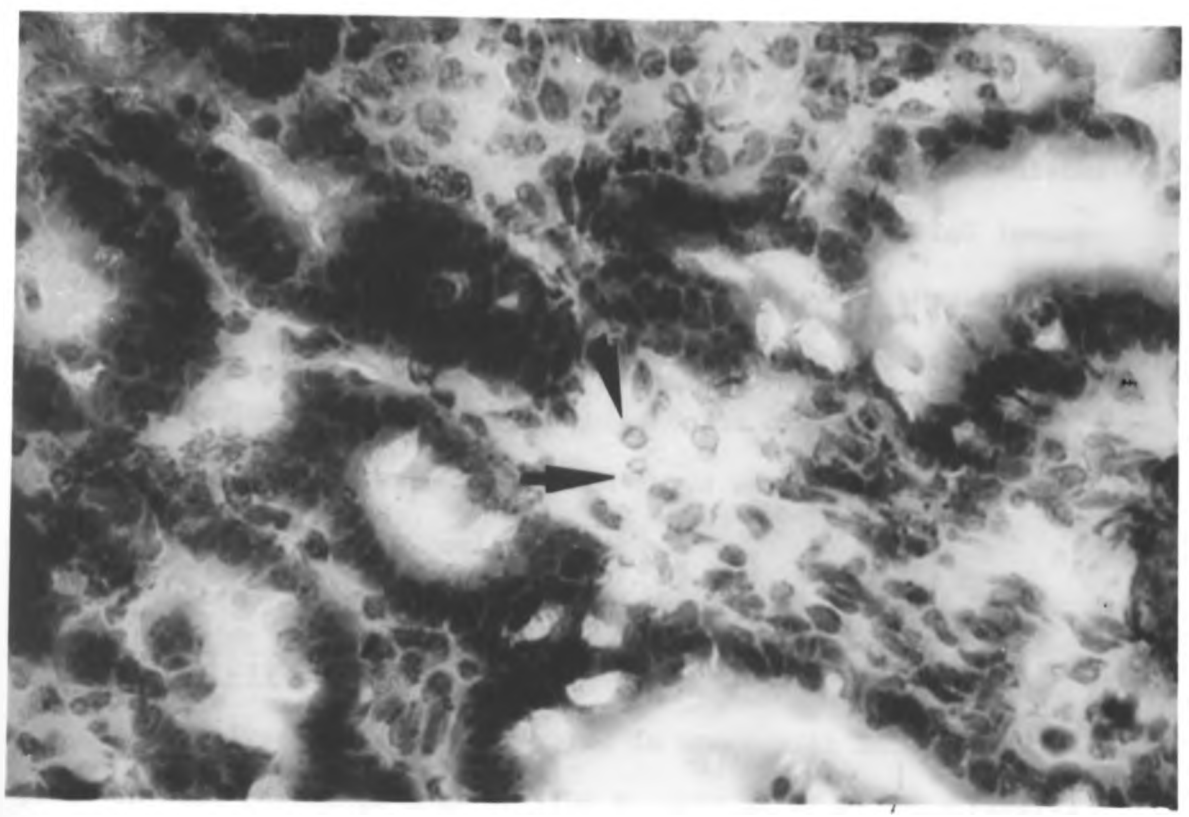

Figure 8

Observations of Calf 1214

This experiment was performed to determine whether or not a calf that received a booster dose at birth after an *in utero* vaccination was more resistant to challenge than the calves receiving only a primary dose. This calf was born 18 days after vaccination *in utero* with *E. coli* antigen. A booster dose was given at birth and 7 days later an oral challenge dose of live *E. coli* was administered. The calf was necropsied 5 days after the challenge.

Sections from each tissue were stained with conjugated antibodies and a count of plasma cells made in 20 consecutive microscope fields in each section. The results are shown in Table 2.

Figures 9, 10 and 11 are photomicrographs illustrating fluorescent plasma cells producing immunoglobulins IgM and IgG classes and antibodies against *E. coli*, respectively. These photomicrographs were taken from the jejunal lymph node.

