IMMUNIZATION OF PORCINE FETUSES IN UTERO WITH ESCHERICHIA COLI 0149:K91(B):K88(L) AND RESPONSE OF GNOTOBIOTIC PIGS TO CHALLENGE AT BIRTH

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ABSTRACT

IMMUNIZATION OF PORCINE FETUSES *IN UTERO* WITH *ESCHERICHIA COLI* 0149:K91(B);K88(L) AND RESPONSE OF GNOTOBIOTIC PIGS TO CHALLENGE AT BIRTH

By

Carol A. Thompson

The immunologic response of fetal pigs to *in utero* injection of *Escherichia coli* 0149:K91(B);K88(L) and the response of neonates to challenge at birth were studied in 34 gnotobiotic pigs. A laparotomy was performed at 98 days of gestation. *Escherichia coli* bacterin (5×10^9 organisms) was injected *in utero* into either the amniotic fluid or the muscles of the hind limb of 14 fetuses. The remaining fetuses were either injected *in utero* with saline in the same manner or were uninjected. After delivery by hysterotomy, viable *E. coli* organisms were given orally to 23 of the gnotobiotic pigs (challenged). The remaining 11 gnotobiotic pigs were given saline orally (controls). Eighteen of the 23 challenged pigs (78%) died following exposure. The *in utero* injection of either bacterin or saline did not appear to alter the susceptibility of the neonate at birth to viable *E. coli* of the same serotype. The clinical signs and gross and microscopic lesions were consistent with those associated with colibacillosis.

Antibody to *E. coli* antigen was not detected by using the bacterial agglutination and passive hemagglutination tests in the serum of the pigs at birth. However, antibody to *E. coli* antigen was detected in the serum of 2 challenged pigs at 11 days of age.

The results indicate that with the present techniques, porcine neonates previously injected *in utero* with *E. coli* antigen are not protected when challenged with live organisms of the same strain, nor are detectable antibodies against *E. coli* produced by fetal pigs. However, antibodies to *E. coli* antigen are formed by neonatal pigs before 11 days of age. Thus, one can assume that pigs are immunocompetent to *E. coli* antigen sometime between 98 days of gestation and 11 days of age.

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Carol A. Thompson

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To my Mother and Father

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INTRODUCTION

Active immunization of the fetus *in utero* may be an important means of protecting the neonate against infectious diseases, such as colibacillosis, which are difficult to control using conventional methods. Enteric colibacillosis, caused by enteropathogenic strains of *Escherichia coli*, affects a variety of domestic animals and is a major cause of death of newborn pigs.

Numerous studies on active immunization of the bovine fetus *in utero* for protection of the neonate against colibacillosis have been conducted in recent years. The results of these studies indicate that prenatal immunization may be a means of preventing this disease.

The purpose of the present research was to determine whether previous exposure of the porcine fetus to *E. coli* antigen *in utero* would protect the pig against challenge with the live organism at birth.

OBJECTIVES

The objectives of this research were:

1. To determine whether porcine fetuses could be immunized with a killed inoculum of *Escherichia coli (E. coli)* 0149:K91(B)-K88(L) by *in utero* injection into either the amniotic fluid or muscles of the thigh.

2. To determine whether porcine neonates previously injected *in utero* with *E. coli* bacterin were protected from oral challenge with the live organism of the same strain.

3. To compare the clinical responses and gross and microscopic lesions in porcine neonates given either viable *E. coli* organisms or saline orally.

4. To quantitate the immune responses of pigs by direct bacterial agglutination test.

5. To gain experience in research investigative techniques.

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LITERATURE REVIEW

General Immunology

The immune system is important to the survival of man and animals in an environment containing large numbers of potentially harmful infectious agents. This system enables the animal to produce either specific immunoglobulins, or cells, or both, which will react specifically with foreign substances that enter the body. Lymphocytes are an intrinsic part of the immune system and the principal cells involved in immune reactions.

The peripheral lymphoid tissues of mammals and birds contain 2 different types of lymphocytes which originate from 2 central lymphoid organs (Raff, 1973). The thymus (T) lymphocytes are derived from the thymus and are responsible for the cell-mediated immunity. The bursa (B) lymphocytes, which are responsible for humoral mediated immunity, are derived from the bursa of Fabricius in birds and the equivalent of the bursa in mammals (Raff, 1973). Lymphocytes are produced by the thymus and bursa independently of antigenic stimuli (Raff, 1973).

Lymphocytes first appear in the thymus of the developing fetus. They are derived from hemopoietic stem cells which migrated to the thymus from the fetal liver. In the adult, these stem cells are derived from the bone marrow (Raff, 1973). Once in the thymus, the stem cells proliferate and differentiate into thymus lymphocytes

(Raff, 1973). Some of the lymphocytes in the thymus migrate to peripheral lymphoid organs to establish the T lymphocyte population (Weissman, 1967). In mammals, the B cell precursors or stem cells arise from the bone marrow and, in some unknown tissue, differentiate into B lymphocytes (Eisen, 1974b). These stem cells may undergo differentiation in the gut-associated lymphoid tissues such as tonsils or appendix (Eisen, 1974b; Raff, 1973).

The most widely accepted theory concerning the ability of lymphocytes to recognize specific antigens is the clonal selection theory. The clonal selection theory suggests that

> ...some time in the ontogeny and independently of antigen, individual lymphocytes (or clones of lymphocytes) become committed to responding to one, or a relatively small number of antigens; they express the commitment through antigen-specific receptors on their surface.
> (Raff, 1973)

When antigen enters the body it combines with those B or T lymphocytes which possess the corresponding receptor, thereby stimulating these cells to proliferate and differentiate into blast cells. When the B lymphocytes are stimulated by antigen, some of the resulting blast cells become plasma cells. These cells secrete antibody into the circulating blood (Raff, 1973). The remaining blast cells revert back to B lymphocytes and circulate in the blood and lymph as memory cells to await future contact with the antigen (Eisen, 1974b; Raff, 1973). When T lymphocytes are stimulated by antigen, the resulting blast cells secrete specific lymphokinase, which is important in the cell-mediated response (Raff, 1973).

Immunization of the Fetus

The discovery that the mammalian fetus is capable of an active immune response to antigenic stimuli has led to the development of many new techniques and experimental models to aid in the understanding of the various aspects of immunogenesis (Sterzl and Silverstein, 1967). There is one decided advantage in studying the immune response of the fetus to antigenic stimuli *in utero*. The mammalian placenta protects the developing fetus from a variety of organisms in the environment of the mother (Sterzl and Silverstein, 1967). Therefore, the immune system of the fetus has not been challenged by antigen. Thus, one could be reasonably certain that the immunological response of the fetus to experimentally injected antigen would be caused only by those antigens injected (Sterzl and Silverstein, 1967). Also, in certain animals such as the cow, horse, sheep, and pig there are no maternal antibodies present in the circulation of the fetus to interfere with the immune response of the fetus to the antigen (Soloman, 1971; Sterzl and Silverstein, 1967).

Fennestad and Borg-Petersen (1957) were among the first investigators to study active antibody formation by the fetus *in utero*. A laparotomy was performed in each of 2 cows at 261 and 264 days of gestation. A live suspension of *Leptospira saxkoebing* was injected into the placentome of each cow. Antibodies against this antigen were found in the serum of the newborn. Silverstein et al. (1963) injected fetal lambs *in utero* with bacteriophage ϕX174, horse ferritin, ovalbumin, *Salmonella typhosa*, diphtheria toxoid, and viable *Bacillus Calmette-Guerin* (BCG) at varying times during the gestation period. These fetal lambs were able to produce specific antibody to the first

3 antigens as early as the 66th to 70th day of the 150-day gestation period. However, antibodies were not formed to *Salmonella typhosa*, diphtheria toxoid or BCG at any time during fetal or early neonatal life. The immune response of fetal dogs has also been studied (Jacoby et al., 1969). Fetal dogs were able to produce antibody to bacteriophage ϕX174 and ovine erythrocytes at 40 and 48 days of gestation, respectively. Antibodies were not produced in these fetuses to bovine serum albumin at any time during gestation. These experiments using the fetal lamb and dog indicate that the immunocompetence of the fetus to various antigens does not develop simultaneously but sequentially. The reasons for this are not clear. One possible explanation for the sequential occurrence of antibody is that the clonal lymphocytic precursors may develop or mature at a different rate (Silverstein et al., 1963). Also, whether the lymphocyte is stimulated by its antigen depends on the nature of the antigen and complex interaction with other lymphocytes and macrophages (Raff, 1973).

Fetal pigs *in utero* have been found to be immunocompetent to such antigens as bacteriophage ϕX174 (Hajek et al., 1969), sheep red blood cells (Schultz et al., 1971), swine erysipelas adsorbed bacterin (Wellmann and Reblin, 1972), and parvovirus strain 3060 (Bourne, 1974). To this investigator's knowledge, there are no reports in the literature on the immune response of the fetal pig injected *in utero* with *Escherichia coli* antigen.

Conner et al. (1973) found that ovine and bovine neonates previously given *E. coli* antigen by *in utero* injection into the amniotic fluid were protected when challenged at birth with viable organisms

of the same strain. However, detectable antibody to *E. coli* was not found in the serum of some of these neonates at birth. These investigators postulated that *E. coli* antigen stimulated the intestines to produce antibody locally, thereby protecting the calves and lambs to challenge at birth. Further studies by Wamukoya and Conner (1976) demonstrated that antibodies are produced locally in the intestines of fetal calves following injection of *E. coli* antigen into the amniotic fluid. The vaccination of the bovine fetus with *E. coli* antigen may be a means to protect the neonate against colibacillosis (Conner et al., 1973; Gay, 1975; Olson and Waxler, 1976).

Colibacillosis in Neonatal Pigs

Enteric colibacillosis is caused by enteropathogenic strains of *E. coli*, a gram-negative, nonspore-forming rod belonging to the family Enterobacteriaceae. Enteric colibacillosis is known to occur in pigs, calves, and lambs and is a major cause of losses in the newborn of these species (Blood and Hnerseon, 1968). Poultry and man are also affected (Sojka, 1965a). Enterotoxic colibacillosis, entertic *E. coli* infection, *E. coli* diarrhea, and cholera-like *E. coli* infection are synonymous with enteric colibacillosis (Moon, 1974).

Jensen, in 1893, was the first to observe the association of *E. coli* with diarrhea in calves (white scours). The same investigator, in 1899, suggested that diarrhea in piglets, like white scours in calves, was associated with *E. coli* infection (Sojka, 1965b,c). However, since *E. coli* is a normal inhabitant of the intestinal tract of healthy pigs, there was considerable controversy concerning the importance of this organism in causing colibacillosis (Rutter, 1975; Sojka, 1965a). The controversy continued until serological

procedures were developed for the classification of *E. coli* organisms (Kauffmann, 1947). It then became clear that only a few serotypes of *E. coli* are pathogenic.

