Immunological and pathological alterations in the intestine of cattle following intraluminal inoculation with bovine viral diarrhea virus

Alton Corwin Sandidge Ward

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IMMUNOLOGICAL AND PATHOLOGICAL ALTERATIONS IN THE INTESTINE OF CATTLE FOLLOWING INTRALUMINAL INOCULATION WITH BOVINE VIRAL DIARRHEA VIRUS

Iowa State University Ph.D. 1980

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Immunological and pathological alterations in the intestine of cattle following intraluminal inoculation with bovine viral diarrhea virus

by

Alton Corwin Sandidge Ward

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

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INTRODUCTION

Immunity is defined by Bellanti (1971) to include: "all those physiologic mechanisms which endow the animal with the capacity to recognize materials as foreign to itself and to neutralize, eliminate or metabolize them with or without injury to its own tissues". Immunity may result from innate resistance or passive or active immunization. An important aspect of innate resistance is the body covering which prevents invasion by foreign materials. Although multilayered epidermis of the external layer of vertebrates is quite effective in preventing penetration the mucous membranes of the eye and nose and respiratory, urogenital and alimentary tracts are more readily penetrated. Mechanisms of resistance including mucous and enzyme flushing and immune responses are operational in surveillance of these surfaces. These mechanisms cause local reactions to vary markedly with the site of stimulation in contrast to systemic responses which are essentially the same regardless of the site of initiation.

Infectious processes commonly involve mucous membranes either as a site of initiation or predilection. The intestinal tract of the newborn, gnotobiotic, and immunologically-suppressed animal is extremely susceptible to a wide spectrum of pathogenic agents. Protection of these tissues is primarily dependent upon local immunity. This immunity can be nonspecific in character or involve passive or active specific mechanisms. Active local immunity is most successful when stimulated by antigen acting at that site. Immunization induced by parenteral administration of antigen tends to result in little if any protection of mucosal tissues.
Microbial agents vary in their affinities for the different tissues of a host as well as for the host species. The most evident affinities of bovine viral diarrhea (BVD) virus is for tissues of susceptible cattle include lymphoid tissues and the mucosae of the alimentary tract. Infection of these sites result in alterations of intestinal functions and immunological responses.

The experimentation reported in this dissertation was conducted in an attempt to evaluate the immunological and pathological responses resulting from infection of the intestine of cattle with BVD virus. The specific objectives were to: 1) induce infection in cattle by inoculating BVD virus into the lumen of the intestine; 2) identify the initial target cell (or cells) for viral replication; 3) evaluate the response of lymphoid cells within the lamina propria and circulation, i.e. distruption or stimulation of cells due to viral infection; 4) monitor the spread of the virus from initial site of infection, and 5) determine the levels of immunoglobulins in intestinal secretions and the specific ability of these secretions to neutralize BVD viral infectivity for tissue culture monolayers.
REVIEW OF LITERATURE

The Intestine and Its Immunity

Histology of the intestine

The intestine, composed of its various sections including duodenum, jejunum, ileum, and colon, is uniquely equipped to digest, absorb and transport food as well as to eliminate waste products. Structurally, the intestine is described by Padykula (1973) as being basically the same with minor variations throughout its length. The four concentric tissue layers from the lumen outward consist of the mucosa, submucosa, muscularis and serosa. The mucosa consists of a single layer of epithelium, a highly vascular lamina propria, and a thin muscle layer, the muscularis mucosae. The mucosa is thrown into longitudinal (rugae) and circular (plicae circularis) folds which effectively increase the luminal surface. The most plentiful folds are the finger like villi which project above the glandular crypts.

The intestinal tract, as stated by Walker and Hong (1973) is "among other things, a lymphoreticular organ...." The lymphoreticular component consists of numerous lymphocytes and plasma cells dispersed throughout the lamina propria in addition to the lymphoid cells in Peyer's patches. The attenuated epithelium overlying Peyer's patch germinal centers allows antigen penetration and uptake by migrating mononuclear cells (Mims 1964; Goldschneider and McGregor 1968; Faulk et al. 1971; Sobhon 1971; Bockman and Cooper 1973; Owen and Jones 1974). Antigens may either be processed within the Peyer's patches or allowed
to pass into the lymphoid system where they may then localize in mesenteric lymph nodes and initiate a systemic immune response.

The capacity for local immunity develops early in the fetal intestine as evidenced by the presence of lymphoid follicles in the intestine of bovine fetuses after 20 weeks development (Doughri et al. 1972). The maximum development appears to occur at approximately 8 months of gestation when the mean number of Peyer's patches is approximately 84. However, the total area taken up in the intestine continues to increase throughout gestation. Peyer's patches are numerically most plentiful in the jejunum where the ratio of space occupied by Peyer's patches to the total area of jejunum is greater than 10%. In the ileum one Peyer's patch is continuous along the antimesenteric wall and comprises approximately 80% of the total area. Chapman et al. (1974) observed similar formation of intestinal lymphoid follicles after 50 days fetal development of piglets. They found that the cortico-medullary distinctions characteristic of mature patches only became evident following birth. Peyer's patches are present in low numbers in the human fetus by the third trimester but maximum development does not occur until the age of puberty (Cornes 1965).

Each Peyer's patch consists of numerous follicles arranged side by side in a single layer. The follicles in conventionally reared animals consist of five distinct zones (Fauik et al. 1971; Sobhon 1971): 1) The epithelium which is modified from the villous columnar epithelium; 2) a subepithelial or mixed cell zone; 3) a small lymphocyte zone; 4) a germinal center composed primarily of large lymphocytes; and
5) connective tissue bordering the serosal side of the germinal centers. These centers unlike lymphoid tissues elsewhere in the body have no afferent lymph vessels. The efferent vessels provide direct passage of stimulated lymphocytes to various locations but primarily to the lamina propria where they may mature into plasma cells and establish local immune barriers (Craig and Cebra 1971; Jacobson et al. 1961).

The subepithelial zone contains a mixed cell population consisting of a few small lymphocytes (7 microns in diameter) and a large accumulation of macrophages, plasma cells and medium-sized (8-12 micron) lymphocytes. The cells are loosely arranged in this area in respect to the dense accumulations beneath.

Immediately beneath and fusing with the subepithelial zone is the lymphocyte cuff which consist primarily of small lymphocytes. These cells are identical in appearance with small lymphocytes throughout the body and are characterized by having condensed chromatin within an indented nucleus and only a few organelles dispersed in the scant cytoplasm. Reticular cells, macrophages and medium and large (13-15 micron) lymphocytes are also observed in addition to the small lymphocytes of this zone.

The germinal center is situated beneath a cap formed by the lymphocyte cuff. Five cell types are found in the germinal center, including small, large and medium-sized lymphocytes, dendritic reticular cells and tingible body macrophages. The most abundant cell type is the large lymphocytes which is oval or round, has abundant cytoplasm with numerous free ribosomes, scant rough endoplasmic reticulum (ER), a
moderately well developed Golgi body and mitochondria near one pole of the nucleus. The nuclei are vesiculated and have loosely arranged chromatin and prominent nucleoli. These cells appear to be antibody producers and upon antigenic stimulation proliferate to nearly obliterate other zones. The tingible body macrophages are the largest cells of the germinal centers (20-25 microns). They are oval to round in shape with a large volume of cytoplasm containing rough ER, mitochondria, well developed Golgi apparati, and dense inclusion bodies which resemble lysosomes. Phagocytosis of lymphocytes by these cells has been observed. Stellate dendritic cells provide the scaffolding within the germinal centers and form a sharp demarcation between the germinal center and the lymphocyte cuff. Every lymphocyte within the germinal center contacts several dendritic processes. The reticular cell contains a large amount of euchromatin and a small amount of condensed chromatin within the nucleus. The cytoplasm stains dark with routine stains, has extensively developed rough ER and Golgi systems and many free ribosomes and mitochondria. These cells contact the endothelial cells of the capillaries within the follicles and have been observed to completely wrap some capillaries. Upon antigenic stimulation dendritic cells develop a labyrinth of plasma membrane infoldings which appear to aid in antigen retention. There appears to be a complete segregation of T and B cells into separate compartments, a less well organized reticulum for trapping of antigen, a greater population of suppressor T cells, a deficiency in accessory cells and extensive proliferation of B cells without maturation into plasma cells in the
follicles of Peyer's patches in comparison with other lymphoid tissues in the body (Cebra et al. 1977).

Interfollicular zones contain in addition to blood and lymph vessels, fibroblasts, collagen bundles and numerous plasma cells. Medium and small lymphocytes as well as macrophages and eosinophils are also present. The number of eosinophils is much lower than in villous areas. Active migration of lymphocytes through the endothelium of the post capillary venules has been observed in this area (Sobhon 1971), and provides a means of distribution to other areas.

The connective tissue and muscle layers are very thin beneath the Peyer's patches and as a result offer little resistance to rupture when the overlying lymphoid tissue is eroded. Extensive erosion and ulceration of epithelium and lymphoid follicles particularly of the Peyer's patches are common sequelae to the infection with a variety of pathogens including bovine viral diarrhea virus (Meyling 1970), transmissible gastroenteritis virus (Kent and Moon 1973), and some Salmonella (Sonnenwirth 1970), and E. coli (Formal and Hornick 1978).

Lymphocytes of the Peyer's patches are capable of colonizing other lymphoid organs throughout the body. Lymphoid follicles of the primitive gut contribute to population and development of lymphoid tissue throughout the body during fetal development (Chapman et al. 1974). The Peyer's patch cells of mature animals are also capable of repopulating lymphoid organs which have been depleted by various methods (Jacobson et al. 1961; Goldschneider and McGregor 1968). Cells
which are exposed to antigens in Peyer's patches continue to be released throughout life into the lymphatic system and become established primarily in the lamina propria where they become antibody secreting plasma cells (Bienenstock and Dolezel 1971). Evidence suggests that Peyer's patch primed cells also localize in other tissues such as the mammary gland where some become secretory IgA producers (Goldblum et al. 1975; Gindrat et al. 1972; Montgomery et al. 1973).

The lamina propria is highly vascular with a labyrinth of both blood and lymphatic vessels throughout (Padykula 1973). Crypts between villi are sites of active mitosis and secretion. Undifferentiated cells in the crypts give rise to columnar epithelial and goblet cells which migrate over the villi to the tips where they are sloughed off into the lumen. Both absorptive and excretory processes are normally carried out by epithelial cells. The absorptive process enables uptake of food and antigenic materials. However, these cells do not appear as active in the uptake of particulate material as are the epithelial cells covering Peyer's patches. The excretory process includes release of antibodies which become mixed with the mucus released from goblet cells. The antibody-mucus layer functions in prevention of absorption and uptake of antigens from the gut lumen. Numerous cell types are found in the highly fibrous lamina propria including fibroblast and muscle cells which compose the more stable portion. Plasma cells, monocytes, lymphocytes, eosinophils, mast cells and polymorphonuclear (PMN) leucocytes are present in highly
variable numbers dependent upon stimulation. All cell types contribute to the defense of the organ and extraintestinal tissues.

**Nonspecific Immunity**

The intestine is bombarded with antigenic materials, including components of foods, bacteria, viruses, fungi and parasites, throughout extrauterine life. While numerous bacteria and viruses may be innocuous residents of the gastrointestinal tract and even contribute to disease resistance, others cause disease of the host either by direct action on the intestine or via that organ to infect other tissues. Those microbes having direct action on the intestine may reach the intestine by ingestion and infect from the luminal surface or may infect the intestine from hematogenous or lymphatic spread from other portals of entry.

The term "nonspecific immunity" is neither well-defined nor understood but is used to indicate means of defense which do not require the participation of specific antibodies. The survival of an individual through an acute infection is almost entirely dependent upon nonspecific defense mechanisms which prevent overwhelming invasion and multiplication of pathogens (Morgensen 1979). Therefore, nonspecific immunity constitutes the first line of defense which allows time for a specific immunological response to be made. Numerous factors of nonspecific immunity are recognized and will be discussed below.
Genetic and stress factors

Innate nonspecific resistance to infection varies with species, breed and strain of animal as well as with age, sex, nutrition and the physiological and emotional state of the host. It is well recognized that specific viral and bacterial pathogens have a relatively restricted range of host species (Hutt 1958; Gowen 1960). Animals vary greatly in susceptibility to disease caused by infections with *Salmonella* species (Kent et al. 1966), *Bacillus anthracis* (Lincoln et al. 1967) and enterovirus (Merigan 1974). Similar variances in resistance are also noted between breeds of livestock with some of the most notable documented variances being between the African Zebu and the European breeds of cattle. Genetic variances are also observed within a species and even within individual herds. This has been best demonstrated in dairy herds where dams and sires have been identified which produce daughters with increased resistance to mastitis. Genetic transfer of disease resistance and susceptibility has also been noted in various other animal species and man (Hutt 1958; Soothill 1977).