The observations indicated that exposure to *E. coli* stimulates differentiation of plasma cells producing either IgM or IgG immunoglobulins and that the number of IgG-cells is greater than the number of IgM-cells. The most active tissues in production of these immunoglobulins are the jejunum and its draining lymph node. Of the plasma cells counted, only one-third were shown to contain specific antibodies to *E. coli*.

Observations on Stained Sections from Calves 1534, 2459 and 1377

These calves had been vaccinated *in utero* with *E. coli* and were given an oral challenge dose of live *E. coli* at birth to determine,

Table 2. Plasma cell counts on sections from calf 1214 stained with various conjugated antibodies

Calf no.	Conjugated antibody	Number of plasma cells*					
		Spleen	Duodenum	Jejunum	Jej. l.node	Ileum	Ileal l.node
1214	anti-IgM	12	35	19	82	10	0
1214	anti-IgG	22	111	46	145	34	0
1214	anti-*E. coli*	0	44	45	76	10	0

*Plasma cells were counted in 20 consecutive microscopic fields (X25).

Figure 9. Photomicrographs of sections stained with fluorescein conjugated antibody.

Top - plasma cells in section stained with antibovine IgM (arrow).

Center - the plasma cell in the top photomicrograph magnified several times.

Bottom - plasma cell (arrow) in another field of the same section as in top and center photomicrographs.