The Kauffmann-Knipschildt-Vahlne serological scheme for the classification of *E. coli* is based on the identification of the dominant antigens of the organism, namely the somatic (O), capsular (K) and flagellar (H) antigens (Kauffmann, 1947). The O antigens are heat stable lipopolysaccharide complexes which form part of the cell wall. The K antigens are composed of polysaccharides and form an envelope or capsule around the cell wall. These antigens prevent agglutination of a live suspension of homologous O antigen with its antisera. The K antigens are thermolabile, being inactivated by heating at 100 C or 121 C. The K antigens are subdivided into L, A and B types according to differences in heat stability and antigenicity. The H antigens are thermolabile, are inactivated at 100 C, and appear to be protein in nature. Approximately 150 O, 90 K and 50 H serotypes have been identified (Nielsen et al., 1968). Only a few of these serotypes are enteropathogenic (Moon et al., 1966; Ørskov et al., 1964; Smith and Halls, 1967a).

Pathogenesis

In recent years much work has been done to aid in the understanding of the pathogenesis of enteric colibacillosis. However, more detailed information is needed for complete understanding of the disease. Currently, the most acceptable pathogenesis involves

> ...a susceptible pig infected with an enteropathogenic strain of *E. coli* which has the capacity to proliferate in the proximal small intestine and to produce and

> release enterotoxins in adequate amounts to cause alteration in the normal fluid and electrolyte transport functions of the intestines, with resultant diarrhea, dehydration, and death.
>
> (Kohler, 1972)

Enteropathogenic strains of *E. coli* are widely distributed in the environment and can be found wherever pigs are raised (Kohler, 1972; Nielsen et al., 1968; Rutter, 1975). Heavily populated, poorly ventilated farrowing houses and poor management practices greatly increase the numbers of enteropathogenic *E. coli* organisms in the environment of susceptible pigs (Kohler, 1972; Nielsen et al., 1968). The risk of infection increases with the added numbers of enteropathogens in the environment (Kohler, 1972).

The susceptible pig is infected by ingestion of the enteropathogenic *E. coli* (Rutter, 1975). Many of the *E. coli* organisms are destroyed by the low pH of the stomach contents. However, in the newborn pig the gastric pH is high during the first 48 hours of life. Smith and Jones (1963) examined the pH of the stomach contents of healthy pigs during the first 24 hours after birth and obtained values of 5.5, 5.1, and 4.3 at 6, 12, and 24 hours, respectively. They also found the number of *E. coli* in the intestines of 1-day-old pigs to be quite high. These results indicated that the high gastric pH allowed the organisms to proliferate in the stomach and that these organisms continued to proliferate after passing into the small intestine. This factor may predispose the neonatal pig to infection by the enteropathogenic organisms (Nielsen et al., 1968; Smith and Jones, 1963).

Other factors may be responsible for the ability of the enteropathogenic strains of *E. coli* to proliferate in the anterior part of

the small intestine. Intestinal motility is one factor. Chyme, and the bacteria it contains, are continually moved down the intestinal tract by peristaltic movement. Any factor which decreases or prevents peristalsis will increase the number of organisms in the lumen (Drees and Waxler, 1970; Kohler, 1973; Nielsen et al., 1968). Nutritional factors may also be important as suggested by the occurrence of enteric colibacillosis after weaning (Steven, 1963a; Svendsen et al., 1974). The ingestion of colostrum and the continuous supply of antibodies in the milk of the sow greatly enhance the pig's resistance to colibacillosis (Kohler et al., 1975; Porter et al., 1970a; Svendsen and Wilson, 1971).

The ability of enteropathogenic strains of *E. coli* to proliferate in the small intestine is not unique to these strains. Nonenteropathogenic strains proliferate at the same rate and achieve numbers equal to or greater than those of enteropathogenic strains (Kohler and Bohl, 1966b; Smith and Halls, 1967a). However, enteropathogenic strains of *E. coli* have the ability to adhere to and colonize the intestinal epithelial cells in great numbers (Bertschinger et al., 1972a,b). Nonenteropathogenic strains are mainly observed in the intestinal lumen (Bertschinger et al., 1972a,b). The factors which allow enteropathogenic strains to adhere to the intestinal epithelial cells are not clear. It has been suggested that K88 antigens may play a role in this process (Jones and Rutter, 1972; Smith and Linggood, 1971). A transmissible plasmid controls the production of K88 antigens of strains *E. coli* (Ørskov and Ørskov, 1966; Smith and Linggood, 1971).

Jones and Rutter (1972) conducted a study in which both conventional and germfree pigs were given orally either enteropathogenic strains of *E. coli* which possessed K88 antigen (K88-positive strains) or a strain which lacked K88 antigen (K88-negative strain). These investigators found the K88-positive strain adhered to and colonized the intestinal epithelial surface of the conventional pigs, while K88-negative strain did not. Also, the mortality rate and severity of diarrhea were much greater in those pigs given the K88-positive strain. There was no observable difference between the K88-positive and K88-negative strains' ability to colonize the small intestines of gnotobiotic pigs or in the ability of these strains to cause diarrhea. These researchers suggested that the reduced motility of the germfree gut may have been responsible for the colonization of the intestinal epithelial cells by the K88-negative strain. Also, many *E. coli* enteropathogens which lack K88 antigen colonize the small intestine of pigs (Moon, 1974). More studies are needed to determine the mechanisms of how K88-negative strains colonize the epithelial surface of the small intestine (Moon, 1974). Although proliferation and colonization of the enteropathogenic strains of *E. coli* in the anterior part of the small intestine are important factors in the development of colibacillosis, they alone are not sufficient to cause the fluid accumulation that occurs in the intestines of infected pigs (Jones and Rutter, 1972).

De and Chatterje (1953) and De et al. (1956) discovered that ligated intestinal loops of rabbits became distended with fluid when injected with *Vibrio cholerae* and *E. coli* organisms isolated from human patients with cholera. Smith and Halls (1967b) found that

bacteria-free fluid (enterotoxin) prepared from cultures of *E. coli* isolated from pigs with diarrhea, when injected into ligated intestinal loops of the same species, caused fluid accumulation. Fluid accumulation was not observed in those loops inoculated with bacteria-free fluid from *E. coli* strains which did not cause diarrhea. These investigators postulated that proliferating *E. coli* organisms in the anterior part of the small intestine of the intact host produce enterotoxins and that these enterotoxins induce the diarrhea which occurs in colibacillosis. Other investigators have provided strong evidence that diarrhea seen in this disease is probably enterotoxin induced (Smith and Gyles, 1970; Smith and Linggood, 1971).

Two similar enterotoxins have been isolated from enteropathogenic strains of *E. coli*: heat labile (LT) and heat stable (ST) (Smith and Gyles, 1970; Smith and Halls, 1967b). The LT enterotoxin's ability to cause fluid accumulation in ligated intestinal loops of pigs is greatly reduced when heated at 121 C for 30 minutes and almost completely inactivated when heated at the same temperature for 2 hours (Smith and Halls, 1967b). Antisera prepared against the living organisms neutralizes the dilating effect of this enterotoxin (Smith and Gyles, 1970). The ST enterotoxin is not antigenic or affected by antisera prepared against live *E. coli* organisms (Smith and Gyles, 1970; Smith and Halls, 1967b). A close relationship exists between K88 antigens on a strain of *E. coli* and the ability of the strain to produce enterotoxins (Smith and Gyles, 1970). Both K88 antigen and enterotoxins (ENT) are produced by separate transferrable plasmids (Smith and Gyles, 1970; Smith and Linggood, 1971). However, K88 plasmid can be removed from a strain without affecting

the production of LT or ST enterotoxins by the strain. The reverse is also true: a strain to which the ENT plasmid is transported does not acquire the ability to produce K88 antigens (Smith and Gyles, 1970).

There is probably no difference between enterotoxins produced by different strains of *E. coli* in the same species, but there are differences in those enterotoxins produced in different species (Smith and Halls, 1967b).

Enterotoxins produced by the proliferating *E. coli* enteropathogens induce loss of fluid across an intact mucosa (Moon, 1974; Moon et al., 1971). The enterotoxins of *V. cholerae* have a similar effect on the intestines of people affected with cholera (Carpenter, 1972). Moon et al. (1971) compared the responses of ligated intestinal loops of rabbits and pigs to the injection of enterotoxins from *V. cholerae* and *E. coli*. No differences were observed in the gross and microscopic response of the intestinal loops to enterotoxins from these 2 organisms. The composition of the fluid secreted by the intestine in response to enterotoxins from *E. coli* and *V. cholerae* was also similar. The fluid in comparison to serum was low in protein, Ca^{++}, and Mg^{++} and high in Na^{+}, Cl^{-}, HCO_3^{-}, and K^{+}. These investigators concluded that the response to *V. cholerae* and *E. coli* enterotoxins was similar. Carpenter (1972) reported similar findings in people with cholera.

In most species, the anterior part of the small intestine appears to be more sensitive to the effects of enterotoxins than does the posterior part (Moon, 1974). Carpenter and Greenough (1968) reported that the rate of secretion of fluid in response to

V. cholerae enterotoxins in the duodenum and ileum of the dog was essentially the same and that the lesser net fluid output of the ileum was due to the greater capacity of the ileum to absorb the fluid.

The cells responsible for the secretion of fluids and the exact mechanisms involved in response to *E. coli* enterotoxins are not known. Nielsen et al. (1968) postulated that the crypts of Lieberkuhn secrete fluid in response to injury of the mucosa by enterotoxins. However, there is some evidence that *V. cholerae* enterotoxins stimulate the intracellular enzyme adenylate cyclase, which catalyzes the conversion of adenosine triphosphate (ATP) into 3',5'-cyclic adenosine monophosphate (cAMP), which in turn regulates the secretion by the small intestine exposed to this toxin (Lehninger, 1975; Moon, 1974). The increase of cAMP inhibits the active transport of sodium from the lumen of the small intestine into epithelial cells and stimulates the active secretion of chloride. This results in a net secretion of electrolytes and water (Lehninger, 1975; Moon, 1974). *Escherichia coli* enterotoxins may have the same mechanism of action as those of *V. cholerae* (Evans et al., 1972). Further studies are needed as to the specific mechanism of action of *E. coli* enterotoxins in causing the fluid loss observed in enteric colibacillosis.

The final step in the pathogenesis of enteric colibacillosis is the clinical response of the affected pig. The loss of fluids into the intestinal lumen results in diarrhea and dehydration with eventual acidosis and hemoconcentration. Death usually occurs if the pig is unable to correct the dehydration and electrolyte losses (Kohler, 1972).

Clinical Signs

Enteric colibacillosis usually affects pigs of 3 definite age groups: 1) neonatal pigs 1 to 4 days of age, 2) piglets approximately 3 weeks of age, and 3) pigs 10 to 12 weeks of age (Stevens, 1963a,b).

The clinical signs of colibacillosis in baby pigs usually appear 12 hours after birth. At this time several pigs may be found dead or moribund without showing evidence of diarrhea. Others may appear listless and show varying degrees of diarrhea (Moon, 1969; Stevens, 1963a). The pigs will usually nurse until they are too weak to stand (Moon, 1969). The same clinical signs have been observed in conventional and germfree pigs experimentally infected with enteropathogenic strains of *E. coli* (Christie and Waxler, 1973; Drees and Waxler, 1970; Meyer and Simon, 1972; Miniats and Gyles, 1972).