In most instances it is unknown what factor or factors render one animal, breed, or specie more resistance than another. In the case of some infections it is speculated that inheritance dictates the presence or absence of specific receptor sites, essential enzymes, or substrates (Kunin 1964; Kohn and Fuchs 1973; Merigan 1974; Rutter et al. 1975; Purchase et al. 1977). The factor which results in the refractory state of fowl for anthrax is more easily defined since
reduction of this animal's temperature to that comparable with susceptible animals results in susceptibility. Therefore, it is the susceptibility of the anthrax bacillus to higher temperatures that renders the chicken more resistant to this disease. Enhanced resistance of nude mice for several bacterial pathogens has been noted by Nickol and Bonventre (1977) and is associated with an enhanced macrophage response. Increased macrophage activity is also correlated with greater resistance in other animals (Lindenmann et al. 1978). In regard to the influence of age on resistance a partial understanding of the mechanisms has evolved (Gowen 1960; Bradish et al. 1971; Reinarz et al. 1971; Haller et al. 1977). At birth immunologic capabilities are not fully developed but mature within the first few months of life. These capabilities appear to wane with older age and may result from a change in endogenous hormone production. While a major influence of steroids on the immune response has been speculated, a complete understanding of the complex mechanisms has not been achieved (Wheater and Hurst 1961; Oh 1970). However, the variances of resistance noted between sexes are apparently due to differences in hormone production. Additional factors that reflect hormonal influences are the known physiological and emotional stresses which alter microbial flora and result in greater susceptibility to disease (Webster 1970; Williams and Newell 1970; Gillmore and Gordon 1975; Holdeman et al. 1976). Nutritional deficiencies also affect immunological and hormonal responses and have been shown to contribute to greater susceptibility to disease (Passwell et al. 1974; Coovadia and Soothill 1976; Gyr et al. 1978).
Epithelium

The epithelial layer of the body provides a barrier which protects deeper tissue. This layer varies greatly in relation to location and exogenous influences. While the external epithelium consists of numerous layers of keratinized epithelial cells resistant to bacterial penetration, the epithelial layer lining the intestine characteristically consists of a single layer of columnar cells which function as secretory and absorptive cells as well as a barrier between the blood and lymph rich lamina propria and the bacteria and enzyme rich milieu of the lumen. The integrity of this single-cell barrier is maintained in health by rapid proliferation of undifferentiated cells in the crypts of Lieberkuhn and migration of these cells to the apices of villi. This migration results in a complete replacement of the epithelial layer every 2-4 days in animals with a normal intestinal flora and is dependent upon various factors including type of microbial flora and endocrine influence (Moon 1976). Complete loss of epithelium from villi results from infection with a variety of etiologic agents (Kent and Moon 1973). The villi then become contracted, and fluid and electrolyte flow control is lost. With the removal of the etiological agents by immunological or nonimmunological mediated means the villi return to normal structure and are recovered by epithelium from within crypts which are more resistant to destruction. However, recovery is more protracted where destruction of crypt cells occurs as with feline panleukopenia (Csiza et al. 1971). The epithelial cells throughout the intestinal tract vary little in structure.
between one location and another with the exception of those overlying Peyer's patches (Faulk et al. 1971; Sobhon 1971; Bockman and Cooper 1973; Owens and Jones 1974). Over these areas of lymphoid tissue, the epithelial cells are more cuboidal and in some areas consist only of double membranes separated by a narrow zone of cytoplasm forming a very thin barrier between the contents of the intestinal lumen and the lymphocytes. This thin layer is often penetrated by migrating lymphocytes which may actually contact antigenic material within the gut (Levin et al. 1973; 1974). Antigenic penetration and uptake is most active at these sites and may be aided by migrating lymphocytes, the very thin and relatively easily penetrated epithelial cells, and active pinocytosis of material from the gut lumen. The majority of epithelial cells elsewhere in the intestine are covered by the highly convoluting apical membrane making up a dense microvillus striated border which provides an extensive area for absorption but also provides a barrier between the cell and intestinal contents. Over and within this striated border lies the glycocalyx which consists of mucus, enzymes and antibodies. The number of mucus producing goblet cells dispersed among the absorptive epithelial cells varies somewhat between areas within the intestine and is interrelated with the microbial flora and presence of various irritants.

The final protective role of the epithelial cells appears to be associated with their decomposition and release of bactericidal substances as they are sloughed from the villi (Padykula 1973).
Mucous layer

Just as the epithelial layer provides a barrier between the lamina propria and the intestinal contents, the mucus released from goblet cells lines the luminal surface of the epithelium and inhibits contact and attachment of microbes on the epithelial surface (Springer 1970; Gould et al. 1972). This inhibitory capacity is greatly enhanced by the presence of components of the mucus which bind with particles having an affinity for epithelial receptors and by antibodies secreted into the mucus. Mucins consist primarily of glycoproteins with numerous oligosaccharide units closely packed along the polypeptide chain which render it highly resistant to denaturation by enzymes in the digestive tract (Clamp 1977). Secretory IgA contains a segment which is similar in structure to a portion of the mucous glycoprotein. These segments may be involved in covalent linking between mucus and IgA molecules (Roberts 1974; 1976) thus acting to retain IgA in a strategic location for reacting with potential pathogens (Walker and Isselbacher 1977). It has been demonstrated that antigens bound by antibodies in the mucous layer are acted upon by pancreatic digestive enzymes which enhance antigen breakdown (Walker 1976; Walker et al. 1976). In addition, the immune complexes in the presence of excess antibody stimulate the release of mucus from goblet cells thereby aiding in the removal of the antigens from the epithelial surface. Copious amounts of mucus are also released in response to irritants and some infectious agents (Kent and Moon 1973). In such cases the mucus acts to dilute and wash away the stimulating
factors. Mucins may also prevent interaction of enterotoxins and other biologically active substances with the epithelium (Strombeck and Harrold 1974).

**Peristalsis**

The barrier established by an intact epithelial cell layer covered with mucus provides a degree of immunity which is generally adequate to prevent penetration of microbes from the lumen to the submucosa. This barrier is readily broken through however in the event of stasis of the intestine (Walker 1976). Stasis results in bacterial proliferation and an accumulation of toxic substances and allows closer and prolonged association of microbes with the epithelium. In the lower gut where peristaltic action is normally less than in the upper small intestine the bacterial population is much greater. Peristalsis, therefore, is one of the most important factors involved in controlling the intestinal flora.

Peristalsis causes mixing and distal flow of the *succus entericus* thus reducing antigen uptake particularly in the presence of antibody and aids the cleansing of the epithelium. This action becomes more violent in the presence of various irritants and thereby minimizes their effect upon the gut (Knop and Rowley 1975; Bloom and Rowley 1977).

**Enzymes**

A number of enzymes are released in the alimentary canal where they help to restrict bacterial and viral replication. Lysozyme which is a mucoprotein is present in salivary secretions where it has
lytic action primarily on Gram positive bacteria (Cheville 1976). The level of lysozyme may be relative to bacterial stimulation of the tract since Gordon and Pesti (1971) found more lysozyme present in the saliva of conventional than germ-free mice and Kiess and Neale (1978) observed increased lysozyme levels in the serum and feces of patients with gastrointestinal disease. Its enhanced bactericidal activity in the presence of antibodies of the IgA class and complement was first described by Adindolfi and coworkers (1966a). Lysozyme is also present in gastric, nasal and ocular secretions where it can aid in limiting the bacterial flora (McCarty 1973). Additional enzymes of the digestive tract discussed by Stevens and Sellers (1977) include a variety of carboxylases (salivary, pancreatic and intestinal amylases, and intestinal maltase, isomaltase, sucrase, and lactase) which could conceivably act upon carbohydrate moieties of bacteria, and lipases (gastric, pancreatic and intestinal) which are available to act on lipid moieties of bacteria and viruses. Gastric endopeptidase (pepsin), pancreatic endopeptidase (trypsin, chymotrypsin, and elastase), pancreatic exopeptidase (carboxypeptidase A and B), and intestinal peptidase contribute to further enzymatic activity to which viruses and bacteria must be resistant, or in some way protected, in order to survive in the immediate environment of these enzymes. Although enzymes are lytic for a number of bacteria and viruses they may also reduce the immunological function by degradation of antibodies within the gut lumen (Fubara and Freter 1973).
Bile acids

The normal intestinal flora varies markedly at different levels of the alimentary tract. For example, the stomach and upper small intestine contain a predominance of Lactobacilli and Bacteroides bacteria (Floch et al. 1971) while the number of Escherichia coli is normally low in this portion of the bowel. In contrast E. coli are present in high numbers in the lower bowel while Lactobacilli and Bacteroides species are present in relatively low concentrations. It has been speculated that variances and concentrations of secretions in the tract and secretion by-products are responsible for the varied bacterial flora. Unconjugated bile acids in addition to the enzymes listed above were found by Bertok (1977) to inhibit in vitro replication of certain bacteria and viruses. Growth of Bacteroides, Clostridia and Lactobacilli is reduced markedly by unconjugated bile acids while Streptococcus strains vary in sensitivity and neither E. coli nor Klebsiella species are inhibited (Floch et al. 1971; Binder et al. 1975). Thieler (1957) and Bertok (1977) found that viruses which contain lipoproteins as essential components of integrity and infectivity are inactivated by bile secretions. In addition to the effect bile acids have on growth and inactivation of microbes, bile acids also are able to inactivate endotoxins of bacteria in vivo and in vitro. Such inactivation is irreversible in the presence of proteins. The importance of endotoxin inactivation appears critical in view of the immunosuppressive capabilities of lipopolysaccharides (Springer and Horton 1969; Lagrange and Mackness 1975; Ivanyi 1976).
Relatively low production of bile salts by neonates may contribute to greater susceptibility to colonization of the intestine of the newborn. However, since \textit{E. coli} is not inhibited by bile acids and the pH of the duodenum is relatively basic, thus favorable to the growth of \textit{E. coli}, other undetermined factors appear to be involved in the growth restriction of this group of organisms in that portion of the small intestine.

**Hydrogen ion concentration**

The acid pH of gastric secretions is quite detrimental to the majority of bacteria and viruses (Franklin and Skoryna 1971; Kunstyr et al. 1976). When the stomach environment becomes more alkaline as in cases of pernicious anemia, a large variety of bacteria are able to replicate and cause infections. Viruses of the rhino, calici, alpha, flavo, orthomyxo, paramyxo, rhabdo, leuko, arena, pox, herpes, and irido virus groups are sensitive to a pH of 3 (Wilner 1973) and therefore would not survive the normal pH found in the stomach. A positive correlation has been found to exist between pH and \textit{E. coli} numbers in the upper gastrointestinal tract of piglets (Barrow et al. 1977). Therefore, the higher the pH the greater the \textit{E. coli} population in the gut of piglets. A low pH may normally be established in neonatal animals by colonization with \textit{Lactobacillus} species (Dubos and Schaedler 1962) while an acid pH is maintained in older animals by gastric secretions.
Indigenous microbial flora

Although the normally bacteria-free environment of the uterus prevents colonization of the fetal intestinal tract with the commonly termed "the normal gut bacterial flora", colonization normally begins during the process of birth as bacteria are ingested. Bacterial populations are so much a part of an individual both within the alimentary tract and on exterior surfaces that Rene Dubos et al. (1965) expressed: "indigenous microbiota can influence the morphological and physiological characteristics of its host to such an extent that traits assumed to be unavoidable consequences of the genetic endowment are determined in reality by the microbial environment".

Shedlofsky and Freter (1974) found that while the caecum of germ-free animals was greatly enlarged and Peyer's patches contained few developed germinal centers, introduction of bacteria resulted in a normal gut morphology and a normal immunological response. Lysons et al. (1975) also found that the morphology of the gut in lambs can definitely be affected by bacterial colonization. It can be assumed that viruses of vertebrates also leave their imprint in a similar manner as they become inhabitants with varying degrees of transiency within the gut.

Antagonism between strains of bacteria on the body surfaces are credited with producing greater protection from infection than that mediated by classical immune mechanisms (Freter 1974). Similar antagonistic forces are apparently involved in restricting E. coli and other aerobic bacteria to the lower small and large intestine.
Schaedler and Dubos (1962) found that germ-free mice which had been monocontaminated with coliform bacteria harbored these organisms in high numbers in the stomach and complete intestinal tract. Subsequent inoculation with other bacteria from conventionally raised mice resulted in a marked reduction of coliforms in the upper portions of the digestive tract (Schaedler et al. 1965). Shedlofsky and Freter (1974) noted that the caecal population of *Vibrio cholerae* in germ-free mice could be reduced by a factor of 1.55-2.82 by prior oral or parenteral immunization, and by a factor of 4.72-61.1 when mice were both vaccinated and inoculated with antagonistic bacteria. The antagonistic effect appeared to contribute greatly to *V. cholerae* reduction; however, a possible adjuvant effect should not be ruled out nor should one fail to note that reduction of the caecal population would naturally occur as the size of that organ is reduced due to bacterial action on the gut. The number of organisms as well as the type is important in establishing a normal intestinal state. To convert the gut of the germ-free mouse to the normal state, Freter and Abrams (1972) found that 45 anaerobic and 14 facultative anaerobic strains of bacteria were required. A synergistic role of these bacteria served to suppress various bacterial populations. Certain anaerobes also appeared to react with *E. coli* to suppress still other bacteria, e.g. *Shigella*. The production of volatile fatty acids by anaerobic bacteria was correlated with a 10,000 fold decrease in *E. coli* while elimination of anaerobic bacteria by penicillin treatment resulted in a marked increase of coliform bacteria (Lee and Gemmell 1972). Bacterial antagonism occurs by mechanisms including environmental pH
alteration by specific species which are pH tolerant, competition for specific nutrients, bacteriocins and other bacterial products. Fatty acids produced by various bacteria appear to have a role in microbial control (Meynell 1963; Freter and Abrams 1972).

Saturated fatty acids have been found by Koch et al. (1968) to influence viral uptake in vitro. While considerably less is known about possible antagonism which may occur between viruses than between bacteria, the presence of some viruses within cells has been shown to prevent coviral infection. Varied affinity for viral receptors may also constitute a form of antagonism independent of any immunologic response (Kohn and Fuchs 1973).

Complement

Rowley (1974) concluded that the classical complement system is not functional as an effector pathway of intestinal immunity in mice. This conclusion was substantiated by Benacerraf (1960) who found that levels of specific components of the classical complement system remained essentially the same in irradiated as in nonirradiated mice while properdin levels decreased drastically. The decrease in the properdin levels was correlated with increased susceptibility of the intestine and existed even after the replacement of irradiation damaged epithelium.

Essential components of the alternate complement or properdin pathway include an initiating factor (IF), which upon contact with an activating substance is able to associate with C3, properdin, pro-activator, and its convertase (Gotze and Muller-Eberhard 1971). This
complex in the presence of Mg$$^{++}$$ has C3 convertase activity which results in C3b being deposited on the surface of the target substance and subsequently acts as an opsonin as well as C5 convertase by which the membrane attack complex, C5b-9, is deposited. Although lysis of cells may occur by activation of C3 in the absence of properdin, the presence of properdin increases the duration of C3 and C5 convertase enzymatic activity from 1.5 minutes to 8-10 minutes at 37C.

Bacterial lipopolysaccharides (endotoxins) are some of the most active C3 fixers known. Polysaccharides and aggregates of IgA, IgG and IgE also activate the alternate pathway (Osler and Weiss 1976). The presence of proteolytic resistant secretory IgA in the intestinal lumen where it may act in conjunction with lysozyme maximizes the effective role of properdin. Antibody appears to be required for lysis of virus infected cells by the alternate pathway but is not essential for opsonization of activating particles.