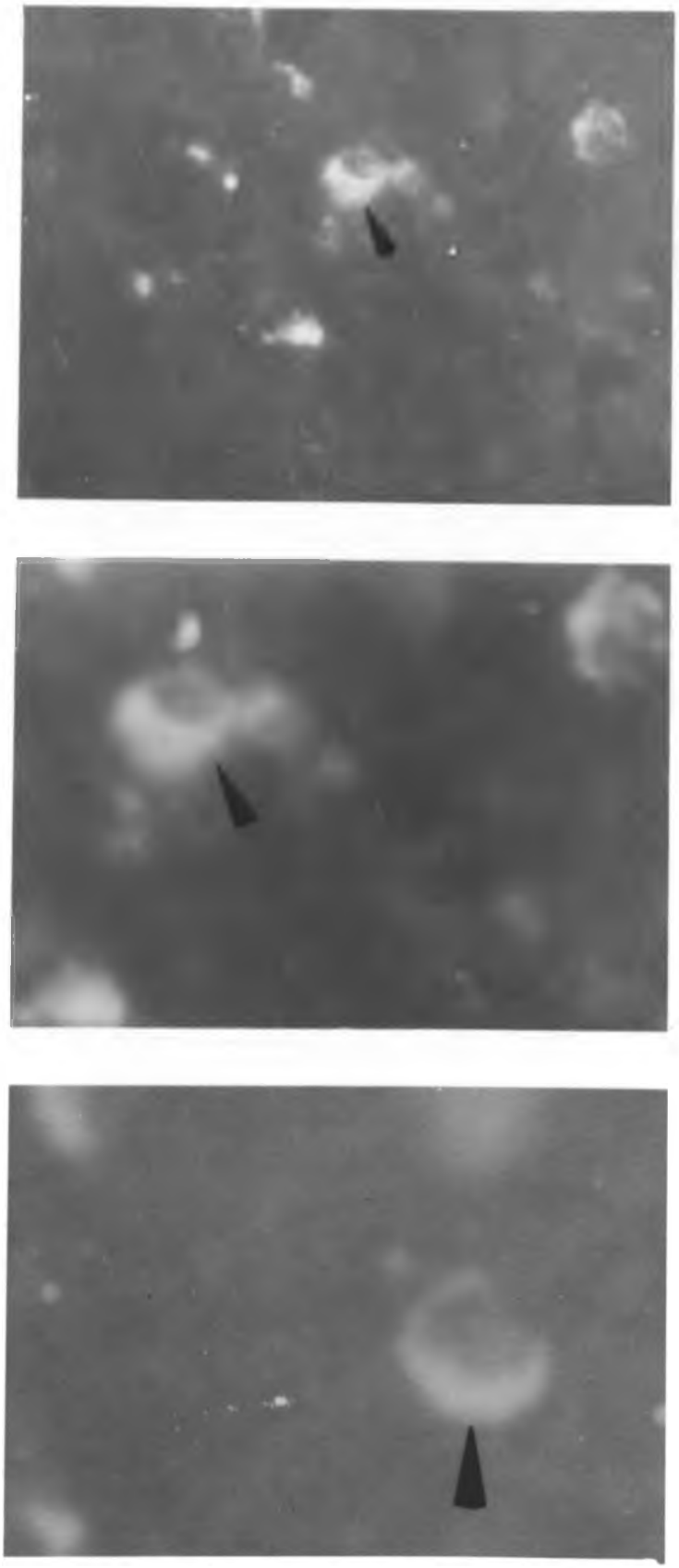

Figure 9

Figure 10. Photomicrographs of section stained with fluorescein conjugated antibody.

Top - plasma cell (arrow) in section stained with antibovine IgG.

Bottom - the same plasma cell (arrow) as in top photomicrograph magnified several times.