Some 3-week-old pigs develop sudden diarrhea which may result in decreased growth rates of these pigs (Sojka, 1965c). Death may occur in a few of the affected pigs (Sojka, 1965c). Changes in management practices, such as the introduction of solid food to the diet of these pigs, may be responsible for the development of colibacillosis in this age group (Stevens, 1963b).

Postweaning enteritis is observed in pigs 2 weeks after weaning. These pigs exhibit a short period of diarrhea. The mortality rate in this age group is usually low (Stevens, 1963a,b).

Gross and Microscopic Findings

The characteristic gross lesions observed in pigs affected with enteric colibacillosis are usually confined to the stomach and

intestinal tract. The stomach is distended with gas and coagulated milk (Christie and Waxler, 1973; Moon, 1969; Stevens, 1963a). The small intestine is dilated and flaccid and contains large quantities of fluid, clots of undigested milk and mucus (Christie and Waxler, 1973; Drees and Waxler, 1970; Kohler, 1967; Meyer and Simon, 1972; Moon, 1969; van Dreumel, 1972; Waxler et al., 1971). Petechial to diffuse hemorrhage and congestion of the intestinal mucosa and engorgement of the mesenteric blood vessels have been reported (Christie and Waxler, 1973; Meyer and Simon, 1972; Miniats and Gyles, 1972; Svendsen et al., 1974). The cecum and colon may also be distended with gas. Gross lesions are not always observed in pigs affected with this disease (Smith and Jones, 1963).

Histopathologic examination of tissues from pigs affected with colibacillosis has revealed such lesions as: severe inflammation and massive numbers of polymorphonuclear leukocytes in mesenteric lymph nodes (Miniats and Gyles, 1972); vacuolation of villous epithelial cells (Miniats and Gyles, 1972); edema of the lamina propria (Christie and Waxler, 1973; Drees and Waxler, 1970; van Dreumel, 1972); and congestion of the submucosa of the small intestine (Svendsen et al., 1974). Hemorrhage and surface necrosis with sloughing of the villi has also been reported (Christie and Waxler, 1973). However, microscopic lesions may not be seen in pigs affected with enteric colibacillosis (Kohler, 1967).

Passive Immunization of the Neonate Against Colibacillosis

The pig is virtually devoid of immunoglobulins at birth. Traces of immunoglobulins antigenically related to IgG have been detected in the serum of the pig at birth (Sterzl and Silverstein, 1967).

This immunoglobulin is probably produced by the fetus *in utero* since there is no transfer of immunoglobulins across the placenta in the pig (Kim et al., 1966; Porter, 1969; Porter and Allen, 1972; Solomon, 1971; Sterzl et al., 1966). Immunoglobulins and antibodies are acquired from the maternal colostrum by absorption from the small intestine within the first 24 to 36 hours of life (Porter, 1973; Porter and Allen, 1972; Wilson and Svendsen, 1972). After this time, no antibodies or immunoglobulins are absorbed by the newborn pig.

The level of passively acquired immunoglobulins and antibodies to *E. coli* in sera of young pigs significantly declines after the first week of life (Porter, 1969; Wilson, 1972; Wilson and Svendsen, 1972). Actively acquired antibodies to *E. coli* are not detected in the sera of neonates until after the third week of life (Wilson and Svendsen, 1972). Thus, after the first week of life, the pig is particularly vulnerable to pathogenic *E. coli* organisms and must rely on local antibodies present in the intestinal tract.

Secretory IgA is the major immunoglobulin and source of antibody to *E. coli* in the intestines of young pigs (Porter et al., 1970a). The function of secretory IgA antibodies is protection of the mucosal surfaces from invasion by pathogenic viruses and bacteria (Tomasi, 1967; Williams and Gibbons, 1972). Secretory IgA present in the intestines of young pigs is obtained mainly from the milk of the sow. Also, some secretory IgA is produced in the intestines of young pigs. Porter et al. (1970b) detected, by the use of immunoelectrophoresis, secretory IgA in the intestinal secretion of young pigs about 10 days of age. Antibodies of the IgA class are produced by immunocytes in the lamina propria of the small intestine and are then transported

through the epithelial cells of the mucosa, where the secretory piece is added (Porter and Allen, 1972; Tomasi, 1967).

Recognition of the importance of *E. coli* antibody in the colostrum and milk of the sow for the passive protection of young pigs against invasion by enteric pathogens has resulted in several studies in active immunization of the sow with *E. coli* antigens. Svendsen and Wilson (1971) found that when colostrum from vaccinated sows was given orally to gnotobiotic pigs, there was some protection against experimentally produced colibacillosis. Protection against colibacillosis was not observed in gnotobiotic pigs given colostrum from nonvaccinated sows. Similar results have been obtained using experimentally infected conventional pigs (Kohler et al., 1975; Rutter and Anderson, 1972).

Kohler and Bohl (1966b) and Miniats et al. (1970) studied the value of feeding antisera to experimentally infected gnotobiotic pigs. These investigators found that these pigs were protected from clinical signs of disease during the time that the antisera were being administered. When the antisera were withdrawn, the pigs developed clinical signs of disease. The antiersa may have interfered with the effects of enterotoxins on the intestinal tract (Kohler and Bohl, 1966b; Miniats et al., 1970).

Gnotobiotic Pigs

In studies concerning the development of immunity, it is of primary importance that there is adequate control of antigenic stimuli (Sterzl and Silverstein, 1967). The porcine fetus *in utero* is usually in a sterile environment and does not come into contact with external

antigens until after birth. The number of antigens in the environment of the newborn pig can be controlled when pigs are raised under gnotobiotic conditions. Also, pigs are virtually devoid of antibodies at birth since maternal antibodies do not cross the placenta in this species (Kim et al., 1966; Porter, 1969; Sterzl et al., 1966; Sterzl and Silverstein, 1967). Thus, one could be nearly certain that the antibody response of gnotobiotic or fetal pigs to the injection of antigen is caused by that antigen without interference of other antigens and antibodies (Kohler and Bohl, 1966b; Porter and Kenworthy, 1970; Sterzl and Silverstein, 1967).

Gnotobiotic pigs are used in the study of certain infectious agents, such as *E. coli*. One can study the effects of specific serotypes and strains of *E. coli* without the interference of intestinal flora or pathogenic organisms or passively acquired antibodies (Sterzl and Silverstein, 1967; Waxler et al., 1971). These factors cannot be controlled under field conditions (Waxler et al., 1971).

Methods in Immunology

Direct Bacterial Agglutination Test

Bacterial cells in suspension will usually clump when mixed with specific antiserum. According to the lattice theory for the precipitation of soluble antibody-antigen complexes, each antibody molecule is linked to more than one antigen molecule, and in turn each antigen molecule is linked to more than one antibody molecule. The aggregates so formed will become large enough to fall out of solution to form a visible precipitate. The principles are more or less the same for the agglutination reaction (Eisen, 1974a).

The bacterial agglutination test can be used to detect and roughly quantitate antibodies in sera when known cells are used (Eisen, 1974a). This test is more sensitive than the precipitin test but not as sensitive as the passive hemagglutination test. The bacterial agglutination test can detect approximately 0.1 µg/ml of antibody (Eisen, 1974a).

The bacterial agglutination test has been used to detect and quantitate antibodies to *E. coli* in both porcine (Corley et al., 1973) and bovine (Conner et al., 1973) species.

Passive Hemagglutination Test

Soluble antigens can be detected by the agglutination reaction when these antigens are attached to the surface of erythrocytes. Erythrocytes will readily absorb polysaccharides and some proteins. This test is very sensitive and can be used to detect as little as 0.01 µg/ml of antibody (Eisen, 1974a). The passive hemagglutination test can be conducted using either the tube method (Sharpe, 1965; Svendsen and Wilson, 1971) or the microtitration method (Sever, 1962). The chief advantages of the microtechnique are the saving of reagents and the rapid performance of microdilution (Sever, 1962). The passive hemagglutination test has been used to detect antibodies against *E. coli* in the serum of pigs (Svendsen and Wilson, 1971).

MATERIALS AND METHODS

Animals

Six gilts (Yorkshire, Hampshire, and mixed breeds), bred by natural service, were obtained as a source of gnotobiotic pigs.[a] The gilts were housed in a barn and were allowed access to a small pasture. Their diet consisted of gestation ration with water *ad libitum*. Food and water were withheld 24 hours before each surgery.

Three Dutch Belted rabbits were used for the production of antisera to be used in the direct bacterial agglutination test and the passive hemagglutination test.

Preparation of Bacterin

Stock cultures[b] of *E. coli* 0149:K91(B);K88(L) were stored in the dark at room temperature in tightly sealed slant tubes containing trypticase soy agar[c] with no dextrose. Subcultures, on the same media, were prepared at regular intervals, incubated overnight at 37 C, sealed, and stored.

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[a]Obtained from the Department of Animal Husbandry, Michigan State University.

[b]Obtained from Dr. H. W. Moon, National Animal Disease Center, Ames, Iowa 50010.

[c]BBL, Division of Broquest, Cockeyville, Maryland.

Preliminary studies were performed to determine the maximum growth curve of the *E. coli* organisms. Sterile test tubes containing 7 ml of brain heart infusion (BHI) broth[d] were inoculated with 1 standard loopful of *E. coli* organisms taken from the stock culture and incubated at 37 C for a variable number of hours. The cultures were washed 2 times in sterile saline and resuspended in sterile saline to their original volume. Serial dilutions were made, and 1 ml from each dilution tube was plated in trypticase soy agar and incubated at 37 C for 24 hours. Those plates containing less than 30 or greater than 300 colonies were discarded, and the remaining plates were counted and tabulated. The preliminary studies revealed the maximum growth to occur at 10 hours.

Bacteria used for injection of the fetuses were prepared with slight modification according to Conner et al. (1973). A culture of *E. coli* organisms was grown for 10 hours at 37 C in BHI broth, washed twice in sterile saline, seeded in bottles containing trypticase soy agar, and incubated at 37 C for 24 hours. The growth was washed off the agar medium with sterile saline and pooled. Formalin (0.4%) was added to the suspension after samples were taken for bacteriologic examination to determine purity and viable cell count. The formalinized cell suspension was incubated in a shaking water bath at 37 C for 18 to 24 hours. The formalinized bacterin was washed 3 times in sterile saline, resuspended in sterile saline to a concentration of 5×10^9 organisms/ml, and stored at 4 C in sterile, heat-sealed ampules. Merthiolate (0.02%, w/v) was added as a preservative.

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[d]Difco Laboratories, Detroit, Michigan.