**Interferon**

A variety of virus inhibiting proteins collectively termed interferon are released by host cells in response to various inducers (Ho et al. 1967; Merigan 1968; Friedman and Sonnabend 1970; Davis et al. 1973). Inducers include double stranded RNA which may either be part of an infecting virus or produced during viral replication, synthetic double stranded polynucleotides, certain bacteria, rickettsiae, bacterial endotoxin and phytohemagglutinin. The interferon released in response to natural and synthetic polynucleotides is of **de novo** production while that released due to the remainder of
the listed inducers is of preformed interferon. A constant background production of interferon occurs in the absence of inducers and serves to enhance production in the event of viral infection. Viral inhibition is a result of an inhibiting protein termed interferon mediated nuclease, which is produced and functional both in the interferon synthesizing cell and interferon activated cells. A small molecule which appears to function in activation of the interferon mediated nuclease has been demonstrated to be present in interferon stimulated cells (Eppstein and Samuel 1978).

Interferon production may vary qualitatively and quantitatively with different inducers and responding tissue (Solov'ev and Bektermirov, 1973; Havell et al. 1978). The response of the interferon activated cells however, does not appear to be influenced by type of interferon. While all cells appear to be capable of interferon production, leucocytes and lymphoid tissues have a major role in production; however, it appears that not all cells are equally sensitive to all inducers (Ho 1967). Solov'ev and Bektermirov (1973) reported that inflammation and phagocytosis by mobilized leucocytes act to increase interferon production. The presence of a rich supply of lymphoid tissue in the intestine and the rapid response of the intestine to inflammatory stimuli may therefore enhance the antiviral activity of interferon in that organ. Although interferon appears to be capable of stimulating all tissues to produce antiviral proteins a gradient of effectiveness appears to exist. For example, epithelial cells have been found to be less responsive to in vitro activation by interferon.
than fibroblastic cells. The presence of some viruses which are not producing obvious pathological effects have also been found to render the infected cells less susceptible to antiviral activity of interferon than noninfected cells (Ito et al. 1978). These factors may play major roles in the susceptibility of the intestinal tract to viral infection since viruses, particularly nonpathogenic viruses, may be present in the intestine. The responsiveness to interferon of epithelial cells so infected would be decreased and could contribute to the denuding effect observed with some viral infections (Hermodsson 1963). However, the denuding event may in itself be a last resort mechanism to rid the mucosa of the virus laden epithelium which would serve as a source of infective viruses for underlying tissues. The dissolution of the epithelial cells would result in release of proteolytic enzymes capable of action on microbial proteins.

Phagocytosis

Numerous cells of a host respond to limit and eliminate the intrusion of foreign materials into and beyond epithelial surfaces. The most responsive cells are the leucocytes which are comprised of two morphologically different groups, viz., the granulocytes and agranulocytes. The agranulocytes consist of the lymphocytes and monocytes, both of which will be discussed later. The granulocytes consist of eosinophils, basophils and polymorphonuclear leucocytes.

The function of eosinophils remain somewhat of an enigma but they are known to be moderately active in phagocytosis (Cline et al.
to carry antimicrobial enzymes (Cotran and Litt 1969) and to react in antibody dependent cytotoxicity reactions (Houba et al. 1976). These cells increase in intestinal tissue and in the circulation in association with food allergies and parasitic infections (Hirsch 1965). Chemotactic substances which attract eosinophils are released by lymphocytes, basophils, and mast cells. The eosinophils phagocytose granules of basophils and mast cells and serve to reduce free histamine in the circulation and therefore may serve to reduce allergic inflammation (Beeson 1977). In addition eosinophils are attracted to and ingest antigen-antibody complexes where the antibody is of the IgG or IgM but not the IgE type. This process serves to prevent activation of complement and subsequent release of phlogistic by-products.

Basophils which normally comprise less than 1% of the circulating leucocytes contain lysosomal constituents similar to those of polymorphonuclear leucocytes and are phagocytic (Weiss 1973). In addition these cells contain heparin, histamine, serotonin and a slow reacting substance (SRS) which is also a vasodilating substance. The surface of basophils attracts IgE antibodies which when combined with their antigens cause degranulation of the cells. Arylsulfatase produced by eosinophils is capable of inactivating SRS thus reducing the strength of the reaction (Wasserman et al. 1975). The chain of events following degranulation induces inflammation accompanied by increased blood flow rich in antibodies and accumulation of phagocytic cells (Rothwell and Huxtable 1976).
The third granulocytic cell, the polymorphonuclear neutrophil, (PMN) is the major phagocytic leucocyte. Normal mean values for neutrophils in domestic livestock and man vary from 30 to 70% of the total leucocytes while the normal miminum total leucocyte counts vary between 4,000 and 9,000 per mm$^3$ (Swenson 1977). While a normal migration of these cells occurs into the tissues, a greater attraction occurs due to a variety of chemotactic substances including C3b and C5b (Ward and Hill 1970), bacterial products (Ward et al. 1958), and antigen-antibody complexes (Henson 1971b). The cells existing in the circulating and capillary and bone marrow maturation pools are first pressed into action in the tissues in the event of infection or injury. The same substances which attract the cells into the tissues also stimulate bone marrow production (Robinson and Mangalik 1975). Approximately 5 days are required from initial stimulation to the release of functional PMNs into the general circulation. Neutrophil migration and accumulation occurs in the intestinal lamina propria due to a variety of conditions including nonspecific inflammation, exposure to endotoxin, and bacterial and viral infections. Neutrophils are phagocytic within the blood stream, tissue and the intestinal lumen where physiological conditions are favorable (Rellamy 1973). These cells contact and ingest bacteria, viruses, and antigen-antibody complexes. Bellamy and Nielsen (1974) found that emigration of neutrophils into the gut was greater when specific antibodies were present for antigens in the gut lumen. Pickering and coworkers (1977) found a significantly (P ≥ .001) greater number of
leucocytes in the stools of human patients suffering from an intestinal invasive pathogen than in the stool of patients with diarrhea caused by noninvasive pathogens. Preformed antibodies have been detected on the surface of neutrophils and appear to be involved in antibody-dependent cytotoxicity (Cooper et al. 1975; Watson 1975). Phagocytosis is enhanced by immune opsonins, complement, and other unidentified serum proteins (Stossel 1975). Phagocytosis can be viewed as having three phases (Bessis 1973); recognition (adsorption), ingestion, and digestion. Surface ionic charges and antibody present on the surface of virus or bacteria function in adsorption. Ingestion is temperature sensitive and may increase with an increase in body temperature (fever). The phagocytized material is exposed to digestive enzymes by the fusion of the membranes of phagosomes and lysosomes. The digestive enzymes listed by Swenson (1977) include alkaline phosphatase, acid phosphatase, ribonuclease, deoxyribonuclease, nucleotidase, B-glucuronidase, lysozyme and cathepsin. Granulocytes are terminal cells and degenerate within a few days after leaving the bone marrow. As these cells lyse, chemotactic substances are released along with hydrolytic enzymes. These enzymes are still functional against viruses, bacteria and other foreign material after cell lysis. Release of leucocytic pyrogen occurs in vitro from exposure of the PMNs to endotoxin, certain viruses, bacteria which they may ingest and an activating factor present in exudate (McCarty 1973). The release of lysosomal contents in areas of inflammatory lesions or generalized infections result in increased body temperature
(Schumacher and Agudelo 1972) which in turn increases phagocytosis. Therefore, both inflammation and fever are recognized as contributing to the immune process but will not be discussed as separate categories.

Of the agranulocytic leucocytes, only macrophages are notably phagocytic. Monocytes which are produced in the bone marrow circulate initially in the blood and are precursors of macrophages which may be found in the tissue as fixed cells and in loose connective tissue (histiocytes), lymphoid tissue (phagocytic reticulocytes), the liver (Kupffer cells), within the walls of lymph and blood vessels (adventitial cells), and as epitheliod or giant cells. In a review by Morgensen (1979), numerous nonspecific functions of the macrophage in response to viruses are discussed. While PMNs are very active in phagocytosis, their activity is not altered by exposure to antigens. In contrast macrophages become activated by exposure to antigen (Hard 1970; North 1970). Activation increases the phagocytic action and rate of destruction not only against the initiating antigen but nonspecifically against unrelated antigens (Ruskin et al. 1969; Hirsch et al. 1970; Landy 1975). Activation results in a modification in the type and relative and total amount of hydrolytic enzymes possessed by the cells. The fact that macrophages from mature mice are more capable of protecting immature mice than are their own macrophages may relate to cell maturity or previous activation (Knop and Rowley 1975). Macrophages also contribute to immunity by interaction with lymphocytes (Argyris 1968; Unanue 1972). The exact mode of interaction has not been conclusively determined but appears to result
from antigen processing and presentation of modified antigen and/or coded information to lymphocytes. Further interaction occurs between activated lymphocytes and macrophages with the former releasing acidic glycoproteins which inhibit migration of macrophages from an area of activated lymphocytes (Leu et al. 1972). This migration inhibition factor (MIF) provides a means of nonspecifically localizing macrophages in the vicinity of antigenic stimulation (Reif et al. 1975) and establishing a long lasting cell-mediated immunity (Cameron et al. 1976).

Macrophages are capable of activity in areas of inflammation which have become too acid for granulocyte activity. The life span of macrophages is from several weeks to several months, therefore they remain active much longer than do PMNs. As long lived cells, macrophages may spare an individual from immunologic paralysis when subjected to excess antigen by modifying and releasing the antigen over an extended time. The ability to retain antigens for a long period of time also results in prolonged exposure of immunocompetent cells and development of delayed hypersensitivity. Cooperation of antibody and complement components enhance the phagocytic and digestive processes of macrophages which have been demonstrated to have Fc and C3 receptor sites (Reynolds et al. 1975). Phagocytosis with the involvement of IgG antibody is enhanced by the presence of complement factors. A specificity of macrophage activity can be imposed by attachment of cytophilic antibody. These antibodies are particularly important in directing specificity of cytotoxic contact which results in destroying cells bearing homologous antigens on their surfaces.
Macrophages comprise a prominent component of cells within the intestinal lamina propria and Peyer's patches. Two hours after feeding adult mice carbon particles, the particles were demonstrated in macrophages of the Peyer's patches (Joel et al. 1970). These cells have also been observed to phagocytose migrating lymphocytes which may have been exposed to antigens within the intestinal lumen (Sawicki et al. 1977). By both processes, the macrophages may have access to antigens which they may process for lymphocyte stimulation. Both macrophages and PMNs act in the lamina propria and deeper tissues to sequester infectious agents which gain entrance into and beyond the mucosa. As a result of antigenic stimulation of gut associated macrophages, a constantly activated population of cells is always present in the normal state (Nickol and Bonventre 1977).

In addition to the phagocytic activity of macrophages may release their digestive enzymes into the surrounding fluid by secretion and release of membrane bound vesicles. Numerous enzymes are present which have varied initiation stimuli and properties (Davies and Allison 1976). Some enzymes occur in extracellular concentrations adequate to control infectious agents on body surfaces and in body fluids. Their extracellular effects appear to facilitate tissue repair as well as microbial elimination. However, excessive release of enzymes whether due to excretion or cell lysis may result in tissue damage.
Macrophages also contribute to nonspecific immunity by augmenting lymphocyte production of various mediators of cellular immunity including interferon, lymphotoxin and migration inhibition, activation, and chemotactic factors affecting macrophages (Epstein 1976). Jullien et al. (1974) concluded that macrophages themselves are interferon producers. It is the interaction of macrophages and their secretions with antigen and lymphocyte populations which determines the type and degree of immune response. This interaction may either cause suppression or stimulation of lymphocyte activities. Multiple contributions of the macrophage are gravely reduced with a low protein diet (Passwell et al. 1974) and may relate to various factors of increased susceptibility associated with poor nutrition.

Cell-mediated immunity

"Cell-mediated immunity" (CMI) is a multi-faceted term denoting the action of cells. Viewed from the broadest perspective all immunological functions require cellular action. The traditional definition however excludes humoral immunity which results from B cell maturation into antibody secreting plasma cells. However, it has been found that T cells also secrete substances which function separately from the cell. In addition, specific T cell functions may be transferred with crude T cell extracts and a more purified component termed transfer factor. Klesius and Kristensen (1977) reported specifically on such factors capable of transfer in cattle. These substances have influences upon a variety of cells including macrophages which become activated for increased phagocytosis and microbial killing. It is
this relationship with macrophages and distinct T cell functions which have influenced the traditional definition of cell-mediated immunity as: "Immunity dependent upon T type lymphocytes and phagocytic cells" (Barrett 1974). However, due to the complex interrelationship of lymphocyte populations a brief discussion of each cell type will be presented under this heading.

Those lymphocytes which bear surface immunoglobulins and receptors for the Fc portion of antibodies complexed with antigen and complement bound to such complexes are termed B cells (Golub 1978). These cells may be separated from other lymphocytes by their adherence to nylon wool (Julius et al. 1973), cotton wool (Hogg and Greaves 1972) or glass beads (Rosenthal et al. 1972). They may be isolated by virtue of their receptors and surface Ig molecules by passage through appropriate columns (Warner 1974; Chess and Schlossman 1977). The major role accredited to B lymphocytes is that of being precursors of plasma cells, the major producers of specific humoral immunity. Möller and Svehag (1972) and Van Boxel et al. (1972) have independently concluded that B cells also function in antibody-dependent cytotoxicity. Since cytotoxicity is not a function normally accredited to B cells, the results of these studies may have been due to the presence of an additional cell type not bearing markers typical of B or T cells. Greenberg et al. (1973) reported on the presence of a third lymphocyte population which they termed "Null" cells. These cells were active in nonspecific antibody-dependent cytotoxicity. Others (Oldham et al. 1977; Chess and Schlossman 1977) have reported on similar or identical
cell population(s) to which they have given various identifications. A contribution to cytotoxicity in cooperation with phagocytic cells is recognized by the contribution of cytophilic antibodies which may direct the activity of both PMNs and macrophages (Gale and Zighelboim 1975; Shin et al. 1972).