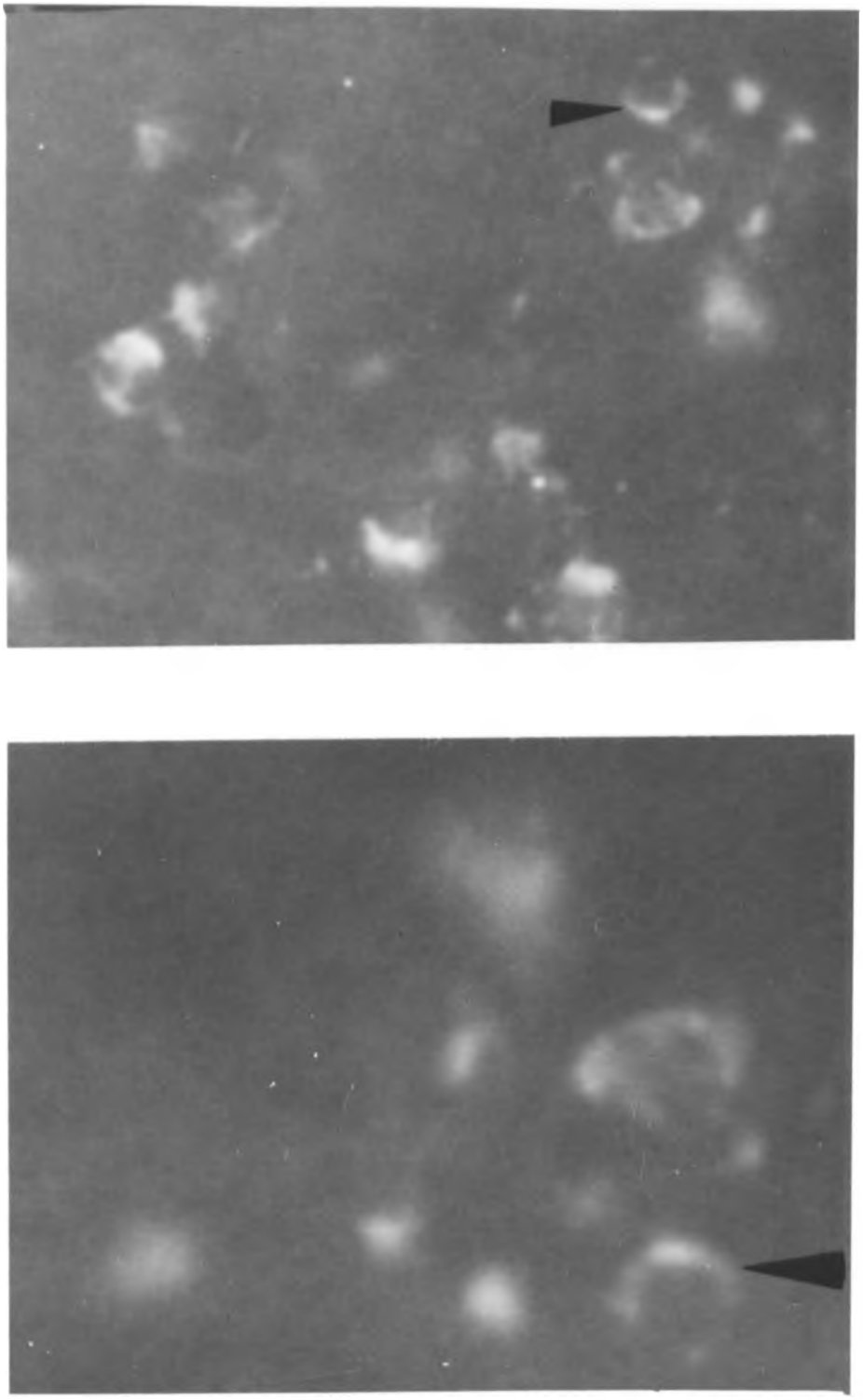

Figure 10

Figure 11. Photomicrographs of sections stained with fluorescein conjugated antibody.

Top - plasma cell (arrow) in section stained with anti-*E. coli*.

Bottom - same plasma cell (arrow) as in top photomicrograph magnified several times.