Fetal Injection

A right flank laparotomy was performed at 98 days of gestation. Anesthesia was produced in the first 3 gilts by epidural injection of 25 ml of 2.5% procaine hydrochloride[e] into the lumbosacral space. A general anesthetic (Halothane 2%,[f] nitrous oxide, oxygen mixture) was given to the last 3 gilts. Five milliliters thiamylal sodium[g] in a 10% solution was given intravenously as preanesthetic medication. The fetuses, enclosed within the uterus, were brought through the surgical incision one at a time and inoculated through the uterine wall. Each fetus and the uterus at the site of the fetus were marked for later identification. Approximately 0.25 ml of sterile India ink was injected into the subcutaneous tissue of the fetus, and suture material was placed in the serosa of the uterus. During the experiment, injections were made into a total of 49 fetuses from 6 litters. The numbers of fetuses, solutions injected, routes of injection and identification of fetuses are summarized in Table 1.

The gilts were allowed to recover from surgery. Their progress and temperature were observed and antibiotics[h,i] were given for several days following the laparotomy. Observations of food and

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[e]Epidural solution, Haver-Lockhart Labs, Kansas City, Missouri.

[f]Ayerst Laboratories, New York, New York.

[g]Surital Sodium, Parke, Davis & Company, Ann Arbor, Michigan.

[h]Combiotic, Charles Pfizer and Co., Inc., New York, New York.

[i]Liquamycin (50 mg/cc), Pfizer, Inc., New York, New York.

Table 1. Numbers of fetuses, solutions injected, routes of injection and identification of fetuses injected *in utero*

Numbers of fetuses injected *in utero*, the solution injected and routes of injection					
Bacterin (5×10^9)		Saline		Sites of India ink injection	Numbers of sutures placed in uterine wall
AF*	IM*	AF	IM		
27				shoulder	1
		18		hip region	2
	3			dorsal midline	3
			1	dorsal midline (lumbar region)	1 (mattress)

*AF = amniotic fluid; IM = intramuscularly.

water consumption, as well as the general condition of the sow, were made throughout the experiment.

Preparation of Gnotobiotic Equipment

The rearing isolators were prepared for receipt of the gnotobiotic pigs according to procedures outlined by Waxler et al. (1966). Each of the 3 isolators was washed with a detergent solution, rinsed with clear water and dried using cheesecloth to prevent stains. Polykleen[j] was used to remove persistent stains and marks and to polish the isolators both inside and outside. The cages, floor mats, caps, bands, and other equipment were washed and dried. The plastic

[j]Schwartz Chemical Company, Inc., New York, N.Y.

outer sleeve of the intake filter of each isolator was removed to inspect the filter for damage. The filters were sterilized, using dry heat, at 150 C for 3 hours. The exhaust valve apparatus of each isolator was checked for fluid level.

The cages were inspected for cleanliness and sharp edges before they were autoclaved at 250 F for 30 minutes. The cages were then wrapped in brown paper and again autoclaved at 250 F for 30 minutes.

After each isolator and gloves were carefully checked for holes, each isolator and the floor mats, bands, and inner cap within each isolator were sprayed with 2% peracetic acid solution[k] with a wetting agent[l] added in a concentration of 0.1%. The cages were unwrapped, and 4 were placed in each isolator and sprayed with peracetic acid. The isolators were allowed to stand for 30 minutes before air flow was started through the filters.

The surgical isolator was washed and sterilized using the same procedures as described above. The surgical instruments, towels, and umbilical clamps were placed in a stainless steel, filter-equipped sterilization cylinder and autoclaved at 250 F for 30 to 45 minutes. A vinyl sleeve, previously sprayed with peracetic acid, was attached to the cylinder and to the stainless steel ring in the wall of the isolator. The instruments and material within the cylinder were then passed directly into the isolators. Blood tubes, towels, needles, syringes, and other equipment which would be needed

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[k] FMC Corporation, Buffalo, New York.

[l] Nacconal NRSF, National Analine Division, Allied Chemical Corporation, New York, N.Y.

during the experiment were placed in a similarly constructed cylinder, autoclaved, and passed into one of the rearing units using the same technique as described above.

Preparation of Live Challenge Organisms

To prepare live challenge organisms, a culture of *E. coli* was grown for 10 hours at 37 C in a tube containing 7 ml of BHI broth. The challenge organisms were washed 2 times in sterile saline. Based upon previous growth of 10-hour cultures, sterile saline was added to the washed challenge organisms to give a concentration of approximately 3×10^6 organisms/ml. Challenge organisms were placed in sterile ampules, heat sealed, and used within a few hours. A viable count was obtained to determine the actual concentration of the challenge organisms.

Gnotobiotic Procedures

A total of 34 pigs from 3 litters was delivered by hysterotomy at 112 days of gestation and raised under gnotobiotic conditions according to techniques described by Waxler et al. (1966). Anesthesia was produced by epidural injection of 25 ml of 2.5% procaine hydrochloride at the lumbosacral space. Promazine hydrochloride[m] (0.5-1.0 mg/lb body weight) was given as a tranquilizing medication. Once the pigs were obtained, the sow was euthanatized.

The pigs from each sow were separated into 3 rearing isolators. Each isolator contained pigs which received *in utero* injection of *E. coli* bacterin or saline or were uninjected. After blood and

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[m]Sparing, Wyeth Laboratories, Inc., Philadelphia, Pennsylvania.

bacterial samples were taken, the pigs in 2 isolators from each litter were given orally 1 ml of live challenge organisms (2-7 x 10^6 organisms). The pigs in the remaining isolator from each litter were given 1 ml of sterile saline and kept as controls.

Specimen Collection

Serum Samples

The gnotobiotic pigs were bled at birth and at the termination of the experiment at 10 or 18 days of age. Those pigs which appeared very weak before the end of the experiment were bled and then euthanatized. Approximately 5 ml of blood was collected from the anterior vena cava into 10 ml plastic syringes, placed in test tubes and allowed to clot. The serum samples were obtained according to accepted procedures and frozen at -24 C until used.

Bacteriologic Samples

Bacteriologic procedures were performed according to Waxler et al. (1966) with minor changes. Swabs were taken from the rectum of pigs and waste material in pans of the cages of each isolator at the beginning and end of the experiment. The specimens from each isolator were streaked on blood agar[n] plates and incubated aerobically and anaerobically at 25 C, 37 C and 55 C. Thioglycollate broth[n] was also inoculated and incubated aerobically at the same temperatures as above. In addition, PPLO broth[n] was inoculated and incubated at 25 C and 37 C. Every 3 days, 1 loopful of the bacterial

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[n]Difco Laboratories, Detroit, Michigan.

sample in PPLO broth was transferred into fresh medium and incubated at the same temperatures. On the 12th day, the bacterial sample in PPLO broth was transferred to PPLO agar and incubated at 25 C and 37 C for 1 month.

Cecal material from pigs which died or were euthanatized was taken with sterile swabs, streaked onto trypticase soy agar, and incubated at 37 C for 24 hours, at which time the plates were inspected for growth.

Tissue Samples

Those pigs which did not die were euthanatized by intravenous injections of pentobarbital sodium[o] or by electrocution. A muscle relaxant was given prior to electrocution. The pigs were placed in dorsal recumbency and a midline incision was made to expose the abdominal and thoracic organs. A general examination of the organs was made at that time. The cecum was opened with sterile instruments and its contents were sampled for bacteriologic examination. A small section of the ileum was taken starting at the ileocecal valve and progressing anteriorly. Samples of mesenteric lymph node adjacent to the ileum were also taken. Other tissue samples were taken from the liver, spleen, right kidney, thymus, and mandibular lymph nodes. Any other tissue which appeared abnormal was taken for histopathologic examination. The tissues were placed in 10% (v/v) formalin solution, processed according to standard procedures, cut at 6 microns, and stained with hematoxylin and eosin (Luna, 1968).

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[o]Haver-Lockhart Labs, Kansas City, Missouri.

Immunologic Procedures

Direct Bacterial Agglutination Test

The antibody levels of sera were determined with slight modification, according to procedures described by Conner et al. (1973). Briefly, the O antigen was prepared by growing *E. coli* cultures in BHI broth at 37 C for 10 hours. The culture was heated in a boiling water bath at 100 C for 1 hour and allowed to cool, and formalin (0.5%) was added. The O antigen was either used immediately without washing or stored at 4 C. The K antigen was prepared from cultures grown in BHI broth at 37 C for 10 hours. Formalin (0.5%) was added, and the antigen was used within 18 hours.

Serial twofold dilutions of sera were prepared in 0.5 ml of saline with the beginning dilution of 1:2, and 0.5 ml of antigen was added. Rabbit antiserum against *E. coli* was used for the positive control. The O agglutination tubes were incubated at 50 C for 20 hours. The K agglutination tubes were incubated at 37 C for 2 hours, then at 4 C overnight, and placed at room temperature for 1 hour before results of the tests were read.

Passive Hemagglutination Tests

The passive hemagglutination test was performed on selected serum samples according to methods described by Shape (1965). A 48-hour growth, on trypticase soy agar, was harvested using 4 ml of saline. The bacterial cell suspension was heated in a boiling water bath at 100 C for 1 hour, allowed to cool, and centrifuged at 3000 rpm for 30 minutes. The resulting supernatant was stored at 4 C until used.

Hemolysins were removed from each serum sample (2 ml) by mixing the sample with 0.1 ml of washed packed sheep red blood cells (SRBC). The mixture was left at room temperature for 3 hours and then centrifuged, and additional SRBC (0.2 ml) were added to each serum sample. The serum samples were then stored overnight at 4 C.

Washed SRBC (1 ml) were added to 5 ml of O antigen in 94 ml of phosphate buffered saline (PBS) and incubated at 22 C for 45 minutes. The coated SRBC were washed 3 times in PBS, and a 1% suspension of washed coated SRBC in PBS was prepared.

Serial twofold dilutions of the serum in 0.025 ml amounts were made in PBS starting with a dilution of 1:2. Control tests included coated SRBC and PBS, uncoated SRBC and PBS, and uncoated SRBC and sera. In addition, rabbit antiserum against *E. coli* was used for a positive control. The plates were incubated at 22 C for 45 minutes and then at 4 C overnight.

RESULTS

Surgical Procedures

The results of the surgical procedures and fetal injections are summarized in Table 2.

Laparotomy Procedures

The epidural procedure produced anesthesia which was adequate initially in that there was minimal reaction to the incision through the abdominal wall. However, the surgical procedure was time consuming and, before it was completed, there was considerable struggling on the part of the sow. Subsequently, 2 of the 3 sows which were given the epidural anesthesia aborted following surgery. The remaining gilt had an uneventful surgical recovery. The general anesthesia administered to the last 3 gilts alleviated the problems encountered with the epidural anesthesia. These gilts had an uneventful recovery from surgery.