The thymus-dependent T cell population appears to be a heterogeneous group. The heterogeneity is expressed in size and apparent function although all bear the theta marker (Guy-Grand et al. 1974; Chess and Schlossman 1977). It is also evident in the homing pattern of cells from various sources in Peyer's patches, mesenteric lymph nodes, and peripheral lymph nodes. A major portion of small cells appear to home to tissues while another portion of cells appear to be sessile and may constitute a pool of cells with memory. The various functions of T cells are numerous, complex and only partially understood. Functions include interaction with and modulation of B cell and macrophage functions. These processes may occur by direct contact or by release of humoral factors collectively termed lymphokines (WHO 1973). The lymphokines affecting macrophages include migration inhibition factor (MIF), macrophage aggregation factor (MAF), and macrophage chemotactic factor (MCF) whose functions are reflected in the nomenclature. An additional substance, the macrophage resistance factor, which would render macrophages nonspecifically resistant to infection with certain bacteria and viruses has been postulated. Similar factors which influence granulocytes, attract them into areas of inflammation and prevent their migration out of an area of activity.
The activity of T cell factors on lymphocytes induces blastogenesis and enhances ongoing transformation of lymphocytes undergoing blastogenesis. T cells also are regulatory cells and may either cooperate with B cells to enhance or modify their response in the presence of antigen or may suppress B cell activity (Katz and Benacerraf 1972). Subpopulations of T cells also appear to exist which modify cell-mediated responses of responding T cells (Chess and Schlossman 1977). A population of antibody coated T cells has also been observed which had a suppressive affect on macrophages (Gershon et al. 1974).

The literature abounds with information on CMI responses of lymphocytes from the peripheral circulation. In vitro tests most commonly employed are the MIF and lymphocyte transformation tests. However, these tests have only been applied relatively recently to lymphocytes from mucosae. The first such tests were conducted with cells washed from alveolar spaces (Henney and Waldman 1970; Galindo and Myrvik 1970; Reif et al. 1975). It has been found that local antigenic stimulation resulted in reactions dependent on systemic responses demonstrated by peripheral and spleen derived lymphocytes. In addition to an increase in IgA producing cells the immune response resulted in development of a population of cells which in the presence of activating antigen inhibited the migration of macrophages.

Evidence for the cell-mediated capacity of intestinal lymphoid tissues has been primarily speculative based on intestinal morphology and studies of other tissues. In vitro stimulation of de novo antibody production has not been conclusively demonstrated in cells from
Peyer's patches although a complete complement of the cell types known to cooperate in antigen recognition and processing and antibody production appear to be present (Levin et al. 1973; Sobhon 1971). It has been speculated that antigen sensitive cells exist in a sequestered state or that one or more cell types necessary for an immune response are absent from Peyer's patches (Katz and Perey 1973; Kagnoff and Campbell 1973; Veldkamp et al. 1973). Suppressor T cells capable of suppressing both antibody production (Mettingly 1978) and delayed-type hypersensitivity reactions (Kagnoff 1978) have been demonstrated in Peyer's patches. However, cells which have been stimulated in Peyer's patches are capable of antibody production in vitro and in the lamina propria (Bienenstock and Dolezel 1971; Robertson and Cooper 1973). Evidence in support of CMI capability of gut associated lymphocytes comes from the work of Frederich and Bohl (1976) who conducted a series of tests to determine the CMI response to the viral agent of transmissible gastroenteritis (TGE) in swine. They found a significantly greater inhibition of macrophage migration by lymphocytes from the intestinal lamina propria of pigs inoculated orally than for spleen cells from these animals. A reverse response was detected for cells from animals given subcutaneous virus inoculations. Their tests conclusively demonstrated the capacity of cells in the lamina propria to function in CMI responses. Muller-Schoop and Good (1975) compared in vitro responses of lymphocytes from Peyer's patches, spleen and mesenteric and inguinal lymph nodes to several antigens. Stimulation by oral inoculation or normal intestinal flora
resulted in greater response of Peyer's patch cells than for cells from other lymphoid organs. Both B and T cell responses were noted in the Peyer's patches and it was concluded that not only were functional cells present but that they were immunocompetent when antigen stimulation was presented from the intestinal lumen. Intragastric immunization has repeatedly been demonstrated to be of value in establishing protection against organisms infecting by the same route. Such infectious agents include *Escherichia coli* (Kohler et al. 1975), *Salmonella* (Collins and Carter 1974) and TGE virus (Bohl and Saif 1975).

Maximum CMI capabilities do not appear fully developed in domestic animals at birth. However, colostrum contains in addition to a high concentration of immunoglobulins, IgA producing lymphocytes and viable T lymphocytes. It has been speculated that both lymphocyte types are capable of *in vivo* activity in the neonate following ingestion (Goldblum et al. 1975; Parmely and Beer 1977). In addition, the secretory IgA antibodies present in colostrum are directed primarily against antigens encountered in the gastrointestinal tract (Goldblum et al. 1975; Gindrat et al. 1972; Montgomery et al. 1973). These results imply that cells stimulated in the gut associated lymphoid tissue migrate to the mammary tissue and secrete into mammary alveoli. If indeed the cells observed in colostrum are of intestinal origin they may be capable of homing once again into the intestinal lamina propria and initiating local immune responses. It is possible that such cells could be analogous with the migrating
theliolymphocytes described by Ficntelius (1968) and may represent a population of T cells which function in antigen recognition.

The presence of CMI capability of intestinal lymphoid tissues is essential for normal intestinal integrity. Nude mice and neonatally thymectomized animals lack T cells in the intestine. This deficiency is associated with a lack of mast cells in the lamina propria and depressed local immune capacity (Ruitenberg and Elgersma 1976). The dependency of mast cells upon an intact T cell system is further demonstrated by subcutaneous thymus cell implants which trigger normal mast cell development and activity in the lamina propria. In the absence of normal mast cell function, immunity dependent upon IgE is suppressed as well as that dependent upon eosinophil functions (Colley 1973).

Numerous viruses including measles, equine infectious anemia and BVD cause immunosuppression by depression of T cell responses (McFarland 1974; McGuire 1976; Muscoplat et al. 1973a; Reggiardo 1975). Selective depression of T cell activity may occur due to the presence of receptor sites on T cells for various viruses (Notkins et al. 1970). However, when the T cell suppression results in selective suppression of B cell helper function as noted by McFarland and McGuire a protracted disease and/or carrier state may result.

While the suppressed or inactive state of T cells results in increased susceptibility and protracted infection, an overactive or internally uncontrolled T cell response system produces a more critical disease state. Such a state is exemplified in food allergy responses,
sprue in man, and various diseases affecting the gastrointestinal tract of animals (Kent and Moon 1973).

Specific Immunity

Cells bearing all immunoglobulin classes: IgA, (Tomasi et al. 1965; Allen and Porter 1973; Grabbe et al. 1965; Parrott and Ferguson 1974; Crandall et al. 1967; Cebra et al. 1977; Husband et al. 1977); IgG (Hanson and Brandtzaeg 1973; Lee and Lascelles 1970); IgM, (Lascelles and McDowell 1970; Brandtzaeg and Baklien 1977); IgD, or IgD-like (Vitetta et al. 1975); and IgE, (Ishizaka et al. 1969; Mayrhofer 1977) have been demonstrated in the intestine. Ontological studies of antibody producing cells have been reported in several species including porcine (Chapman et al. 1974), bovine (Schultz et al. 1973) and murine (Friedberg and Weissman 1974). Schultz reported that lymphoid cell development was evident first in the thymus of bovine fetuses at 42 days of gestation while such development was not evident in the gastrointestinal tract until approximately 175 days. IgM producing cells were first observed at 59 days presumably due to antigenic stimulation. Silverstein and Parshall (1966) found similar ability of fetal lambs to produce an IgM immune response to viral antigens. The first demonstrated IgG production was much later with 145 and 140 days of gestation being recorded for bovine (Schultz 1973) and human (Thorbecke and Van Furth 1967) fetal tissues respectively. However, others (Gitlin and Biasucci 1969) have found IgG producing cells in human fetal liver and gastrointestinal tissue cultures at 84
days while approximately 19 days were required for spleen cells. The variance in age at which IgM and IgG producing cells were first noted appears to be dependent upon antigenic stimulation and sequence of immunological response as well as gestational age. Measurable quantities of immunoglobulins in the serum have been reported to occur later than demonstration of immunoglobulin bearing cells in both human and bovine fetuses (Cooper et al. 1968a; Schultz et al. 1971; Gitlin and Biasucci 1969).

Chapman and coworkers (1974) worked with porcine fetuses and found IgM producing cells first in the spleen at 55 days of gestation. However, IgG producing cells were not observed until 15 days later. The occurrence of IgG producing cells preceded that of IgM cells in the thymus. In contrast Allen and Porter (1973) did not detect IgG or IgM in fetal or newborn porcine intestine and found that IgA production was not detectable until 2 weeks after birth. Although Ig production was not noted in Peyer's patches by Chapman et al. (1974) lymphoid development within patches was noted as early as 50 days gestation. These workers concluded that Peyer's patch cells may be progenitors for lymphocytes in other areas. This hypothesis is supported by Jacobson and coworkers (1961) who found that Peyer's patches which were shielded from irradiation were capable of repopulation of other lymphoid tissues. Further evidence is provided by the results of Cooper et al. (1966) who found that Peyer's patches show follicular development without obvious antigenic stimulation and removal of Peyer's patch cells from total body X-irradiated rabbits selectively
inhibited humoral antibody production. Although typical morphology of Peyer's patches does not develop without microbial population of the intestine (Cooper et al. 1968a; Pollard and Sharon 1970), lymphoid development is evident early in fetal development and the lack of Ig bearing cells may result due to migration into other areas prior to detectable antibody production. Additional evidence of early migration was noted by Guy-Grand et al. (1974) who observed that cells with surface Ig were evident in Peyer's patches but Ig was not evident within these cells until they were localizing in other tissues. However, Friedberg and Weissman (1974) contended that the mitotic rate of Peyer's patch cells is too low to contribute significantly to the ontological development of other lymphoid organs. The capability of antibody production is indicated by in vitro tests conducted by Vitetta et al. (1975) who observed IgA, IgG and IgM protein secretion by Peyer's patch cells. Cebra and colleagues (1977) were able to transfer cells capable of antibody production into syngeneic mice by transfer of Peyer's patch, thymus and spleen cells into sublethally irradiated mice. It was conclusively shown that IgA producing cells were from the donor Peyer's patch cells which also appeared capable of giving rise to IgM, IgG1 and IgG2 plasma cells.

Since Hanson (1961) and Tomasi and Zigelbaum (1963) first stated that IgA was the major immunoglobulin present in secretions, numerous studies have been conducted in an effort to evaluate its role in immunity. IgA producing cells appear later in development than do IgM or IgG producing cells (Allen and Porter 1973) but rapidly become the
predominant Ig producing cell type in the mucosa (Crandall et al. 1967; Heremans 1975).

The richest source of precursors for IgA producing cells appears to be the Peyer's patches (Craig and Cebra 1971; Cebra et al. 1977; Husband et al. 1977). It is postulated that these cells become sensitized by antigens penetrating or trapped by epithelial cells over the Peyer's patch follicles. The lymphoid cells thus "sensitized" are released into the lymphatics from which they may home to other sites including the mammary glands, spleen, and mesenteric lymph nodes but primarily to the lamina propria of the intestine (Hall and Smith 1970; Hall et al. 1972). It has been further postulated that homing into the lamina propria was due to the presence of antigen which had produced the initial stimulation. However, Halstead and Hall (1972), Moore and Hall (1972), Parrott and Ferguson (1974), and Guy-Grand et al. (1974) found that these cells homed into the lamina propria of antigen free fetal intestine as well. An alternate hypothesis is that homing results from the influence of other cells in the intestine which cooperate with IgA producing plasma cells in the release of secretory IgA (SIgA). These cells, identified primarily as the epithelial cells comprising the outer surface of the mucosa, produce the secretory component (SC), a glycoprotein which binds to the alpha chains of dimeric IgA by disulfide bonds. In man, the IgA in serum is primarily of the monomeric form and may be produced by a different plasma cell line than the dimeric form. The SC portion appears to attach to IgA molecules joined together by the J component produced by the same
plasma cells which secrete the IgA. The functions proposed for SC include transport of SIgA across cell membranes into secretions and protection of the immunoglobulin from proteolytic enzymes within the lumen of the intestine. SIgA appears to bind to mucus glycoproteins in the glycocalyx (Clamp 1977) where it may bathe the epithelial layer. Although numerous workers have credited SIgA with a major role in the local immunity of secretory surfaces the various modes of action are not clearly defined. It appears well established that SIgA alone does not function in responses including the classical pathway of complement fixation (Adindolfi et al. 1966a; Ishizaka et al. 1965; Vaerman and Heremans 1968); however, in the presence of lysozyme, SIgA and complement components do produce bacteriolysis (Adindolfi et al. 1966a; Burdon 1973; Hill and Porter 1974). More emphasis has recently been given to the role of SIgA antibody interaction with components of the alternate complement pathway (Gotze and Muller-Eberhard 1971; Colten and Bienenstock 1973). It has been observed that SIgA aggregated with antigen will trigger this system. In addition SIgA aids in opsonization of bacteria (Henson et al. 1972; Kaplan et al. 1972) although there are contradictory reports by Eddie et al. (1971) and Wilson (1972). SIgA is also capable of virus neutralization (Bellantini et al. 1969; Ogra and Karzon 1969), prevention of microbial adsorption to and penetration of the epithelial layer (Williams and Gibbons 1972; Freret 1972; Parry et al. 1977), and the prevention of absorption of toxins (Kaur et al. 1972). It is in its action at the epithelial level that the greatest contribution may be made to mucosal immunity.
Local antibody production appears evident by the results of numerous studies which indicate favored immunity for sites of initial stimulation with separation of respiratory, urogenital and intestinal responses. However, the immune response does not appear to localize to specific sites within a particular organ. This has been exemplified in work reported by Robertson and Cebra (1976) and Cebra et al. (1977). They found that the introduction of antigen into one of a pair of ileal Thiry-Vella fistulae resulted in antibody production in both if a Peyer's patch was present in the loop exposed to antigen. In the absence of Peyer's patch in the exposed loop only a slight antibody response was noted. Similar reports have been made of studies conducted in sheep (Husband and Lascelles 1974). In addition, infused lymphoid cells from mesenteric lymph nodes selectively migrated to the intestinal lamina propria (Griscelli et al. 1969).