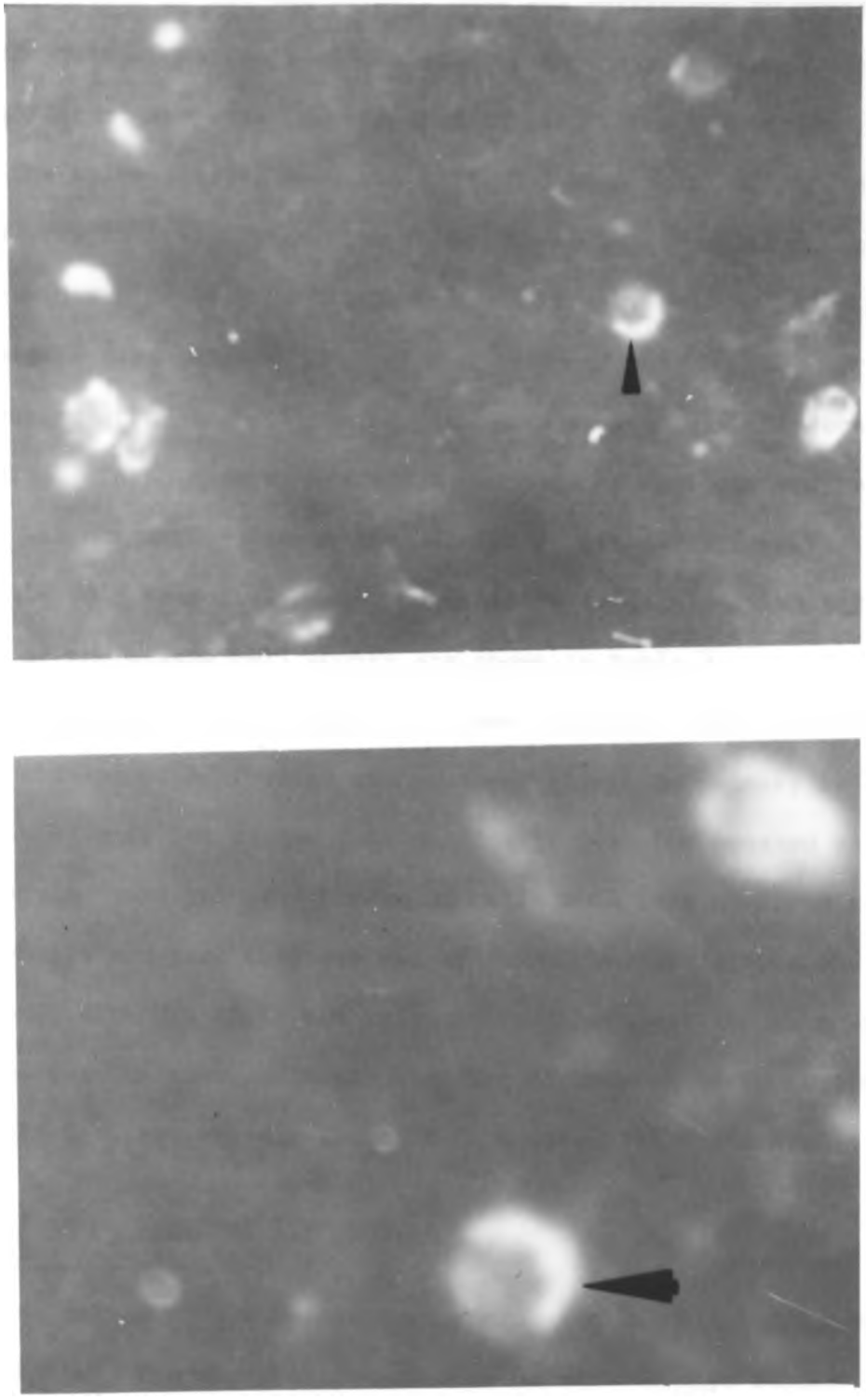

Figure 11

firstly, whether or not the primary dose was sufficient to protect the calf against challenge infection and, secondly, if there was any significant increase in plasma cell counts from the boosted calf. The calves, 1534, 2459 and 1377, had been born 38, 34 and 18 days, respectively, after the *in utero* vaccination with *E. coli*. They were given an oral challenge dose of live *E. coli* at birth and necropsied 5 days later.

Sections from each tissue sampled were stained with conjugated antibodies. In this group of calves, conjugated antibody against *E. coli* was not used to stain the sections. Plasma cells were counted in 20 consecutive microscopic fields in each section. The results of the plasma cell counts are shown in Table 3.

The findings in this group of calves indicated that both IgM and IgG classes of antibodies are produced after the primary injection with *E. coli* but in lower quantities than in the boosted calf. It was again found that IgG producing cells were more numerous than the IgM-cells and that they are mainly formed in the jejunum and the ileum. Although there was only one calf born earlier than 20 days, the results also indicated that the production of antibodies to *E. coli* declines rapidly about 20 days after vaccination.

Observations on Sections from Calves 2290, 1518, 2452 and 1690

These calves were vaccinated *in utero* with *E. coli* and necropsied at birth. The vaccination was done on various days before parturition ranging from 10 to 30 days. Variations in length of intrauterine exposure to *E. coli* antigen served to determine whether

Table 3. Plasma cell counts on sections from calves 1534, 2459 and 1377 stained with fluorescein conjugated antibodies

Calf no.	Conjugated antibody	Number of plasma cells*					
		Spleen	Duo-denum	Je-junum	Jej. l.node	Ileum	Ileal l.node
1534	antibovine IgM	0	0	47	0	8	0
1534	antibovine IgG	0	0	47	34	0	0
2459	antibovine IgM	0	0	0	0	21	0
2459	antibovine IgG	13	0	86	0	46	0
1377	antibovine IgM	26	0	0	0	31	0
1377	antibovine IgG	0	0	95	0	69	0

*The plasma cells were counted in 20 consecutive microscopic fields (X25).

Table 4. Plasma cells counted from sections from calves 2290, 1518, 2452 and 1690 stained with fluorescein conjugated antibodies

Calf no.	Conjugated antibody	Number of plasma cells*					
		Spleen	Duo-denum	Je-junum	Jej. l.node	Ileum	Ileal l.node
2290	antibovine IgM	0	0	0	0	0	0
2290	antibovine IgG	0	0	0	0	0	0
1518	antibovine IgM	20	0	30	0	0	0
1518	antibovine IgG	0	0	36	27	0	0
2452	antibovine IgM	0	0	0	0	15	0
2452	antibovine IgG	0	0	71	0	0	0
1690	antibovine IgM	0	0	24	0	0	0
1690	antibovine IgG	0	0	26	0	0	0

* The plasma cells were counted in 20 consecutive microscopic fields (X25).

or not the length of period of exposure to the antigen affects the animal's resistance to the antigen and if it affected the number of plasma cells produced. The calves, 2290, 1518, 1690 and 2452, were born 10, 20, 20 and 28 days, respectively, after *in utero* vaccination. These calves were necropsied at birth and sections were stained with either conjugated IgM or IgG. The results are shown in Table 4.

The results indicate that the period of maximum plasma cell production is about 20 days after vaccination.