Hysterotomy Procedures

The epidural anesthesia was adequate for this procedure. Forty-five live and 1 mummified fetuses were delivered by hysterotomy at 112 days of gestation. Four pigs from Litter 4 were euthanatized. Two fetuses could not be identified by using the uterine and fetal markings. It was assumed that 1 pig was given saline by *in utero* injection into the amniotic fluid. However, since neither pig could

Table 2. Results of laparotomy and hysterotomy and fetal injection

Gilt no.	Type of anesthesia for laparotomy	No. of fetuses injected *in utero*				Recovery of gilt	Total no. of fetuses in litter	Total no. of pigs used in experiment
		Bacterin		Saline				
		A.F.*	IM**	A.F.	IM			
1	epidural	4	---	3	---	uneventful	7***	---
2	epidural	7	---	5	---	aborted 4 days after surgery	16	---
3	epidural	4	---	2	---	aborted 10 days after surgery	10	---
4	general (Halothane)	5†	---	3	---	uneventful	17††	12
5	general (Halothane)	3	2	2	1	uneventful	12	12
6	general (Halothane)	4	1	3†††	---	uneventful	10	10

*A.F. = amniotic fluid; **IM = intramuscular; ***The pigs were 3 weeks premature; †One pig was mummified; ††Four were euthanatized; †††Could not identify one of the saline-injected pigs at birth.

be identified, they were considered to be uninjected controls. The remaining fetuses given either saline or *E. coli* bacterin *in utero* were identified by their fetal or uterine markings, or both. The 7 pigs of Litter 1 were found to be approximately 21 days premature at the time of the surgical procedure. These pigs died within 1 hour after birth.

Clinical Response of Pigs to Challenge

The clinical responses of the 23 neonatal pigs to challenge with viable *E. coli* are summarized in Table 3. Eighteen of the 23 challenged pigs died. This was a mortality rate of approximately 78%. Fourteen (61%) of these pigs were either found dead or became moribund and were euthanatized within 48 hours after challenge. Clinical signs were observed in 12 (52%) of the challenged pigs and were those associated with colibacillosis, as evidenced by anorexia, depression, and generalized weakness. These signs were usually observed within 24 to 36 hours after challenge. Diarrhea was observed in 8 challenged pigs and appeared within 24 to 36 hours after challenge, persisting in 7 of these pigs until death occurred at 2 to 3 days of age. Diarrhea was observed in the remaining pig (No. 28) for about 3 days, subsided, and was not again observed during the experiment. Five challenged pigs survived until euthanatized at the termination of the experiment at 10 to 18 days of age. Clinical signs of colibacillosis were observed in 1 of these pigs (No. 28).

Clinical signs associated with colibacillosis were not observed in the 11 control pigs. However, 8 of these pigs died before the termination of the experiment.

Table 3. Responses of neonatal pigs to oral exposure to *E. coli* 0149:K91(B);K88(L) following *in utero* injection of bacterin or saline

Pig no.	Litter no.	Isolator or group no.	Type of *in utero* injection*	Oral challenge dose (viable *E. coli* organisms)	Signs of clinical disease after challenge**	Age of pigs at death (days)
1	4	1	---	6.09×10^6	-	2
2	4	1	B	6.09×10^6	+	3
3	4	1	S	6.09×10^6	+	2
4	4	1	B	6.09×10^6	-	2
5	4	2	B	6.09×10^6	-	2
6	4	2	S	6.09×10^6	-	2
7	4	2	---	6.09×10^6	+	2
8	4	2	---	6.09×10^6	+	1
9	4	3	---	---	-	2
10	4	3	B	---	-	4
11	4	3	---	---	-	8
12	4	3	S	---	-	2
13	5	1	S	7.25×10^6	--	2†
14	5	1	B	7.25×10^6	+	3†
15	5	1	B(im)††	7.25×10^6	-	2†
16	5	1	---†††	7.25×10^6	+	2†
17	5	2	S(im)	7.25×10^6	-	10†
18	5	2	B	7.25×10^6	-	4†
19	5	2	B(im)	7.25×10^6	+	2†
20	5	2	---	7.25×10^6	+	3†
21	5	3	S	---	-	10†
22	5	3	---	---	-	4

Table 3 (cont'd.)

Pig no.	Litter no.	Isolator or group no.	Type of *in utero* injection*	Oral challenge dose (viable *E. coli* organisms)	Signs of clinical disease after challenge**	Age of pigs at death (days)
23	5	3	B	---	-	1
24	5	3	---	---	-	2
25	6	1	---	2.06×10^6	+	2
26	6	1	B	2.06×10^6	+	2
27	6	1	B	2.06×10^6	+	2†
28	6	1	S	2.06×10^6	+	11†
29	6	2	B(im)	2.06×10^6 3.3×10^6***	-	11†
30	6	2	---	2.06×10^6 3.3×10^6 2.31×10^6Δ	-	18†
31	6	2	B	2.06×10^6 3.3×10^6 2.31×10^6	-	18†
32	6	3	B	---	-	11†
33	6	3	S	---	-	1†
34	6	3	---	---	-	18†

*B = bacterin injected intra-amniotically, S = saline intra-amniotically, --- = noninjected; **- = no clinical disease, + = clinical disease; ***Challenged at 5 days of age; †Age of pigs at euthanasia; ††B(im) = bacterin injected intramuscularly; †††S(im) = saline injected intramuscularly; ΔChallenged at 13 days of age.

Direct Bacterial Agglutination and Passive Hemagglutination Tests

The results of the direct bacterial agglutination test for pig sera with K and O antigens, as well as the passive hemagglutination test with O antigens, are summarized in Table 4. All the pigs had negative O and K agglutinin titers at birth. The sera of pigs No. 28 and 31 had O agglutinin titers at 11 days of age. The reciprocal of the titers was 16 and 4, respectively, with the bacterial agglutination test and equal to or greater than 256 with the passive hemagglutination test. The serum of pig No. 31 was tested again at 18 days of age using the bacterial agglutination test. The O agglutinin titer had not changed. Only the serum of pig No. 28 had K agglutinin titer at 11 days of age.

Gross Findings

The changes observed in most of the pigs given live challenge organisms orally were confined to the organs of the digestive system and were those associated with colibacillosis. The stomach, posterior 1/3 of the jejunum, cecum, and spiral colon were distended with gas. The stomach was filled with undigested coagulated milk. The intestine of some pigs also contained an abundant yellowish ingesta. Other gross lesions were observed in only a few pigs. These lesions consisted of hemorrhagic enteritis and colitis (pig No. 15), hyperemia of the stomach and jejunum (pig No. 18), and hyperemia of the jejunum, ileum and spiral colon (pigs No. 25 and 26). Also, pig No. 25 had hemorrhagic areas involving the mucosa of the fundus of the stomach. Partial atelectasis of the apical lobe of the right lung was observed

Table 4. Direct bacterial agglutination and passive hemagglutination titers* of serum from pigs injected *in utero* with *E. coli* bacterin (Group 1) or saline (Group 2) or uninjected (Group 3)

Group no.	Pig no.	Age at time sample taken (days)	O149 antigens		K antigens
			Direct†	Passive††	Direct
1	2	0**	-	NT***	-
	4	0	-	-	-
	5	0	-	NT	-
	10	0	-	NT	-
	14	0	-	NT	-
		3	-	NT	-
	15	0	-	-	-
	18	0	-	-	-
		4	-	NT	-
	19	0	-	NT	-
		2	-	NT	-
	23	0	-	NT	-
	26	0	-	-	-
	27	0	-	NT	-
	29	0	-	-	-
		11	-	NT	-
		18	-	NT	-
	31	0	-	-	-
		11	4	256†††	-
		18	4	NT	-
	32	0	-	NT	-
		11	-	NT	-
2	3	0		NT	-
	6	0	-	NT	-
	12	0	-	NT	-
	13	0	-	NT	-
	17	0	-	NT	-
		10	-	NT	-
	21	0	-	NT	-
		10	-	NT	-
	28	0	-	-	-
		11	16	256†††	4
	33	0	-	NT	-

Table 4 (cont'd.)

Group no.	Pig no.	Age at time sample taken (days)	0149 antigens		K antigens
			Direct	Passive	Direct
3	1	0	-	NT	-
	7	0	-	NT	-
	8	0	-	NT	-
	9	0	-	NT	-
	11	0	-	NT	-
	16	0	-	NT	-
		2	-	NT	-
	20	0	-	NT	-
	22	0	-	NT	-
	24	0	-	NT	-
	25	0	-	-	-
	30	0	-	NT	-
		11	-	NT	-
	34	0	-	NT	-

*Titer expressed as reciprocal of the highest dilution showing agglutination.

**0 = at birth.

***NT = not tested.

†Direct bacterial agglutination.

††Passive hemagglutination.

†††Titer was equal to or greater than 256.

in pig No. 7. No gross changes were observed in challenged pigs that survived 10 days or more after challenge.

The stomach and entire intestinal tract of 2 of the pigs given sterile saline orally at birth were distended with gas. Organisms were not isolated from the cecal contents of these pigs. It is possible that the gas entered the stomach and intestine of these pigs with food ingested or during the process of dying, or both. Also, the gas may have been produced by organisms not detected with the bacteriologic procedures used. One control pig (No. 23) died when fetal membranes lodged in the larynx and upper 1/3 of the trachea. The entire right lung of pig No. 33 was collapsed. No other gross changes were observed in control pigs at necropsy.

Histopathologic Findings

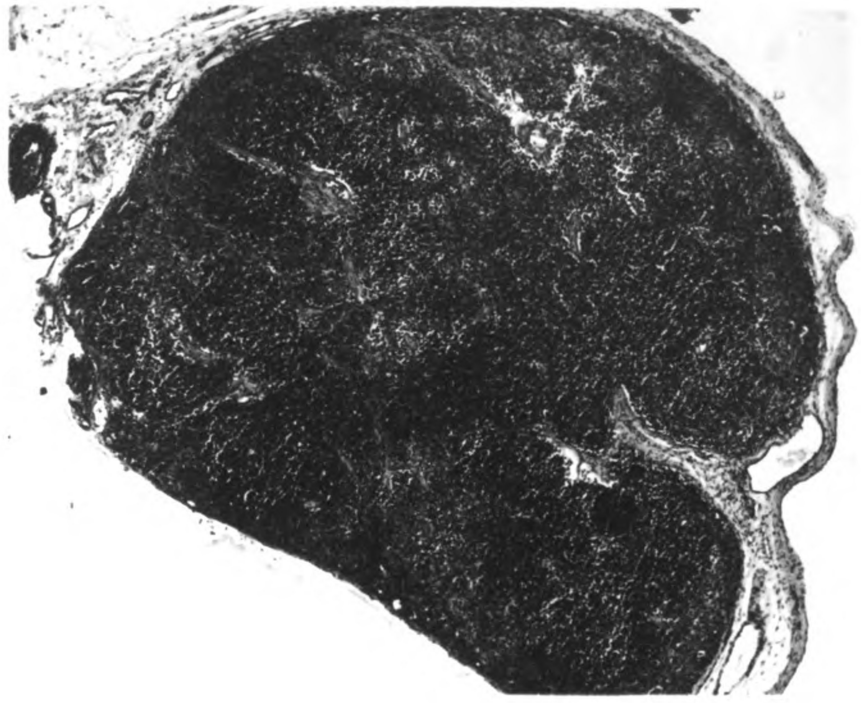
Sections from mandibular and mesenteric lymph nodes, thymus, ileum, liver, spleen, and kidney were evaluated.