In summation, the Peyer's patches are a rich source of IgA precursor cells. These cells appear to become sensitized to antigens prior to emigration from the patches and may then be viewed as large lymphocytes in the thoracic lymph. These cells home primarily to the intestinal lamina propria where they become antibody secreting plasma cells. Secretory antibodies function in numerous but presently poorly defined roles within the glycocalyx and gut lumen.

Although IgA is the major immunoglobulin present in normal secretions, it has been found that in selective IgA deficiencies IgM may contribute significantly to secretory immunity (Brandtzaeg 1975). This is particularly evident where a deficiency in IgA production is
evident (Savilahti 1973). As with IgA, IgM in the polymeric form is joined together by the J chain which appears to be a prerequisite for attachment to SC. The union between polymeric IgM and SC is less stable than is that between IgA and SC but appears adequate for a functional role in secretory immunity. The recognized biological functions for IgM include complement fixation by the classical pathway and opsonization. Although in developed countries with improved sanitation, IgA deficient people do not appear more susceptible to disease than the IgA competent, a greater association with food allergies is noted in these individuals. This fact would suggest that the secretory IgM system is less efficient in preventing penetration of antigen (Butler and Oskvig 1974) allowing a subsequent IgE antibody response to be elicited (Stokes et al. 1974). Brandtzaeg and Baklien (1977) have demonstrated the presence of IgM producing cells in the lamina propria in numbers less than demonstrated for IgA, and IgM as well as IgA antibodies present in the epithelial cell border. Brandtzaeg (1975) found IgM distribution in the epithelium equivalent with that for IgA. Allen and Porter (1970), Allen et al. (1976), and Crandall et al. (1967) have shown similar distribution of IgM and IgA secreting cells in animals as that reported above for man. Initial IgM responses to antigenic stimulation are noted in intestinal tissues in a manner comparable to that expected elsewhere in the body (Crandall et al. 1967). Similarly, IgG producing cells in the lamina propria increase following an initial IgM response.
Although IgG is not generally accredited a highly active role in intestinal immunity due to its susceptibility to proteolytic enzymes, approximately 2-6 percent of plasma cells in lamina propria secrete IgG (Brandtzaeg and Baklien 1976). These cells in relation to IgA and IgM producing cells are more centrally located in the villous lamina propria. It appears that this immunoglobulin may be active in the gut of the neonate prior to secretion of a level of enzymes which would cause degradation. IgG, particularly is present in high concentration in colostrum of species which supply passive immunity to their young via colostrum (Porter et al. 1977). Immunoglobulins are absorbed intact and supply systemic humoral immunity. Due to their smaller molecular size IgG molecules pass through vascular walls more readily than do larger immunoglobulins. IgG antibodies have been found to supplement the secretory immune system in secretions of the respiratory tract (Wells et al. 1977) of lambs following suckling. Similarly, IgG may be the predominant immunoglobulin in nasal secretions when the immunization bypasses the mucosal tissues. IgG antibodies are detectable in intestinal secretions as well and in the absence of proteolytic enzymes would appear to aid in complement fixation, opsonization and virus neutralization. The level of IgG in the serum has been found to increase in individuals with enteric allergies (Ahlstedt et al. 1977). During an inflammatory response IgG antibodies flow into the intestinal lumen where they may function in the diluted intestinal milieu reacting specifically with antigen. Aggregated and antigen-complexed IgG is capable of causing degranulation.
of neutrophils thus stimulating microbial killing and inflammatory responses (Henson 1971a; Henson 1971b).

Cells bearing IgD have also been reported in the intestinal lamina propria and Peyer's patches. However, the significance of this finding is uncertain. Vitetta and Uhr (1975) speculated that IgD antibodies on cell surface may be necessary for antigen recognition and initiation of antibody secretion. Vitetta and colleagues (1975) found that 85 to 95 percent of lymphocytes in Peyer's patches had surface IgD detectable by iodination. Vitetta and Uhr postulated that IgD which is the most vulnerable immunoglobulin to enzyme action is present on the surface of antibody precursor cells and when combined with antigen functions in triggering. Therefore, the Peyer's patches which appear to be the primary source for precursor cells for the lamina propria would consist predominantly of virgin cells bearing the antibody synthesis triggering mechanism, IgD.

The fifth immunoglobulin class, IgE, is actively involved in intestinal immunity as well as pathological conditions. Peyer's patches appear to be the source of precursor cells for IgE producing plasma cells (Ogilvie and Parrott 1977). The production of IgE is T cell dependent (Colley 1973; Ruitenburg and Elgersma 1975); therefore, in T cell deficiencies there is also an IgE deficiency. The greatest percentage of IgE is cell associated and that released from plasma cells is rapidly attached to mast cells and basophilic leucocytes. Upon contact of antigen with specific cell bound antibody, degranulation of both cell types may occur resulting in release of potent
vasoactive substances which increase fluid extravasation. In addition
substances are released which cause contraction of smooth muscles and
chemotaxis of eosinophils. The effect upon the intestine would there­
fore be to cause exudation of fluid with increased antibody release
and an accumulation of phagocytic cells. Contraction of smooth muscles
would aid in expulsion of the initiating factors within the gut lumen
(Bloch et al. 1972).

The involvement of IgE antibody is most evident in self-cure proc­
esses in response to parasite infestations. Initial exposure results
in depletion of mast cells in the associated tissue and an increase
in IgE plasma cells and serum IgE. Mast cells which invade into
intraepithelial spaces increase in number 10-14 days later.
As degranulation of the latter mast cell population occurs the sur­
rounding epithelium, endothelium and lamina propria are damaged. An
increased intraluminal flow occurs resulting in exposure of parasites
to enzymes and specific antibodies, primarily IgM and IgG1. The
contraction of smooth muscles then results in expulsion. Damage to
parasites, presumably antibody mediated, precedes this process. The
antibody class involved is unknown although IgA does not appear to
be involved in resistance to intestinal parasites. Although the
major role of IgE antibody appears to be in degranulation of mast
cells and basophilic leucocytes, this antibody in the aggregated form
which would occur in combination with eliciting antigen is capable of
complement activation by the alternate pathway (Ishizaka and Ishizaka
1972). IgE mediated pathological conditions may occur in the absence
of functionally normal suppressor T cells which would modify T cell stimulation of IgE plasma cells and/or their precursors. The condition termed sprue in man may result from such a disorder.

Bovine Viral Diarrhea

The first reports of what was termed "an apparently new transmissible disease in cattle" by Olafson et al. (1946) by review appears to have been a new disease in this country or the expression of a virus variant not previously recognized. This conclusion is made in view of the high incidence of morbidity and mortality of cattle affected with the disease at that time. An alternate conclusion which could equally well be defended is that the herds in which the disease was described had previously been free of infection and therefore were completely susceptible to disease. Clinical signs recognized for the disease at that time included: febrile responses, salivation, nasal discharge, depression, anorexia, diarrhea, dehydration, ulceration of the lips, checks, tongue, pharynx and esophagus, and abortion. The lack of a bacterial etiological agent, the transmissibility of disease with bacteria free material, and the most evident symptom of diarrhea led to the name of "virus diarrhea of cattle" (Olafson and Rickard 1947). Similar symptoms were described by Ramsey and Chivers (1953) and Pritchard (1954-1955) in Iowa cattle. Severe lesions of the mucous tissues were recognized by these researchers, who termed the condition "mucosal disease" and "transmissible erosive gastroenteritis" respectively. Subsequent studies which demonstrated cross
neutralization between causative agents for virus diarrhea and mucosal disease resulted in the modified name of "bovine virus diarrhea - mucosal disease complex" (BVD-MD) (Kniazeff and Pritchard 1960; Gillespie et al. 1961).

Following the isolation of the first cytopathogenic strain of BVD-MD virus by Gillespie and coworkers in 1960, studies on characterization commenced. Infective units were found to pass through a 50 millimicron filter, to be lipid solvent sensitive indicating the presence of an envelope, and capable of replication in the presence of 5-iododeoxyuridine which inhibits DNA synthesis, thereby indicating an RNA core (Hermodsson and Dinter 1962). These virions have a sedimentation coefficient of 80-90S and a buoyant density between 1.075 and 1.115 g/cm³ and are susceptible to a pH of 5.0 or less (Parks et al. 1972; Lambert et al. 1974). Further characterization by electron microscopy by Ritchie and Fernelius (1969) revealed "ribosome-like" virus specific precursor particles 15-20 nm in diameter and larger units, 30-50 nm and 80-100 nm in diameter, which combined with specific antibody and were assumed to be the infectious units. They concluded that BVD virus could not be classified on the basis of information available at that time but described the morphology as resembling rubella virus which is ragged in appearance with an amorphous envelope. Other authors (Burki 1966; Castrucci et al. 1968; Tanaka et al. 1958) also pointed out difficulties with classifying this group of viruses. Results of electron microscopy and density gradient studies led Maess and Reczko (1970) to suggest that the BVD-MD virus be grouped
with the myxovirus group. However, similarities of BVD-MD virus with viruses of the Togaviridae family have resulted in a more recent but unofficial classification in the proposed genus of Pestivirus (Fenner 1974) along with the similar causative agents of hog cholera and equine arteritis which are elsewhere grouped as unclassified viruses of vertebrates (Wilner 1973).

The pathological responses to hog cholera, equine arteritis and bovine viral diarrhea viruses are similar (Kent and Moon 1973). That of bovine viral diarrhea virus infections has been reported by numerous workers including Olafson et al. (1946), Ramsey and Chivers (1953), Baker et al. (1954), Pritchard et al. (1955), Seibold (1956), Carlson et al. (1957), Trapp (1960), Peter et al. (1967), Jubb and Kennedy (1970), and Thomson (1972). It has been found that, while most cattle develop antibodies to BVD virus without clinical manifestations of disease, a relatively small (2-50 percent) morbidity rate is noted. Experimental infection generally results in a quiescent manifestation with possible nasal discharge, a biphasic febrile response 2-4 and 6-8 days following inoculation and leucopenia following or coinciding with febrile spikes. All ages are susceptible to natural infection although clinical illness is most commonly evident in animals 6-18 months of age with those 10-15 months of age having the most severe lesions. Infection of the fetus may result in abortion or other sequelae dependent upon fatal age and immunocompetence (Casaro et al. 1971). Animals which develop severe diarrhea generally die. Lesions observed in such animals include isolated pinpoint to almost complete
erosion of the epithelium in the oral cavity with lesions being most extensive on the soft palate. Similar lesions are noted in the esophagus, being most pronounced in the distal portion. Erosions extending over pillars are noted in the rumen. The abomasal mucosa is commonly slightly hyperemic with diffuse petechial hemorrhages and larger shallow ulcerations. Catarrhal enteritis may involve the entire length of the small and large intestine but is most severe in the ileum which has the greatest area of Peyer's patches. Peripheral and mesenteric lymph nodes may be enlarged and have depletion of germinal centers with neutrophil and eosinophil infiltration. The spleen may also have similar lesions. Peyer's patches become clearly visible from the serosal side and hyperemic as viewed on the luminal surface. These lymphoid centers become necrotic and may become cryptlike if healing occurs.

Endothelial damage results in submucosal edema in equine arteritis and hog cholera (Estes 1972; Jubb and Kennedy 1970). Infarction occurs with mucosal disease but appears to result from secondary mycotic invasion and thrombosis (Kent and Moon 1973). Endothelial cell damage does not appear to be consistently present with BVD-MD infection as with equine arteritis and hog cholera.

Leucopenia (Peter et al. 1967; Baker et al. 1954) and depression of lymphocyte functions are other common sequelae to BVD infection (Muscoplat et al. 1973a; Pospisil et al. 1975a; Reggiardo 1975). Muscoplat et al. (1973a) and Pospisil et al. (1975a) found that lymphocytes infected with BVD virus in vivo or in vitro were less responsive
to phytohemagglutin (PHA) than noninfected lymphocytes. In addition Muscoplat et al. (1973b) found that immunoglobulin bearing cells were also reduced in infected versus noninfected cattle indicating B cell as well as T cell suppression. Since transformation due to PHA is primarily an indicator of T cell response, it is postulated that cell mediated responses would be reduced in BVD infected cattle. Macrophages as well as lymphocytes support replication of BVD viruses and therefore may be less effective in immune responses (Truitt and Shechmeister 1973). These facts appear to give a basis for the altered immunological responses noted by Peter et al. (1967) and McKercher et al. (1968). Chronic infections may occur in which virus can continually be isolated from the blood (Malmquist 1968). Such a tolerent state may result from elimination of T cell populations which would react against the virus and may also allow secondary bacterial invasion.

The predilection of BVD virus for the lymphoid tissues and the gastrointestinal tract results in infections which lend themselves to extensive study of the immunological response within the intestine. The goal of this study was to evaluate the pathological as well as the immune response and to identify target cells in the intestinal mucosa.
MATERIALS AND METHODS

Experimental Animals

Two Holstein-Friesian (#1 and #2) and one Ayrshire (#3) bull calves which were free of serum neutralizing antibodies to BVD virus by standard serological methods, were isolated from other cattle during the period of the study. A Thiry fistula was surgically established from a section of the ileum of #1 when 6 months of age. Single Thiry-Vella fistulae were established with ileal segments of #2 and #3 when approximately 10 months old. Bulls #2 and #3 were subsequently infected with BVD virus while #1 served as a control.

Two heifer (#35 and #555) and one steer (#118) Hereford calves were purchased from a commercial herd following weaning. Thiry-Vella fistulae were established with ileal segments of each of these calves. Prior to purchase all calves were serologically negative for BVD virus neutralizing antibody. Calves #35 and #556 were serologically negative following surgery while #118 had increasing titers to BVD virus. Heifer #35 was experimentally infected while #118 served as a naturally infected control in the recovering state and #556 served as a negative control.