Statistical Analysis

t_1 = control (saline) calves

t_2 = calves vaccinated *in utero* and necropsied at birth

t_3 = calves vaccinated *in utero*, challenged at birth and necropsied 5 days after challenge

t_4 = calf vaccinated *in utero*, boosted at birth, challenged 7 days later and necropsied 5 days after challenge

Data

Tissue	Plasma cell	t_1	t_2	t_3	t_4
spleen	IgM	0	5	9	12
	IgG	0	0	4	22
duodenum	IgM	0	0	0	35
	IgG	0	0	0	111
jejunum	IgM	0	14	16	19
	IgG	0	33	76	46
jej. l.node	IgM	0	0	0	82
	IgG	0	7	11	145

Tissue	Plasma cell	t_1	t_2	t_3	t_4	
ileum	IgM	0	4	20	10	
	IgG	0	0	38	34	
ileal l.node	IgM	0	0	0	0	
	IgG	0	0	0	0	
	Sums i.	0	63	174	516	total $Y_{.}=753$
	replication ri	12	12	12	12	n=48
	mean $(\bar{Y}i.)$	0	6	15	43	

	t_1	t_2	t_3	t_4	Σ (sums)	
$\left(\sum_{i=1}^{12} Y_{ij}^2\right)$	0	1375	8094	45656	55125	[B]

	t_1	t_2	t_3	t_4	Σ (sums)	
$(Y_{i.}^2/ri) =$	0	330.75	2523	22188	25041.75	[C]

	t_1	t_2	t_3	t_4	
difference (SSi) =	0	1044.25	5571	23468	SSE = 30083.25

	t_1	t_2	t_3	t_4	
$(df)i$ =	11	11	11	11	VE = 44

	t_1	t_2	t_3	t_4
S_i^2 =	0	94.932	506.455	2133.455

	t_1	t_2	t_3	t_4
Si =	0	9.743	22.506	46.189

$Y_{..}^2/n$ = 11812.688 [A]

The sample variances of treatments are significantly different (ranging from 0 to 2133.455).

Sums of Squares

SSY = [B] - [A] = 55125 - 11812.688 = 43312.312

SST = [C] - [A] = 25041.75 - 11812.688 = 13229.062

MST = SST/(t-1) = 13229.062/3 = 4409.687

MSE = SSE/VE = 30082.25/44 = 683.710

Upper Percentage Points of Dunnett's t-Distribution: Two-sided Comparisons with a Control

a) Treatment 2 compared with treatment 1 (control)

$$t_D = (\bar{Y}_2 - \bar{Y}_c) \; / \; \sqrt{MSE \; (\frac{1}{r_2} + \frac{1}{r_c})}$$

$$= (6 - 0) \; / \; \sqrt{683.710 \; (\frac{1}{12} + \frac{1}{12})}$$

$$= 6 \; / \; \sqrt{683.710 \; (0.16667)}$$

$$= 6 \; / \; 10.67$$

$$= 0.56$$

b) Treatment 3 compared with treatment 1 (control)

$$t_D = (\bar{Y}_3 - \bar{Y}_c) \; / \; \sqrt{MSE \; (\frac{1}{r_3} + \frac{1}{r_c})}$$

$$= (15 - 0) \; / \; \sqrt{683.710 \; (0.16667)}$$

$$= 15 \; / \; 10.67$$

$$= 1.4$$

c) Treatment 4 compared with treatment 1 (control)

$$t_D = (\bar{Y}_4 - \bar{Y}_c) \; / \; \sqrt{MSE \; (\frac{1}{r_4} + \frac{1}{r_c})}$$

$$= (43 - 0) \; / \; \sqrt{683.710 \; (0.16667)}$$

$$= 43 \; / \; 10.67$$

$$= 4.03$$

Critical Values

$\pm t_D$, , M, VE

$\pm t_D$, 0.05, 4, 44 = 2.534

$\pm t_D$, 0.01, 4, 44 = 3.19

Statistical analysis shows that the boosted calf had significantly higher number of plasma cells than calves in the other treatment groups. The analysis is shown in Table 5.

Table 5. Statistical analysis

Treatment	Mean ± SD	SE	No. of sections	P value
t_2	6 ± 9.7	± 2.8	12	N.S.*
t_3	15 ± 22.5	± 6.5	12	N.S.*
t_4	43 ± 46.2	± 13.3	12	<0.01

*Not significant.