Mandibular and Mesenteric Lymph Nodes and Thymus

Evaluations were made of the mandibular and mesenteric lymph nodes of all but 4 experimental pigs. There were no significant morphologic differences observed between these 2 lymph nodes. The lymph nodes taken from most of the pigs had only poor demarcation between cortex and medulla. Follicular development of lymphoid tissue was observed in 11 pigs (Figure 1A, 1B). Germinal centers were observed in mandibular lymph nodes taken from pigs No. 21 and 28 and mesenteric lymph nodes of pigs No. 17, 21, 28, 29 and 31 (Figure 2A, 2B). All of these pigs, with the exception of pig No.

Figure 1A, 1B. Photomicrographs of the mesenteric lymph node of pig No. 14, previously injected *in utero* with *E. coli* bacterin, challenged with viable *E. coli* at birth, and euthanatized at 3 days of age. Notice the poor demarcation between the cortex and medulla, the absence of lymphoid follicles and germinal centers, and the relatively small numbers of lymphocytes in the cortical area. H&E stain; x 50 (A) and x 125 (B).

A



B

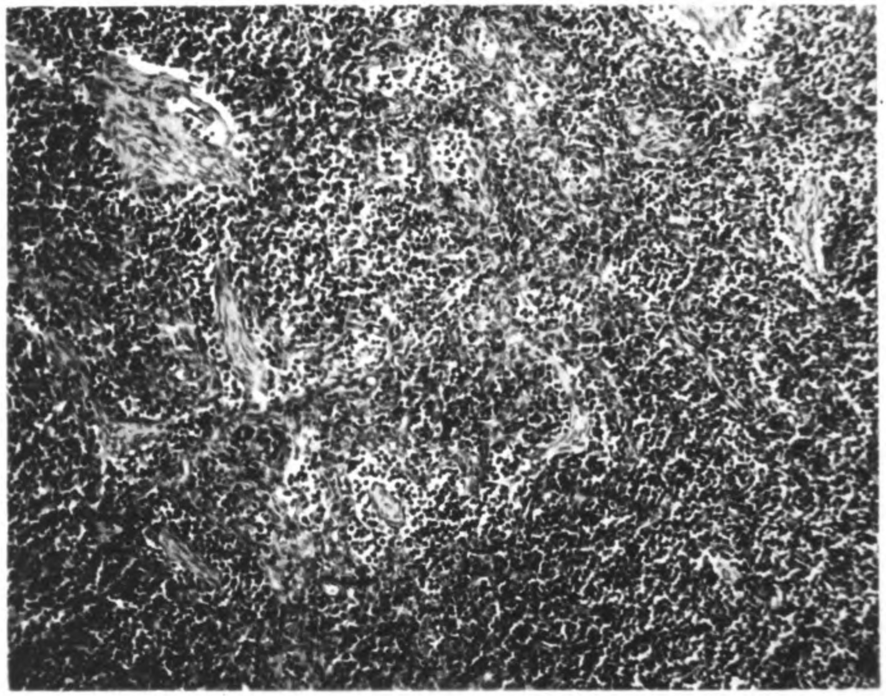
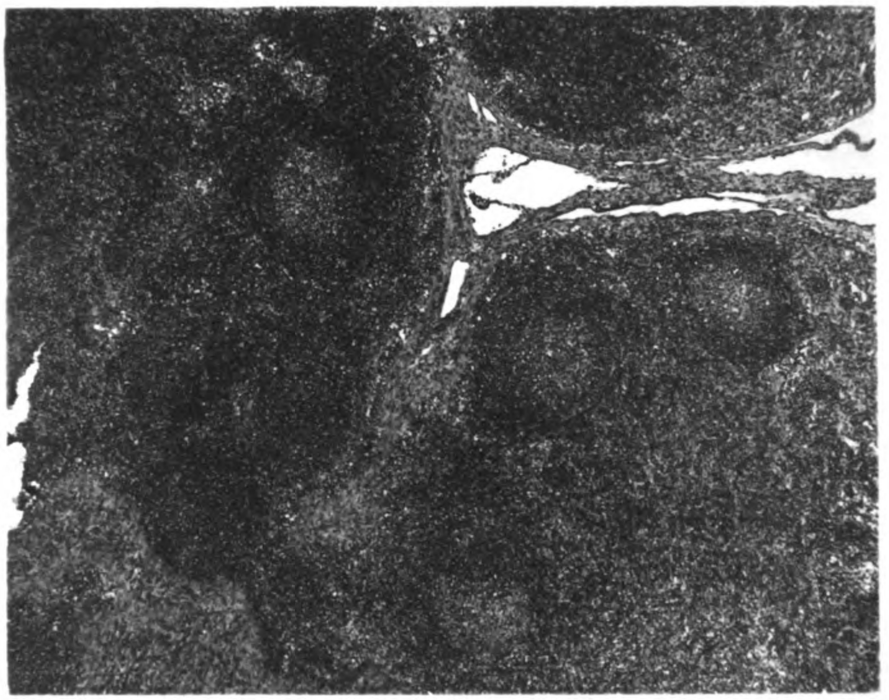


Figure 1

Figure 2A, 2B. Photomicrographs of the mesenteric lymph node of pig No. 31, previously injected *in utero* with *E. coli* bacterin, challenged with viable *E. coli* at birth, and euthanatized at 18 days of age. Antibodies were detected in the serum of this pig at 11 and 18 days of age. Notice the lymphoid follicles with germinal centers and numerous lymphocytes in the cortical areas. H&E stain; x 50 (A) and x 125 (B).

A



B

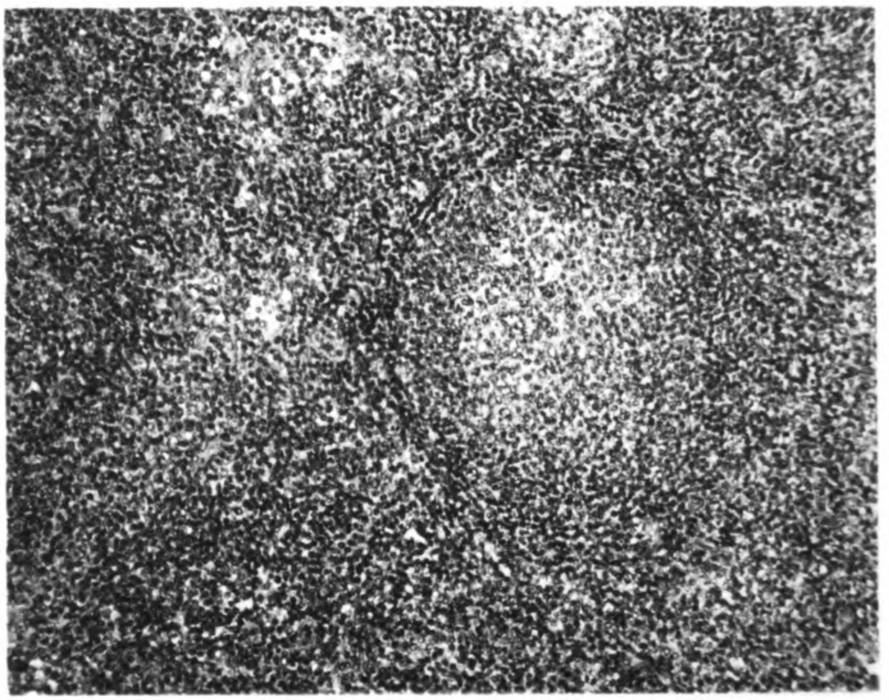


Figure 2

21, were given orally the live challenge organisms and survived 10 days or more postchallenge. Pig No. 21 was given saline at birth and survived until the termination of the experiment at 10 days of age. Numerous eosinophils and neutrophils were observed in the medullary regions of many of the lymph nodes examined. Massive hemorrhage was observed in the mesenteric lymph node of pig No. 15. No differences in the appearance of the mesenteric or mandibular lymph nodes could be attributed to *in utero* injection of *E. coli* bacterin. However, the development of germinal centers in pigs No. 17, 28, 29 and 31 may have been due to the administration of viable *E. coli* organisms at birth.

The thymuses of 33 pigs were evaluated. The thymus of all these pigs was well developed with distinct cortical and medullary regions. Hassall's corpuscles were observed in the medulla.

Ileum

Morphologic changes were observed in the ileum of 11 challenged pigs. These changes consisted of sloughing and erosion of the epithelium of the villi. Extensive hemorrhage and sloughing of villous epithelium were observed in sections of the ileum of pig No. 15 (Figure 3). The same lesions were observed to a lesser extent in pig No. 13. Vacuolation of villous epithelial cells and numerous goblet cells were observed in all sections examined. However, in most instances vacuoles were more numerous in epithelial cells of the ileum taken from the control pigs that survived until the termination of the experiment (pigs No. 21, 22, 32, and 34) (Figure 4). Lesions were not observed in sections of ileum of 4 of the 5 challenged

Figure 3. Photomicrograph of the ileum of pig No. 15, previously injected *in utero* with *E. coli* bacterin, challenged at birth with viable *E. coli* and dying at 2 days of age. Notice the extensive hemorrhage with sloughing of the villous epithelial cells and the edema and congestion of blood vessels in the submucosa. H&E stain; x 50.

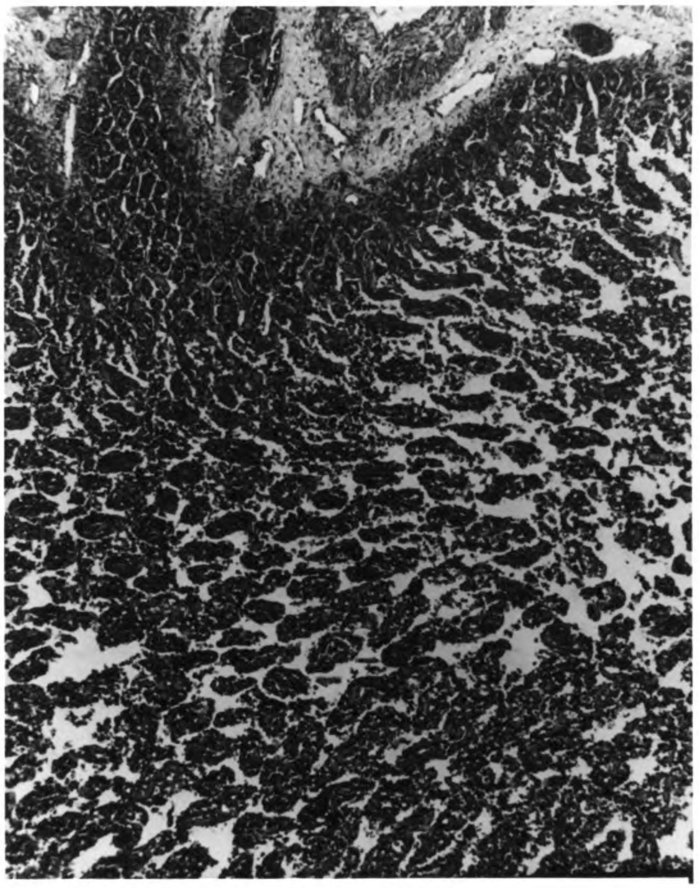
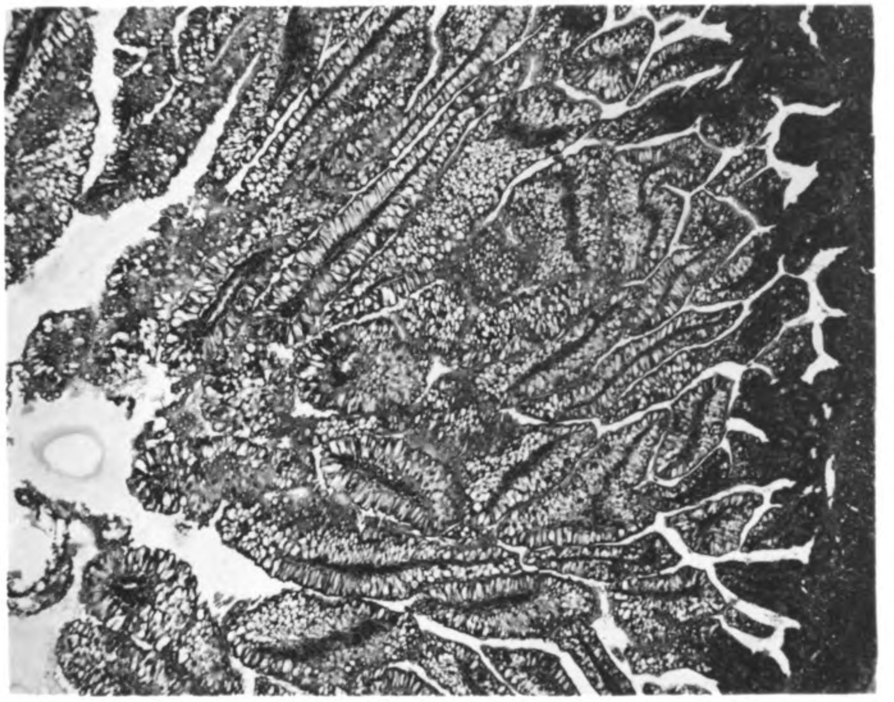