Two colostrum-deprived Holstein-Friesian bull calves (#4 and #5) were obtained from a commercial dairy farm soon after birth and raised in isolation for 7 months prior to experimental infection. One liter of colostrum from a closed herd with no history of recent BVD virus infection was fed to each calf at its first feeding. They were subsequently fed powdered milk. Low serum virus neutralizing titers were detected
in sera collected when 2 days of age but were undetectable three months prior to and one day following infection. A reenterable laparotomy was conducted on both of these animals to allow temporary ligation of a section of the ileum for infection and subsequent biopsy.

Surgical procedures

The Thiry and Thiry-Vella fistulae were established by Dr. G. M. Shires, and surgical teams using the procedure described by Markowitz et al. (1964). Anesthesia was maintained with Halothane while the animals were maintained on their left sides for surgery. A single vertical incision was made in the upper right flank region through which a portion of the ileum was exteriorized. A segment of ileum, approximately 30-45 cm long with its blood supply was separated from the intestine for fistulation. The anterior and posterior portions of the intestine were anastomosed and returned to the peritoneal cavity. Two additional openings were then made in the abdominal wall lateral to the initial incision. Each end of the isolated intestinal segment was sutured to a flange of teflon gauze embedded in the middle of a 7 cm long cannula made of Slygard encapsulating resin (Figure 1). The lower end of the cannula was secured within the intestinal lumen. Each cannula was then passed into the peritoneal cavity and the upper end passed through an opening

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1 Ayerst Laboratories Incorporated, New York, New York.
2 V. Mueller Hospital Supply Corporation, Rochester, Minnesota.
3 EnSCO Distributing Corporation, St. Louis, Missouri
Figure 1. Components, mold and finished cannula used to retain open ends of Thiry-Vella fistulae.

A. Slygard resin
B. Curing agent to cause resin to develop relative firm state
C. Diluent used to reduce viscosity of resin
D. Mold for retaining teflon gauze and resin during curing
E. Teflon gauze
F. Finished cannula with embedded skirting of teflon gauze

Figure 2. Implanted cannulas, the lower one third of which were inserted in and retained the opening of the intestinal segment forming the Thiry-Vella fistula. Secretions and biopsies were readily obtained by passage of a biopsy instrument through the cannulas.
lateral to the initial incision. Additional sutures secured the intestine and teflon gauze to the abdominal wall. Following local application of approximately 5 gm chlortetracycline hydrochloride powder the incision was closed thus completing the surgical procedure (Figure 2). The newly formed fistulae were then flushed with sterile physiologic saline solution (PSS).

Post surgical care included intramuscular injections containing 12,000,000 IU penicillin G and 1.5 gm dihydrostreptomycin twice daily for four days. Fistulae were flushed with PSS the day following surgery and every 2 or 3 days thereafter.

Laparotomies on bull calves #4 and #5 were conducted by Dr. Peter South, a member of the University of Idaho Veterinary Science faculty. General anesthesia was accomplished with Rompun. Local anesthesia was produced initially and prior to reentry by injection of 2% lidocaine without epinephrine. A vertical incision approximately 15 cm in length was made in the upper right flank area. A portion of the ileum was brought to the incision site and ligated with sterile 0.5 cm 0.0 latex tubing. Two such ligations were made approximately 38 cm apart including a portion to which a strong blood supply was evident. A wedge shaped biopsy was removed anterior to the ligated portion. The section between ligatures was injected with a suspension of BVD virus and returned to the peritoneal cavity while the surgical procedure was

1 Haver-Lochart, Bayvet Div., Cutter Laboratories, Shawnee, Kansas.
continued. A portion of sterile latex glove was inserted through the incision to prevent reattachment of the opposing sides. The portions of latex remaining externally were laid flat vertically against the flank area. A 5x21 cm section of sterile Velcro hook material\(^1\) was sutured on each side over the latex material (Figure 3). Thirty minutes after inoculation the ligatures were removed from the gut and a section of sterile umbilical tape was tied loosely around the site of the previous anterior ligature so the same section could be retrieved for subsequent study. Approximately 225 ml of a 0.2% solution of nitrofurazone\(^2\) in Ringer's solution was placed in the cavity. Temporary closure was accomplished by close apposition of the Velcro strips on each side of the incision and binding these together with a 5x21 cm strip of Velcro loop material (Figure 4). The surgical area was covered with a sterile 46x56 cm surgical pack which was held in place by adhesive tape and tightly wrapped bandages.

The incision was reopened 24 hours and again 72 hours following surgery, the injection site was examined and biopsies were taken from within the formerly ligated area. No additional samples were taken due to the deteriorating condition of the animals. After the final biopsy was obtained, the Velcro strips and latex glove material were removed and the incised tissues were sutured together. Daily 15 ml

\(^1\)Velcro USA, Inc., Manchester, New Hampshire.

\(^2\)Med-Tech, Inc., Elwood, Kansas.
View of reenterable laparotomy as Velcro loop (A) material is pulled from opposing sides of two strips of hook material (B). The hook material was sutured to the skin over flat latex material which prevented contact between the apposing sides of the major incision.

Closed surgical site of reenterable laparotomy. Velcro loop strip pressed over the apposing edges of strips Velcro hook material provided adequate support for temporary closure.
intramuscular injections of Combiotic\textsuperscript{1} containing 200,000 units penicillin G and 250 mg dihydrostreptomycin per ml were given for 1 week following surgery.

\textbf{Inocula preparation}

A field strain of bovine viral diarrhea (BVD) virus designated 74-1015 was used to inoculate experimental animals. This isolate had been obtained from the tissues of an animal submitted to the Iowa State Veterinary Diagnostic Laboratory. Inoculation of this virus into cattle in previous studies elicited BVD virus neutralizing antibody production.

Secondary monolayers of embryonic bovine testicle (EBTe) cells were seeded with the BVD 74-1015 strain from its 3rd and 4th tissue culture passages. Cytopathic effects (CPE) on monolayers began to develop between the 3rd and 5th postinoculation (PI) days. On the 10th PI day tissue cultures were rapidly frozen and thawed twice at -70 C and 37 C respectively, the cell suspension was decanted into sterile centrifuge tubes and centrifuged at 150 x g for 10 minutes at 4 C. The supernatant was frozen at -70 C in 10 ml aliquots for subsequent inoculation of test animals. The concentration of virus in the suspension was estimated to be $10^{4.5}$ tissue culture infective doses (TCID) per ml by microtiter titration procedures.

Inoculum for calves \#4 and \#5 consisted of 5th passage BVD 74-1015 virus which had been propagated in EBTe cells just prior to inoculation. The virus titer as determined by microtitration was

\textsuperscript{1}Pfizer, Inc., New York, New York.
approximately the same as that for the harvested 4th and 5th tissue culture passages.

Inoculum for control animals #118 and #556 was prepared from noninoculated EBTe monolayers processed as above. The supernatant fluid was frozen at -70°C in 10 ml aliquots prior to use.

**Experimental infection**

Animals #2, #3, and #35 were inoculated with 10 ml of the BVD 74-1015 virus suspension on post-surgical day 30, 25, and 7 respectively. The inoculum was placed directly in the lumen of the Thiry-Vella fistulae immediately after flushing with PSS. Control animals #118 and #556 were similarly inoculated with tissue culture supernatant fluid from noninfected EBTe monolayers on post-surgical days 9 and 11 respectively.

Ten milliliters of viral suspension was injected into the lumen of the ligated ileum of calves #4 and #5. The intestinal contents had been partially removed by gently passing the section of intestine between two fingers prior to ligation. The viral suspension was retained within the section for 30 minutes to allow adsorption prior to removal of the ligatures.

**Collection of data and samples**

Rectal temperatures were measured twice daily (between 6:00 and 9:00 a.m. and 5:00 and 8:00 p.m.). Total and differential peripheral blood leucocyte counts were determined daily during the first 5-7 days postinoculation (PI) and every 2-3 days following to 14 days PI.
Blood was collected without the use of anticoagulants for lymphocyte transformation studies and virus neutralization tests. The blood was defibrinated according to the method of Kay and Kaeberle (1972) and the cellular and fluid portions separated by centrifugation at 750 x g for 40 minutes in a swinging bucket-type centrifuge. Buffy coat layers of leucocytes were obtained from blood collected with ethylenediaminetetraacetate (EDTA) for virus isolation.

Blood was also collected for bacteriological culture. Following removal of the hair overlying the jugular vein the area was sponged with 70% alcohol and 2% tincture of iodine. Approximately 5 ml of freshly collected blood was transferred to each of two vacuum bottles which contained 50 ml of Columbia broth\textsuperscript{1} with added CO\textsubscript{2}. In addition one of the vacuum bottles contained sodium polyanethol sulfonate (SPS) which is anticomplementary and interferes with bactericidal activity of blood.

Fistular secretions were collected periodically for evaluation of immunoglobulin and specific BVD virus neutralizing antibody levels. Biopsies were obtained from the luminal surface of the fistulae using a Quinton Multi-purpose Suction Biopsy Instrument\textsuperscript{2} fitted with a capsule designed for collection from animals with thick mucosa. The capsule has one side hole with a diameter of 3.18 mm into which the

\textsuperscript{1}Difco Laboratories, Detroit, Michigan.

\textsuperscript{2}Quinton Instrument Company, Seattle, Washington
tissue is pulled by approximately 12 pounds of vacuum before activation of the enclosed knife. Biopsies were immediately placed into cold 2.5% glutaraldehyde in 1.0 M phosphate buffer (PB) pH 7.4. After a 15-30 minute period for initial fixation the biopsies were cut into blocks approximately 2.0 mm$^3$ and transferred to fresh glutaraldehyde where they remained for 24 hours at 4 C. The fixative was then washed off and replaced with cold PB. Biopsies were maintained in cold PB until processed in the laboratory. Biopsies were taken from animals #4 and #5 by cutting wedge sections approximately 1 x 0.5 cm from the serosal surface into the lumen. These samples were processed by the same procedures as those obtained from fistulae.

Laboratory Procedures

Preparation of special reagents

Bovine immunoglobulins were prepared according to the procedure of Mach and Pahud (1971). Saliva collected without chemical stimulation served as the source of secretory IgA. IgM and IgG were isolated from blood serum. Antiserum was produced against each immunoglobulin in rabbits by standard procedures and was purified by adsorption with fetal calf serum and specific bovine immunoglobulin fractions.

Specific anti-BVD virus antibody was obtained from bovine serum with a neutralizing titer of 64. The immunoglobulin portion of the serum was precipitated by 50% saturation with ammonium sulfate. The precipitate was dissolved and reprecipitated once with ammonium sulfate to remove unreacted proteins. The precipitate was dissolved in PSS
and extensively dialyzed against phosphate buffered saline (PBS) pH 7.2. Subsequent dialysis was conducted against 0.3 M borate buffer pH 8.0. An affinity column of cyanogen bromide activated 4B Sepharose prepared by the method of Rejnek et al. (1969) to which BVD viruses were bound, was used to adsorb specific antibodies from the serum globulin fraction. The column was then flushed with borate buffer until the absorbancy of the effluent at 280 nm was essentially nil. Adsorbed antibodies were then eluted with 0.05 M pH 2.4 glycine-HCl buffer into a flask containing 5% ammonium bicarbonate solution. The final eluant was concentrated using negative pressure dialysis and dialyzed against PBS. Virus neutralization tests indicated the continued activity of serum fractions following elution from the column.

This procedure was repeated several times to acquire an adequate amount of purified antibody. The virus specific antiserum fractions were pooled and concentrated for subsequent procedures.

Portions of the above antisera specific for BVD virus and the various bovine immunoglobulins were conjugated with horseradish peroxidase (RZ3) (15 mg peroxidase per 5 mg serum protein) by the method of Avrameas and Ternynck (1971). These reagents were subsequently used to label the specific antigens in intestinal tissue.

Hematology

Blood was collected into tubes containing EDTA. Smears were made from the blood when it arrived in the laboratory and stained

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1 Sigma Chemical Company, St. Louis, Missouri.
with Wright's\(^1\) stain for differential evaluations. Total leucocyte counts were conducted by standard procedures using a hemocytometer.

**Serology**

Blood serum was tested for the presence of virus neutralizing antibodies in a microtiter system after the procedure of Jenney and Wessman\(^2\). Fistular secretions were passed through a 0.22 micron filter\(^3\) prior to testing so as to eliminate bacteria. However, fistular secretions prevented the formation of monolayers and were unsuitable for the above technique. Therefore, these secretions were tested for virus neutralization in 15 x 60 mm tissue culture plates in which a monolayer of bovine turbinate cells had previously been established. A 50% plaque reduction (TCPR\(_{50}\)) compared to control plates was interpreted as the end point. The reciprocal of the highest dilution in which 50% reduction occurred was expressed as the titer. All samples were tested in duplicate and a control in which virus was not included was tested simultaneously to test toxicity.

**Quantitation of immunoglobulins**

Radial immunodiffusion tests were conducted to quantitate the various immunoglobulins present in fistular secretions. Specific antiserums to bovine IgA and IgM prepared as discussed above were

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\(^1\)Sigma Chemical Company, St. Louis, Missouri.


\(^3\)Millipore Corporation, Bedford, Maryland.
diluted and added to agar at 45°C according to established procedure (Mancini et al. 1965). The agar was poured on warm agar coated 8.3 x 10.3 cm glass plates and cooled. Wells with a 3 mm inside diameter were then cut in the agar and 10 microliters of the secretion was added. Immunodiffusion kits specific for bovine IgG1 and IgG2 were obtained from a commercial source and separate dilutions of serum containing known quantities of the specific immunoglobulin were added to wells on the plates to serve as controls. The plates were incubated in a moist chamber at room temperature for 24 hours for IgM and at 4°C for 16-20 hours for IgG1, IgG2, and IgA and the diameter of the subsequent band of precipitation was measured. These measurements were compared to the measurements for known quantities of immunoglobulins and the unknown values were extrapolated.

**Virus isolation procedures**

Embryonic bovine testicle (EBTe) cultures in the 2nd to 5th passage were used for all virus isolation procedures. Eagle minimal essential medium (MEM) with 100 units penicillin, 100μg of streptomycin and 0.25μg of Fungizone per ml of medium was used for all tissue cultures. Monolayers were established using medium with 10% virus screened fetal calf serum (FCS). The concentration of calf serum was reduced to 2% following inoculation with specimens for virus isolation.