DISCUSSION

The objective of this research was to determine whether or not local antibody was produced in the gastrointestinal mucosa, mesenteric lymph nodes and spleen following exposure of the bovine fetus to *E. coli* antigen. To do this, fluorescent antibody tests, using conjugated antibovine IgM and IgG and anti-*E. coli* to detect and enumerate antibody producing plasma cells, were applied. The results clearly show that there were significant differences in the plasma cell counts in the tissues of the control and immunized animals. Whereas the control calves had no detectable cells using fluorescent antibody tests, even though a few plasma cells were found in histologic sections, the immunized calves had varying numbers of antibody producing plasma cells in the tissues examined. The finding of plasma cells in the H&E sections of control calves is in agreement with South (1971), who reported that all species phylogenically more advanced than amphibians have lamina propria plasma cells. These are the cells that differentiate and produce specific antibodies when stimulated by an antigen. It is likely that if more microscopic fields were examined using fluorescent antibody tests in the control sections, some plasma cells would have been detected. Alternatively, the H&E staining may be a more sensitive method to enumerate cellular components in a section.

The jejunum and its draining lymph node and the ileum had more plasma cells than any of the other tissues examined in the three groups vaccinated with *E. coli* antigens. This finding is in support of the clinical observations of Dade and Trapp (personal communication, 1975) that *E. coli* most of the time multiplies in the jejunum and the ileum. In addition, in the boosted calf, the duodenum and spleen became active in formation of plasma cells. This suggests that, under optimal conditions, the entire length of the small intestine, its draining lymph nodes and the spleen can be sites of antibody formation although the ileal lymph node showed no detectable plasma cells in any of the groups. Calves boosted at birth had specific antibodies to *E. coli* in all the tissues examined except the spleen and the ileal lymph node. Calves in the other two vaccinated groups had detectable plasma cells in the jejunum and ileum and occasionally in the spleen.

Gay (1971) observed that calves vaccinated for the longest time produced heterogeneic antibody to *E. coli* and also to *S. typhimurium* whereas calves vaccinated for 16 days showed only a partial response in this respect and the calf vaccinated for only 14 days showed a serotype-specific response. In the present study, it was demonstrated that calves born 10 days after vaccination had no detectable plasma cells. The highest number of cells was found in sections of calves born 18 to 20 days after vaccination. This may be explained by the fact that animals begin to produce detectable antibodies to bacterial antigens 14 days after the primary injection. The peak production period follows in the few succeeding

days. It was also observed that the number of cells declined if tissues were obtained from calves born more than 20 days after vaccination.

Gay (1971) proposed that since IgG and IgM are absorbed non-selectively in the calf and since IgM fractions may contain antibodies against *E. coli*, it is possible that a deficiency of antibody in the IgM class rather than in the IgG class might render the calf susceptible to colisepticemia and diarrhea. Previous work has shown that there is more IgG than IgM in colostrum and in this study more IgG- than IgM-producing plasma cells were formed. However, the relative biological activity of IgM and IgG antibodies against *E. coli* are important since it has been shown that fewer molecules of IgM are required for complement fixation and opsonization activity than IgG (Robbins *et al.*, 1965). This might explain why, although IgM is produced in less quantity, it is still a more important antibody against *E. coli* than the abundant IgG antibody. This observation does not imply that IgG is unimportant in protecting the calf against *E. coli* but that more molecules of IgG than IgM may be required to protect against the same number of *E. coli*. Alternatively, the finding of high numbers of IgG than IgM plasma cells could be due to the shift of plasma cell antibody production from IgM to IgG. Murphy *et al.* (1966) reported that all the 5 cattle they experimentally infected with *Anaplasma marginale* had IgM globulin agglutinating activity at the times of peak parasitemia and hemolytic crisis. As the carrier phase developed, it appeared that antibody activity shifted to IgG globulin. This finding is

in agreement with the observations by Brambell (1970) that the first antibodies to appear, when ruminant fetuses were challenged with a viable antigen, were of the IgM class. The high number of IgG-producing cells counted in this study could also be due to the strain of *E. coli* used in this work. Glantz (1971) found that 026 serotype is noted for producing septicemias and it is possible that the antibody which functions better in serum than on local mucosal surfaces should be produced in larger amounts; hence the higher numbers of IgG- than IgM-producing plasma cells. This could also account for the generally low numbers of plasma cells detected in all groups of vaccinated calves.