Figure 3

Figure 4A, 4B. Photomicrographs of the ileum of pig No. 32, previously injected *in utero* with *E. coli* bacterin, given saline orally at birth and euthanatized at 11 days of age. Notice extensive vacuolation of the villous epithelial cells and the long fingerlike projections of the villi. H&E stain; x 50 (A) and x 125 (B).

A



B

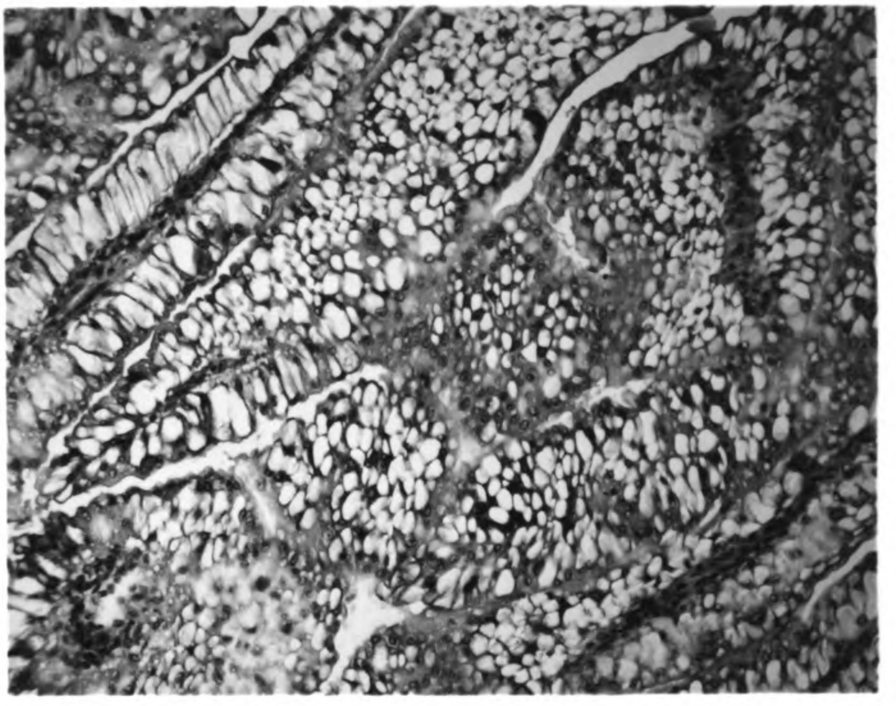


Figure 4

pigs that survived until 10 or 18 days of age. Slight erosions of villous epithelium were observed in sections taken from challenged pig No. 28. Also, the least number of vacuolated epithelial cells were observed in the ileum of this pig (Figure 5). Neutrophils were observed among the epithelial cells of the villi, around the crypts, and in the lamina propria of some of the sections evaluated. Lesions were not observed in sections of the ileum taken from control pigs.

Liver, Spleen, and Kidney

Vacuolation of the hepatic cells and numerous foci of extramedullary hematopoiesis were observed in all sections examined.

The splenic lymphoid nodules of all the experimental pigs were generally quite small and lacked germinal centers.

Changes observed in sections of the kidney were cloudy swelling and hydropic degeneration of the cortical tubular epithelial cells.

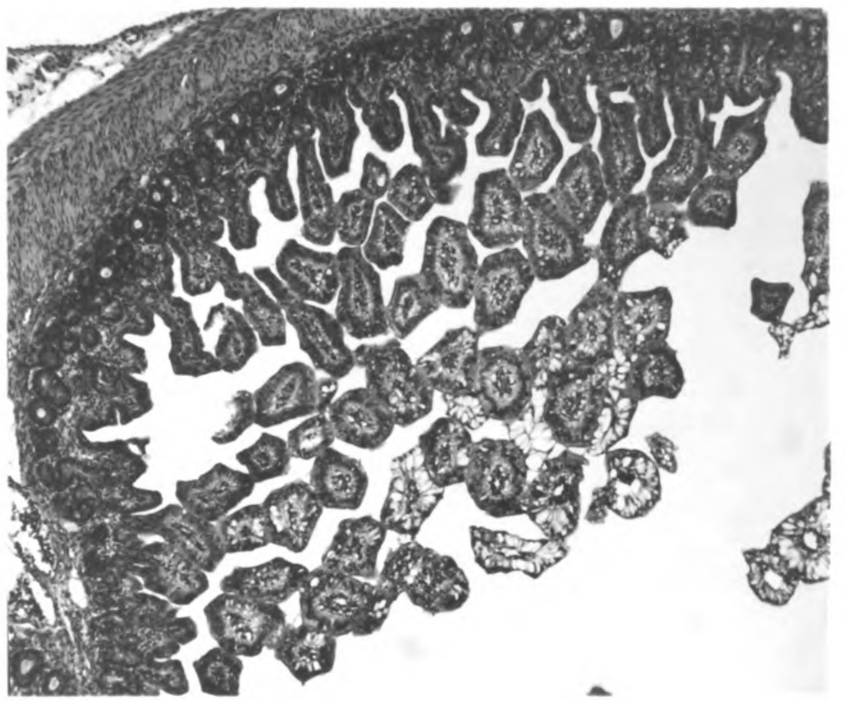
No differences were observed in the liver, spleen, or kidney of these pigs which could be attributed to the *in utero* injection of *E. coli* antigen or to viable *E. coli* organisms given to pigs at birth.

Bacteriologic Findings

No growth was obtained from the isolators at the beginning of any of the experiments. Bacteriologic cultures indicated growth of *E. coli* in the isolators housing challenged pigs. Also, gram-positive cocci were isolated from fecal samples collected from isolator 2 that housed Group 2 of Litter 6. No growth was obtained from isolators housing the control pigs.

Figure 5A, 5B. Photomicrographs of the ileum of pig No. 28, previously injected *in utero* with saline, challenged at birth with viable *E. coli* and euthanatized at 11 days of age. Notice the relatively small numbers of vacuolated epithelial cells when compared to the ileum of pig No. 32. H&E stain; x 50 (A) and x 125 (B).

A



B

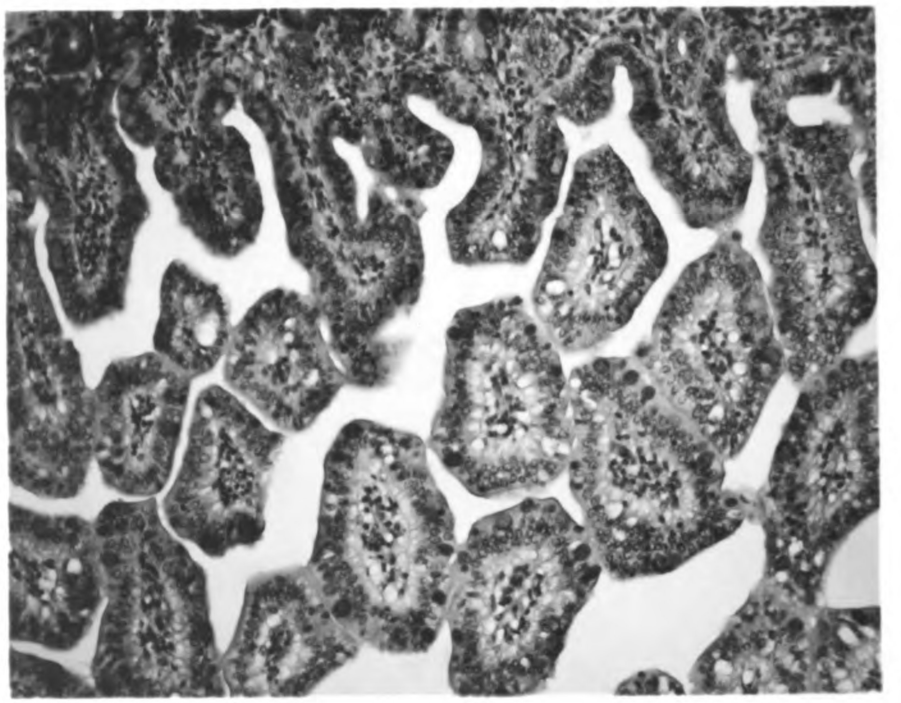


Figure 5

Cecal contents from each pig were bacteriologically evaluated. Heavy to moderate growth of *E. coli* was isolated from 17 of the challenged pigs. Slight growth of *E. coli* was isolated from challenged pigs No. 19, 29, 30 and 31. Bacteria were not isolated from challenged pigs No. 17 and 18, nor from any of the control pigs. Gram-positive cocci were isolated from the cecal contents of pigs No. 30 and 31 at 18 days of age. These organisms were present in the isolator housing these pigs, and probably entered the isolator between the 10th and 18th day of the experiment, since these organisms were not isolated from the cecal contents of pig No. 29 at 10 days of age.

DISCUSSION

The results of the direct bacterial agglutination and passive hemagglutination tests indicate that antibody against *E. coli* was not produced in those pigs previously injected *in utero* with the *E. coli* Also, the *in utero* injection of either the bacterin or saline did not appear to alter the susceptibility of the neonate to challenge at birth. The responses of these pigs to challenge suggest that local antibodies against *E. coli* were probably not produced by the intestines.