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1. Miles Laboratories, Inc., Elkhart, Indiana.
2. Grand Island Biological Company, Santa Clara, California.
Buffy coats obtained from blood collected with EDTA were washed twice with sterile physiological saline (0.85% NaCl). The washed cells were then frozen at -70 C and thawed at 37 C twice to aid in release of virus from infected lymphocytes. Each specimen was suspended in 1.0 ml of MEM with 2% FCS and placed on a monolayer in 25 cm² flasks.

The cellular components collected with fistular secretions were centrifuged down and, after the supernatant fluid was removed, were stored at -70 C until virus isolation procedures could be conducted. When these samples were processed for virus isolation, MEM with 2% FCS was added and the suspension agitated vigorously. Following centrifugation at 750 x g for 10 minutes the supernatant fluid was collected and filtered through 0.22 micron pore size Millipore membranes.¹ A 0.5 to 1.0 ml portion of filtrate was placed on EBTE monolayers in 25 cm² flasks. Following a 1 hour incubation period at 37 C, 5 ml of MEM with 2% FCS was added. Tissue cultures were maintained for 7-10 days PI and viewed daily with an inverted microscope for cellular alterations from day 2 to the conclusion of incubation period. Each culture was then frozen and thawed before material was transferred to additional monolayers. Subsequent tissue culture passages were conducted by transferring 0.5 ml of the tissue culture suspension to a fresh EBTE monolayer. Each specimen was taken through 3 such tissue culture passages. Monolayers without any inoculation and others inoculated with the NADC BVD-MD virus were concurrently studied with each passage of test materials.

¹Millipore Corporation, Bedford, Maryland.
A final tissue culture passage of the above material was conducted in duplicate on glass coverslips in Leighton tubes. Noninfected and NADC and 74-1015 BVD virus infected monolayers were also prepared. These were harvested on PI day 5 and following a brief wash in PBS were fixed in acetone. One set of coverslips was stained with fluorescein isothiocyanate-conjugated anti-BVD virus serum¹ by standard procedures. These coverslips were evaluated with a Leitz Wetzlar² microscope equipped with ultraviolet light source. The second set of coverslips was stained with peroxidase-conjugated anti-BVD serum. The peroxidase conjugate was reacted with the acetone fixed monolayers for 18-24 hours at 4°C in a moist chamber. The coverslips were washed in four one minute and one five minute washes of PBS to remove unbound conjugate. They were then allowed to dry and were overlayed with Karnovsky's solution (15 mg of 3-3' diaminobenzidine in 20 ml 0.05 M TRIS-HCl buffer pH 7.6 with H₂O₂ added immediately before use to give a 0.003% H₂O₂ solution) for 10 minutes. The Karnovsky's solution was washed off with a brief rinse in PBS and a final distilled water rinse and the coverslips were allowed to dry at room temperature. Mounting medium was used to fix the coverslips to microscope slides. After the mounting medium was dried, the cells were evaluated by light microscopy at 400X.

¹Anti-BVD FA conjugate obtained from Veterinary Services and Diagnostic Laboratory, NADC, Ames, Iowa.

²Ernst Leitz, Wetzlar, Germany.
Bacteriological cultures

Columbia broth blood culture bottles were incubated at 37 C following addition of blood. At 24 and 72 hours of incubation approximately 0.5 ml of broth was removed and transferred to the surface of blood agar (5% citrated bovine blood in trypticase soy agar) in two 100 mm petri plates. The broth was spread over the surface of the plates; one plate was incubated at 37 C in a candle jar to provide an atmosphere of reduced O₂ and increased CO₂ while the other was incubated at 37 C in a conventional bacteriological incubator. Isolated bacteria were identified by Dr. Roger Hogle.

Lymphocyte transformation studies

Blood was aseptically collected from the jugular vein into sterile 60 ml syringes and immediately transferred to sterile Erlenmeyer flasks fitted with screw caps. Prior to sterilization 7 applicator sticks bound in a cone shape were placed in each flask for defibrination (Kay and Kaeberle 1972). Defibrination of the blood was accomplished by rapidly rotating the flask for 5 to 10 minutes following collection. The flasks were then allowed to set for approximately 10 minutes and the defibrinated blood was decanted into sterile 50 ml screw-cap tubes in which it was centrifuged for 40 minutes at 750 x g in a swinging bucket-type centrifuge. The buffy coat was aseptically removed and suspended in two volumes of Hanks' balanced salt solution (HBSS). The lymphocytes were separated from this suspension by layering 8 ml

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1Grand Island Biological Company, Santa Clara, California.
onto 4 ml of Ficoll\textsuperscript{1}-Hypaque\textsuperscript{2} mixture with a specific gravity of 1.080 and centrifugation at 400 x g for 30 minutes. The mononuclear cells which were layered at the interface of the solutions following centrifugation were removed with a sterile pipette and added to 5 volumes of HBSS. Cells were sedimented by centrifugation at 200 x g for 10 minutes, and resuspended in 5 ml of fresh HBSS. Following a final centrifugation at 200 x g for 10 minutes, cells were suspended in 1 ml of M199 medium\textsuperscript{3} containing 20% fetal calf serum and antibiotics (100 units of penicillin,\textsuperscript{3} 100\mu g streptomycin,\textsuperscript{3} 0.25\mu g fungizone\textsuperscript{3} and 0.5 mg gentamycin\textsuperscript{3} per ml of medium). After the cells were enumerated, the concentration was adjusted with M199 so that 1.5 ml medium contained approximately $2 \times 10^6$ lymphocytes and that quantity was transferred to sterile tubes to which was added 0.1 ml of a phytohemmagglutinin-P\textsuperscript{4} (PHA-P) dilution. Duplicate tubes without PHA-P were set up for controls.

The lymphocyte cultures were incubated at 37 C for 48 hours in an atmosphere containing 5% CO\textsubscript{2}. At the end of this period, 0.1 ml of tritiated thymidine\textsuperscript{5} ($^{3}$H-thymidine) was added to all of the tubes. Following 20-24 hours further incubation at 37 C, the cells were

\textsuperscript{1}Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey.
\textsuperscript{2}Winthrop Laboratories, New York, New York.
\textsuperscript{3}Grand Island Biological Company, Santa Clara, California.
\textsuperscript{4}Difco Laboratories, Detroit, Michigan.
\textsuperscript{5}New England Nuclear Corp., Boston, Massachusetts.
harvested by centrifugation and precipitated with trichloracetic acid. The precipitate containing the nucleic acid was dried and dissolved in Soluene-100\(^1\) which was then transferred to scintillation vials with 10 ml of scintillation fluid (160 ml Permaflour\(^1\) in 3.78 liters of toluene). Emissions of beta particles from \(^3\)H thymidine incorporated in nucleic acids in each culture were counted for a 10 minute period in a Packard Liquid Scintillation Counter.\(^1\)

**Histological studies**

Processing of glutaraldehyde-fixed intestinal biopsies included careful sectioning with a scalpel or razor blade to cut from the mucosal surface through the underlying connective tissue. A portion of the biopsy was then processed by standard dehydration and paraffin embedding procedures. Sections 4 \(\mu\)m thick were cut and stained with hematoxylin and eosin stain. These sections were evaluated by light microscopy with a magnification range of 60X to 400X.

Additional 1.5 mm sections of glutaraldehyde-fixed tissue were cut and washed in three changes of PB at 4 C. A portion of the biopsies at this stage were dehydrated with a graded series of ethanol and then transferred to absolute methanol for 15 minutes. Inactivation of endogenous peroxidase activity in some samples was attempted by a subsequent 15 minute treatment in absolute methanol containing 1\% sodium nitroferricyanide\(^2\) according to the method of

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\(^1\)Packard Instrument Company, Downers Grove, Illinois.

\(^2\)Sigma Chemical Company, St. Louis, Missouri.
Strauss (1971). Tissue fragments with and without peroxidase inactivation were incubated for 24 hours in peroxidase conjugates specific for bovine IgA, IgG and IgM and BVD virus. Unbound conjugates were removed by three 5 minute washes in PB and the tissues were post-fixed in 2.5% glutaraldehyde for 15 minutes followed by three additional 30 minute rinses in PB. A subsequent 30 minute incubation in Karnovsky's solution without H₂O₂ followed by 15 minutes in Karnovsky's solution with 0.003% concentration of H₂O₂ resulted in reaction with peroxidase and formation of optically detectable and electron opaque deposits. The tissue fragments were then washed in two 5 minute PB rinses to remove excess Karnovsky's solution and post-fixed for 1 hour in 2% osmium tetroxide in pH 7.4 phosphate buffer. Tissue fragments were then washed in three 5-10 minute changes of PB, dehydrated by standard procedures in an increasing concentration series of ethanol and embedded in an epon-araldite resin.

Thick (2 μm) sections of plastic embedded tissues were cut using a Sorvall Porter-Blum MT-2 ultra-microtome equipped with glass knives. These sections were spread on glass slides with water and allowed to dry. A coverslip was mounted over the dried sections with mounting medium. Sections were viewed by light microscopy at 400X and the areas of peroxidase positive staining were noted.

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1 Polysciences, Inc., Warrington, Pennsylvania.

Sections 80-100 nm thick were cut with glass knives on the Sorvall Microtome for electron microscopy. Sections were examined both without further staining and with double staining with uranyl acetate and lead citrate (Venable and Coggeshell 1965) in a Zeiss EM10 electron microscope\(^1\) using 80 KV.

\(^1\)Carl Zeiss, Inc., Oberkochen, West Germany.
RESULTS

Clinical Observations

A diphasic febrile response was noted in each animal experimentally inoculated with BVD virus. Febrile responses were noted in animal #2 on PI days 3, 6, 7, 9, and 12-14 (Figure 5). On day 11 PI this animal was observed to have a severe orchitis and a biopsy was taken from the left testicle. Animal #3 had acute febrile responses on PI days 3, 7, and 12 (Figure 6). The latter response was associated with acute tympany. Following treatment with a gas reducing agent _per os_ and 20 cc Combiotic, the temperature returned to normal and no further complications were noted until PI day 18 when a temperature of 104.3 was recorded. Orchitis with moderate swelling was evident on the following day and a treatment program of 8 cc Combiotic given twice daily was initiated. The rectal temperature had again returned to the normal range on PI day 22. The left testicle was removed on PI day 23 for bacteriological culture. Animal #35 had an elevated temperature on days 2-4 and again on PI days 7 and 8 (Figure 7). A mild febrile response was also observed on PI day 19 without other observable complications.

Animals #4 and #5 were specifically studied to determine if testicular involvement might again be associated with BVD virus infections of young bulls. No testicular involvement was grossly evident. Semen samples collected by electroejaculation and placed on EBTrs monolayers proved to be highly toxic for monolayers and resulted in complete
Figure 5. Graphs indicating total leucocyte percentages in relation to preinfecion levels, absolute lymphocytes/mm$^3$ of blood, lymphocyte transformation activity in presence of PHA and the rectal temperatures of animal #2 which was infected with BVD virus.
Figure 6. Graphs indicating total leucocyte percentages in relation to preinfection levels, absolute lymphocytes/mm³ of blood, lymphocyte transformation activity in presence of PHA and the rectal temperatures of animal #3 which was infected with BVD virus.
LYMPHOCYTE TRANSFORMATION ACTIVITY/DAY 0 ACTIVITY (x-x)

DAYS PRIOR TO AND AFTER BYV INOCULATION

RECTAL TEMPERATURE (0-0)

TOTAL LEUCOCYTES (% OF VALUE RECORDED ON DAY 0) (0-0)

ABSOLUTE LYMPHOCYTE NUMBERS (x-x)
Figure 7. Graphs indicating total leucocyte percentages in relation to preinfection levels, absolute lymphocytes/mm$^3$ of blood, lymphocyte transformation activity in presence of PHA and the rectal temperatures of animal #35 which was infected with BVD virus.
destruction of the cell layer in 30 minutes. Therefore, no conclusive evidence was obtained to determine the presence or absence of BVD infection involving the testicles of these animals.

A biphasic febrile response was noted in both #4 and #5 with peaks on PI days 3 and 4 and again on days 7-9 (Figure 8 and 9). No febrile responses were noted after that time through 3 weeks PI.

Animals #118 and #556 had elevated temperatures throughout the period of study (Figures 10 and 11). The temperatures were checked with two rectal thermometers to determine if readings were comparable. Temperatures for two other animals in the same section of the animal research facility were determined and found to be similarly elevated. The constant elevated temperatures may be reflective of a high ambient temperature.

None of the animals developed diarrhea. A small amount of blood tinged mucus was noted in the fecal material from animals #2 and #3 at 7-10 days and #556 at 7 days following inoculation. All experimentally infected animals were noted to have a dry unproductive cough, a condition which was most severe in #2. Animal #4 became depressed and showed moderate ataxia with anorexia during the 72 hours following surgery. Aminobolic boluses were given per os and approximately 5 liters of warm water were given by stomach intubation. On the 6th day following surgery soft feces were passed but true diarrhea was not observed.