Logan *et al.* (1974) have shown that colostral IgM and IgG offer greater protection against *E. coli* in calves than IgA. Dixon *et al.* (1961) reported that colostral IgG levels were considerably higher than those of the maternal serum and that the IgM levels in colostrum were not different from the levels in serum. Blakemore *et al.* (1956) and Garner *et al.* (1958) had shown that homologous gammaglobulins, given intravenously to a pregnant cow, were transferred to mammary gland secretions during the weeks immediately preceding calving. They also found that whereas there was almost complete exclusion of IgG2 from the immunoglobulins of milk, there was no selective absorption by the intestinal mucosa of the calf. All these findings seem to suggest that more IgG1 antibodies are produced during the colostral period and that IgG1 is more likely to be responsible for protection against enteric diseases than IgG2. This is supported by the observation that there were more IgG- than IgM-producing plasma cells.

Immunoglobulin-A-producing plasma cells were not studied in this work, but Gordon *et al.* (1971) reported that calves form IgA on their gastrointestinal mucosa and Porter (1973) and Mach *et al.* (1971) observed that IgA is a major immunoglobulin in the washings from the epithelium of the intestinal tract in calves. Furthermore, IgA has been found to have some activity against *E. coli* although not as much as IgM and IgG (Logan *et al.*, 1974). However, these findings are not in accordance with reports that IgG1 predominates both in the bovine mucosal secretions and also in plasma cells locally situated in the external mucosa (Curtain, 1971) and that no IgA-producing cells are present in the lamina propria of bovine intestinal tissue (Vaerman, 1970). It seems reasonable to propose that IgA might be playing a role in protection of the calf against *E. coli* although the role might be less significant than that played by IgM and IgG.

None of the beef calves used in this study, including control calves, succumbed to challenge with live *E. coli*. This observation is in sharp contrast with the findings of Conner *et al.* (1973) and Gay (1971). They reported complete mortality in control animals (dairy breed) despite the fact that the challenge dose they used was lower. The finding is also in contrast with our own observation on dairy calves where the control calf died within 36 hours after challenge while the calf vaccinated *in utero* survived. This difference could not have resulted from a different route of antigen administration since a dairy calf similarly injected with saline in this study succumbed to challenge infection. The discrepancy may be a reflection of breed differences.

SUMMARY AND CONCLUSIONS

Fetuses were immunized or vaccinated *in utero* by depositing either killed *E. coli* or sterile physiological saline in the amniotic cavity. Of the calves vaccinated with physiological saline, some were necropsied at birth and others were given an oral challenge dose of live *E. coli* and necropsied 5 days later. Of the calves that received *E. coli in utero*, some were necropsied at birth, others were given an oral challenge dose and necropsied 5 days later, and a third group was given a booster dose at birth, challenged orally 7 days later and necropsied 5 days after the challenge.

Clinically, both control and *E. coli* vaccinated calves showed no signs of colibacillosis.

Using fluorescent antibody tests, the highest number of antibody-producing plasma cells were counted, in the jejunum, its draining lymph node and the ileum. Most plasma cells were detected in the calf that had been boosted. Plasma cell numbers were greatest in the calves which were born 18 to 20 days after vaccination *in utero*. More IgG- than IgM-producing plasma cells were counted and the cells producing specific antibodies against *E. coli* comprised only one-third of the total number of the cells counted.

While there were no obvious differences in clinical signs between the control and the immunized calves, the finding of IgM- and IgG-producing plasma cells in the intestinal mucosa and draining lymph nodes of immunized calves is of interest. Further study may provide a better understanding of the type of antibody that is involved in the protection of the calf against *E. coli*.

The observations that the highest numbers of plasma cells are produced about 20 days after vaccination, mainly in the jejunum, jejunal lymph node and the ileum, may be of value and more work in this area may be considered an important advance in understanding the immunologic response of the calf to *E. coli*.

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