Clinical Responses of Gnotobiotic Pigs to Challenge at Birth

There was one primary factor which may have influenced clinical response of challenged pigs. The viable bacterial count of the challenge dose administered to gnotobiotic pigs at birth in Litters 4, 5 and 6 ranged from 2×10^6 to 7.25×10^6 (Table 3). However, the pigs in Group 2 of Litters 5 and 6 probably received a much smaller number of viable organisms. Clinical signs of disease were observed in pigs No. 19 and 20, but only a few *E. coli* organisms were isolated from the cecal contents of these pigs. *Escherichia coli* organisms were not isolated from the cecal contents of pigs No. 17 and 18, nor from the cages and waste pans of the isolator at the termination of the experiment. Peracetic acid used to sterilize the ampules containing the viable organisms may have entered when the ampule was

opened, killing many of the bacteria present. The reason that only pigs No. 19 and 20 appeared to receive the challenge organisms is not known. One could postulate that these 2 pigs may have been the first pigs in the isolator challenged and that some of the organisms were still viable at that time. However, this explanation does not account for the inability to recover *E. coli* organisms from the waste pans or cages of the isolator housing these pigs. One would have thought that the remaining organisms in the isolator would have multiplied. The bacteriologic samples may have been taken from the cage and waste pans used by one of the pigs that had no *E. coli* organisms in the cecal contents. Also, peracetic acid may have entered the tube containing the bacteriologic sample while the tube was being passed out of the isolator.

Escherichia coli organisms were not isolated from the ampule containing the viable cell suspension used to infect the pigs in Group 2 of Litter 6 at birth. After all the pigs in Group 2 of Litter 6 were reinfected at 5 days of age and 2 pigs (Nos. 30 and 31) again at 13 days of age, only a few *E. coli* organisms could be isolated from the cecal contents and from the cages and waste pans of the isolator. Peracetic acid may have entered the ampule killing many of the *E. coli* organisms. Also, the pigs may have been more resistant to infection at 13 days of age. Another possible explanation for the difficulties encountered in challenging these pigs is that the organism may have been losing its virulence.

Another factor that may have influenced clinical responses of the challenged pigs was the number of pigs in Litter 4. There were 17 pigs in this litter. These pigs were very small and weak at birth.

How much of an effect this had on the clinical responses of these pigs to challenge is not known. However, there appeared to be no difference in the clinical responses of these pigs when compared to clinical responses of pigs that received the intended dose of challenge organism in Litters 5 and 6. The weakness of the pigs in Litter 4 at birth may have been a factor in the death of all the control pigs of this litter.

The clinical signs of disease observed in challenged pigs were consistent with the observation reported by other investigators (Christie and Waxler, 1973; Drees and Waxler, 1970; Meyer and Simon, 1972; Miniats and Gyles, 1972). Organisms of the strain of *E. coli* used in this experiment seem to be quite virulent, as evidenced by the death of 14 of the challenged pigs within 48 hours after challenge. Also, many of these pigs died peracutely before the appearance of diarrhea. The survival of pigs No. 17, 29, 30 and 31 is difficult to interpret since the challenge doses given to these pigs were probably much too low. The results from challenge pig No. 28 indicate that this pig was able to survive the injurious effects of the organisms upon the intestines. Antibodies against *E. coli* were detected in this pig at 10 days of age. The antibodies present in the serum probably had little effect on the organisms present in the intestinal lumen of this pig (Porter, 1973). It is possible that local antibodies were produced by cells in the intestines of this pig which enabled the pig to survive infection (Porter and Allen, 1972; Tomasi, 1967; Wamukoya and Conner, 1976). However, the investigation of the development of a local immune response by this pig is beyond the scope of the present research.

Histopathologic and Gross Findings

The poor development of the mesenteric and mandibular lymph nodes and lymphoid structures of the spleen of all pigs that died before 5 days of age appeared to be independent of the *in utero* injection of either bacterin or saline. This is a further indication that the *in utero* injection of the bacterin did not cause an immune response in these pigs, since in the absence of antigenic stimuli the lymphoid tissues of fetal and germfree pigs mature slowly (Kruml et al., 1970; Sterzl and Silverstein, 1967). It is possible that pigs given live challenge organisms orally at birth died before the *E. coli* antigen could stimulate maturation of the lymphoid tissue and subsequent production of antibodies.

The maturation of the mesenteric and mandibular lymph nodes of pigs that survived until the termination of the experiment appeared to be dependent on the administration of challenge organisms, age and individual variations. The challenge organisms probably stimulated development of lymphoid tissues of pigs No. 17, 21, 29 and 31 but not pig No. 30. Age seemed to be a factor in the development of lymphoid tissues of pig No. 21, since this pig was given saline at birth (Waxler and Drees, 1973).

The significance of the sloughing of villous epithelial cells of the ileum was difficult to interpret due to the autolytic changes observed (Cross and Kohler, 1969). The extensive vacuolation observed in the ileum of pigs No. 17, 21, 22, 32, and 34 was probably due to slow replacement of absorptive epithelium that occurs in the germfree ileum (Moon et al., 1973). The least number of vacuolated epithelial cells were observed in the ileum of pig No. 28. The

presence of *E. coli* organisms probably caused the epithelial cells to turn over at a faster rate with the eventual loss of much of the vacuolated epithelial cells (Moon, 1972).

The gross lesions observed in the challenged pigs were consistent with those of colibacillosis (Christie and Waxler, 1973; Drees and Waxler, 1970; Meyer and Simon, 1972; Waxler et al., 1971).

Immunologic Response of Fetal Pigs to Injection

As previously stated, the results of the direct bacterial agglutination test and passive hemagglutination test and the clinical response of the neonate to challenge indicate that antibody against *E. coli* was not produced in those pigs previously injected *in utero* with the *E. coli* bacterin. The failure of these fetuses to produce antibody to *E. coli* bacterin could stem from several possibilities: (1) the immunologic unresponsiveness of the fetal pigs to *E. coli*; (2) the dose of the antigen; (3) the routes of injection; and (4) the sensitivity of the immunologic tests employed.

Previous studies of fetal immunization have shown that immune competence to antigen develops sequentially rather than simultaneously (Jacoby et al., 1969; Silverstein et al., 1963). According to the clonal selection hypothesis, when antigen enters the body it binds to those lymphocytes which already have receptors for the antigen on their surface. The interaction of antigen with its receptor initiates the activation of these cells to produce antibody (Eisen, 1974b; Raff, 1973). It has been postulated that clonal lymphocytic precursors may develop or mature at different rates (Sterzl and Silverstein, 1967). If this is true, then perhaps the clonal lymphocytic

precursors for *E. coli* antigen had not developed in these fetal pigs when the antigen was injected at 98 days of gestation. However, if all the lymphocytic precursors developed and matured at the same rate, then perhaps enzymes needed to catabolize *E. coli* antigen before it is presented to the specific lymphocytes may be absent in fetal pigs.

A second consideration is the dose of the antigen administered to fetal pigs *in utero*. The reaction of lymphocytes to antigen depends largely on the concentration and nature of the antigen. It is not known whether the number of organisms given to the fetuses by *in utero* injection into either the amniotic fluid or muscles of the thigh caused high dose tolerance. Germfree piglets have been found to have small numbers of immunocompetent cells (Sterzl and Silverstein, 1967). If the same can be said of the fetal pig, then a high concentration of antigen would be required to make contact with the few cells. However, *E. coli* is a complex antigen having repeating identical determinants and is poorly catabolized (Raff, 1973). This antigen chiefly stimulates B cells. More antigen is needed to stimulate B cells than T cells, but B cells may be paralyzed by very high concentrations of antigen.

Bovine fetuses have been stimulated to produce antibody to *E. coli* antigen by *in utero* injection of *E. coli* antigen into the amniotic fluid (Conner et al., 1973) and muscles of the body (Gay, 1975). Recent studies by Wamukoya and Conner (1976), with the bovine fetus, have shown that injection of *E. coli* antigen into the amniotic fluid probably offers the best protection of the neonate against colibacillosis. The swallowing of the amniotic fluid containing

antigen stimulated the intestine to produce antibodies locally. Thus, one could speculate that the *in utero* injection of antigen into either the amniotic fluid or muscles of the hind limb of porcine fetuses would have caused a response similar to that of bovine fetuses if the fetal pigs were immunocompetent to *E. coli* antigen at the time of injection.

The final consideration is the immunologic test used. The bacterial agglutination test is not as sensitive in detecting antibody as the passive hemagglutination test (Eisen, 1974a). This was shown by the marked differences in the O agglutinin titer of pigs No. 28 and 31 when determined by the 2 methods. It is possible that the bacterial agglutination test failed to detect antibody in the sera of pigs taken at birth. However, this possibility is not likely since antibodies were not detected in sera of pigs at this age when the passive hemagglutination test was used. This investigator is aware that the antibody binding property of the KB antigen of the *E. coli* organisms was not inactivated by heating at 100 C for 1 hour. Thus the KB antigen was present along with the O antigen in the supernate used in the passive hemagglutination test. It is possible that part of the O agglutinin titer of these 2 pigs was attributed to the presence of KB antigen.

The neonatal pig is able to produce antibodies to *E. coli* as indicated by the detection of antibody in sera of pigs No. 28 and 31 at 10 days of age. Since these pigs were in constant contact with the *E. coli* organisms, it is difficult to postulate at what time between birth and 11 days of age these antibodies were produced.

SUMMARY AND CONCLUSIONS

The immunologic response of fetal pigs to *in utero* injection of *E. coli* 0149:K91(B);K88(L) and the response of neonates to challenge at birth were studied in 34 gnotobiotic pigs. A laparotomy was performed at 98 days of gestation. *Escherichia coli* bacterin (5×10^9 organisms) was injected *in utero* into either the amniotic fluid or the muscles of the hind limb of 14 fetuses. The remaining fetuses were either injected *in utero* with saline in the same manner or were uninjected. After delivery by hysterotomy procedure, viable *E. coli* organisms were given orally to 23 of the gnotobiotic pigs (challenged). The reamining 11 were given saline orally (controls). Eighteen of the 23 challenged pigs (78%) died following exposure. The *in utero* injection of either bacterin or saline did not appear to alter the susceptibility of the neonate at birth to viable *E. coli* of the same serotype. The clinical signs, gross and microscopic lesions were consistent with those associated with colibacillosis.

Antibody to *E. coli* antigen was not detected using the bacterial agglutination and passive hemagglutination tests in the serum of the pigs at birth. However, antibody to the *E. coli* antigen was detected in the serum of 2 challenged pigs at 11 days of age.

The results indicate that, with the present techniques, porcine neonates previously injected *in utero* with *E. coli* antigen are not protected when challenged with live organisms of the same strain, nor

are detectable antibodies against *E. coli* produced by fetal pigs. However, antibodies to *E. coli* antigen are formed by neonatal pigs before 10 days of age. Thus, one can assume that pigs are immunocompetent to *E. coli* antigen sometime between 98 days of gestation and 11 days of age.

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