Figure 8. Graphs indicating total leucocyte and absolute lymphocyte levels with febrile responses of animal #4 which was inoculated with BVD virus in a ligated intestinal loop on day 0.
The diagram shows the total leucocytes (% of value recorded on day 0) and rectal temperature over days prior to and after BVD inoculation. The y-axis represents the percentage of leucocytes, with values ranging from 0 to 110. The x-axis represents the number of days, ranging from -1 to 17. The rectal temperature is also plotted, with values ranging from 100 to 108. The graph indicates a significant drop in leucocytes and a rise in temperature after the BVD inoculation.
Figure 9. Graphs showing total leucocyte and absolute lymphocyte levels with febrile responses of animal #5 which was inoculated with BVD virus in a ligated intestinal loop on day 0.
RECTAL TEMPERATURE (°C)

TOTAL LEUCOCYTES (% of value recorded on day 0)

ABSOLUTE LYMPHOCYTE NUMBERS (x-x)
Figure 10. Graphs showing total leucocyte and absolute lymphocyte levels with febrile responses and lymphocyte transformation activity of animal #118 which was inoculated in intestinal fistulae with virus free tissue culture supernatant fluid.
RECTAL TEMPERATURE (°C - °C)

DAYS PRIOR TO AND AFTER BVD INOCULATION

LYMPHOCYTE TRANSFORMATION ACTIVITY/DAY 0 ACTIVITY (x-x)

TOTAL

LEUCOCYTES (% OF VALUE RECORDED ON DAY 0) (°-°)

ABSOLUTE LYMPHOCYTE NUMBERS (x-x)
Figure ??: Graphs showing total leucocyte and absolute lymphocyte levels with febrile responses and lymphocyte transformation activity of animal #556 which was inoculated in intestinal fistulae with virus free tissue culture supernatant.
Experimental Observations

Leucocyte evaluations

A diphasic leukopenia corresponding in time with early febrile responses was also noted in infected animals (Figures 5-9) and was most pronounced in animals #2 and #3. The percentages of polymorphonuclear (PMN) cells and lymphocytes varied considerably between animals so that no trend was observable based on these values. Bull #2 was observed to have 55% lymphocytes prior to infection and 90% between the first two febrile phases (i.e. on PI day 5). Absolute lymphocyte values however decreased moderately between these dates from 9,200 to 8,200 mm$^3$. Absolute lymphocyte values on PI days 0 vs 5 for animals #3 and #35 were 7,081 vs 4,160/mm$^3$ (59% of that detected on day 0) and 6,773 vs 5,206/mm$^3$ (77% of that detected on day 0) respectively and therefore obviously represented decreased absolute numbers. During this time period PMN values decreased from 5,880 to 549/mm$^3$ and 2,425 to 1,702/mm$^3$ in animals #2 and #3 respectively but increased from 622 to 1,702/mm$^3$ in animal #35 during a comparable period.

Total and differential leucocyte evaluations were not determined on blood from animals #4 and #5 until the second and first days respectively after inoculation. The lowest total number of leucocytes were observed on day PI 4 for #4 followed by a slow increase to PI day 8. Absolute lymphocyte numbers decreased from 3,298/mm$^3$ on PI day 2 to 2,800/mm$^3$ on PI day 4. The total leucocyte count dropped to 52% of day 2 values by day 4 with the greatest reduction in PMNs (Figure 6).
The lowest total leucocyte reduction in blood of #5 occurred on day 3 with the major cell type involved being the PMNs which had dropped to 55% of the PI day 1 value. Absolute PMN values were reduced further to 15% of PI day 1 values on PI day 7. Absolute values thus reflected for PMNs dropped from 5,544/mm³ on PI day 1 to 828/mm³ on PI day 7. However, the cell type having the greatest reduction on PI day 4 was the lymphocyte at 28% of the PI day 1 value. Absolute values for lymphocytes then increased to values greater than those observed on PI day 1 and remained high during the remainder of the study (Figure 9).

Both total and differential leucocyte counts remained relatively constant for control animals #118 and #555 (Figures 10 and 11) during the time of study although #556 did have a drop of total leucocyte values from PI day 2 to PI day 4 reflecting approximately a 35% reduction. This corresponded in time with a slight febrile response. A second febrile response was noted in this animal on PI day 8 but no corresponding depression of leucocytes was observed.

**Lymphocyte transformation**

A reduced transformation response of lymphocytes was detected in infected animals. The greatest degree of depression compared to activity on the day of inoculation was noted on PI day 5 (9 fold) for #2, PI day 3 (5 fold) for #3, and PI day 8 (3 fold) for #35. Lymphocytes from control animals also had depressed transformation indices as indicated in Figures 10 and 11 but were of a lower magnitude than that observed in infected animals. Lymphocyte transformation studies were not conducted on cells from animals #4 and #5.
**Virus isolation**

Tissue culture monolayers which were inoculated with fistular material underwent moderate cellular changes during the first passage presumable due to cytotoxicity. These changes included granular appearances of cells and a more rounded outline of individual cells although cells remained attached to the culture flask. These cellular alterations were not evident in tissue passages 2 through 4 for the majority of samples.

A relatively small number of tissue culture monolayers developed typical cytopathic effects (CPE) characterized by elongated areas from which cells had detached from the flasks. The ISU-74-1015 virus strain used for experimental infection of fistulae does not produce as rapid or marked CPE as does the NADC strain. Both of these strains were included as controls for evaluating test materials with fluorescent antibody and peroxidase antibody staining and proved to give equivalent results.

The results of coverslip monolayers inoculated with test material originating from buffy coats and fistular secretions and stained with the conjugates are presented in Table 1. Of the 20 buffy coat samples obtained from animals #2, #3, and #35 and tested for BVD virus using both immunofluorescent (IF) and immunoperoxidase (IP) conjugates on monolayers, 11/20 (55%) were positive with one or both stains. A greater number, 11/14 or 79%, of monolayers inoculated with fistular samples were positive with the conjugates. The lowest incidence of viral isolation from experimentally infected animals was from animal
Table 1. Isolation of virus from blood buffy coats and fistular secretion samples of cattle inoculated with BVD virus as indicated by immunofluorescence (IF) and immunoperoxidase (IP) stains

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Days PI</th>
<th>Buffy Coat</th>
<th>Fistular Secretions</th>
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</table>

<sup>a</sup>Animals #2, #3 and #35 inoculated with virus within Thiry-Vella fistulae. Animals #4 and #5 inoculated with virus in ligated section of ileum. Animals #118 and #556 were sham inoculated into Thiry-Vella fistulae.

<sup>b</sup>PI, indicates the number of days postinoculation.

<sup>c</sup>IF, test results with immunofluorescent conjugated anti-BVD serum.

<sup>d</sup>IP, test results with immunoperoxidase conjugated anti-BVD serum.

<sup>e</sup>NT, samples were not collected or were not tested for indicated PI day.
Table 1 (continued)

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Days PI</th>
<th>Buffy Coat</th>
<th>Fistular Secretions</th>
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<td></td>
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<td>NT</td>
</tr>
<tr>
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<tr>
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<tr>
<td></td>
<td>4</td>
<td>-</td>
<td>NT</td>
</tr>
</tbody>
</table>
#2 which had 1/5 buffy coat cultures in which BVD virus was detected although 4/5 fistular samples had detectable BVD virus. Animal #3 had 4/6 buffy coat samples and 3/5 fistular samples in which BVD virus was detected while #35 had 5/8 buffy coat and 4/4 fistular samples which were BVD virus positive. Virus was not demonstrated by any of the test procedures in monolayers inoculated with either buffy coat cells or fistular material from control animals #118 and #556. Buffy coat samples only were cultured for BVD virus from animals #4 and #5 and resultant coverslip monolayers were tested with IF conjugates only. Both animals #4 and #5 had 3/7 postinoculation buffy coat samples which were positive for BVD virus.

Additional samples from which virus isolation was attempted were testicular tissue fragments surgically removed from #2 on PI day 12 and testicular fluid collected on PI day 16. Monolayers inoculated with testicular fragments after four tissue culture passages were positive with both the peroxidase and fluorescein conjugates. No viral antigens were demonstrated in monolayers inoculated with testicular fluid from this animal or semen from animals #4 and #5.

**Bacteriological cultures**

Blood was cultured from animals #1, #2, #3, #35 and #556 following BVD virus inoculation. Four blood agar (BA) plates were inoculated with culture medium (Table 2). Bacteria were present on 1/12 and 0/4 plates inoculated from control animals #1 and #556 respectively. The organism in these cases were *Bacillus*
Table 2. Results of blood cultures from control and virus inoculated cattle following inoculation into broth with and without SPS

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Day PI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CB with CO&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CB with CO&lt;sub&gt;2&lt;/sub&gt; and SPS&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>CO&lt;sub&gt;2&lt;/sub&gt; BAP</td>
<td></td>
</tr>
<tr>
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<td>CO&lt;sub&gt;2&lt;/sub&gt; BAP</td>
<td>Aerobic BAP</td>
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<td>Bacillus</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
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<tr>
<td></td>
<td>5</td>
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<tr>
<td></td>
<td>7 Bacillus</td>
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</tr>
<tr>
<td></td>
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<td>NG</td>
</tr>
<tr>
<td></td>
<td>6 NG</td>
<td>NG</td>
<td>Micrococcus</td>
</tr>
</tbody>
</table>

<sup>a</sup>Days postinoculation.

<sup>b</sup>Columbia broth with CO<sub>2</sub> added. (Difco)

<sup>c</sup>Columbia broth with CO<sub>2</sub> and sodium polyanethol sulfonate (SPS) added. (Difco)

<sup>d</sup>Blood agar plates were inoculated from broth and incubated aerobically and in CO<sub>2</sub>.

<sup>e</sup>Animals #1 and #556 served as controls, #1 was not inoculated, #556 was sham inoculated with virus-free tissue culture preparation.
and considered a contaminate. *Bacillus* was also isolated from animal #2 on PI day 7 on both BA plates set up from the Columbia broth bottle without SPS added, but not from SPS-Columbia broth. A *Bacillus* was also isolated from animal #3 at 5 days PI and was isolated from both SPS and non SPS containing bottles. On PI day 9 an alpha hemolytic *Streptococcus* was isolated on all 4 BA plates from animal #3. Four blood cultures from animal #35 were negative for bacteria on all BA plates. *Micrococcus* was isolated from broth containing SPS from this animal on the fifth culture conducted on PI day 7.

Cultures of the testicular biopsy taken from animal #2, 12 days PI yielded no bacteria; however, *Pasteurella multocida* in pure culture was isolated from the testicle following surgical removal on PI day 16.

**Virus neutralizing tests**

All animals experimentally infected with BVD virus were free of neutralizing antibodies in their serum to this virus at the time of inoculation. Animals #1, #2, and #3 had respective titers of 4, 4 and 2 at approximately 4 months of age. These antibodies were assumed to be of maternal origin and were no longer detectable at the time of surgery. Animals #35, #118, and #556 were seronegative for BVD at the time of purchase. Following purchase recorded titers were 8, 128, and 64 respectively.

Neutralization tests were conducted repeatedly on all animals following inoculation. The results of these tests are recorded in Table 3. Serum antibodies were first detected at 11 days PI in
Table 3. Virus neutralizing antibodies against Bovine Viral Diarrhea Virus (BVD) in serum of cattle inoculated with BVD (#2, #3, #4, #5, and #35) naturally infected (#118) and noninfected control (#556)

<table>
<thead>
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<th>Animal No.</th>
<th>Age (days)(^{a})</th>
<th>VN(^{b}) Titer</th>
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<td>&lt;4</td>
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<tr>
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\(^{a}\)Age from birth when dates were known and day postinoculation (PI) following inoculation.

\(^{b}\)Virus neutralization titer of serum.
animal #2 and 15 days PI in #35 while no antibodies were detected in serum from calf #3. High serum titers of 512 and 128 were present in animals #4 and #5 respectively when tested at 21 and 20 days PI.

Although numerous fistular samples were collected the quantity was not sufficient in all cases to permit the necessary centrifugation, filtration, and quantitative studies desired. Increasing titers were demonstrated in fistular secretions from experimentally infected animals (Table 4). Virus neutralization was evident in the 1:4 dilution of the earliest samples collected from calves #2 and #3 but was not demonstrated in samples taken at 2 and 8 days PI, respectively. Possible reasons for these variances will be discussed later. The time required for local antibody production varied between animals. The most rapid production was demonstrated in the secretions of animal #35 at 7 days PI (8 days earlier than first detectable serum antibodies) while 12 days (comparable to 11 days for detectable serum antibody production) were required for animal #2. It is of interest to note that animals with fistulae of longest duration required a slightly longer time for evidence of antibody formation. The titers of animals #2 and #35 were already decreasing by 20-23 days PI but were still at the maximum detected in secretions from #3.

The neutralizing titers in fistular secretions from #118 remained constant over the time tested and were at a level which might be expected in the serum of a convalescent animal. No neutralizing antibodies were detected in secretions from #556.
Table 4. Virus neutralizing activity against bovine virus diarrhea virus in secretions from Thiry-Vella fistulae secretions of cattle

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Days PI&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup>Days postinoculation.

<sup>b</sup>Titer is the reciprocal of highest dilution of secretion capable of neutralizing 50% or more of virus in test system.

<sup>c</sup>Animals were inoculated in Thiry-Vella fistulae with BVD virus (#2, #3 and #35) or virus-free material (#118 and #556).
Quantitation of immunoglobulins in fistular secretions

Radial immunodiffusion assays were conducted to determine the quantity of immunoglobulins IgA, IgG_1, IgG_2, and IgM (Table 5).

IgA values varied from 48 to 350 mg/100ml. Values appeared to decrease following virus introduction into the lumens of fistulae. The lowest values observed in animals #2 and #3 were on PI day 2. A second depression of IgA values was observed at 10 days PI in animal #3. Animal #35 had low IgA values from PI day 1 through 10.

Increasing IgA levels were observed in fistular secretions of the naturally BVD infected control animal #118. This animal also had increasing serum virus neutralizing antibodies during this time. Animal #556 did not have virus neutralizing antibodies in either serum or fistular secretions subsequent to tests conducted at the time of transfer to the university facilities. The IgA level in the fistular secretion collected on the day of inoculation was 125 mg/100ml, but subsequently fell to approximately half that value until PI day 11 when 125 mg/100ml was again present. Fistular secretions from animal #1 were not tested due to the duration (6 months) of the fistula and its apparent deterioration judged by the cell mass present with the secretions.

IgM values remained relatively stable in the fistular secretions of all animals with values ranging from 30 to 120 and a mean of 53 mg/100ml. Two samples with IgM values of 92 and 120 mg/100ml were collected at 13 and 11 days PI from animals #3 and #35 respectively.
Table 5. Immunoglobulin values (mg/100ml) in secretions from Thiry-Vella fistulæ of cattle inoculated intrafistularly with bovine virus diarrhea virus suspensions or virus-free cell culture material

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<sup>a</sup>Days postinoculation.

<sup>b</sup>Animals #2, #3, and #35 were inoculated with BVD virus in the lumen of the fistulæ. Animals #118 and #555 received similar inoculation with virus free cell culture medium.
Values for IgG₁ and IgG₂ varied considerably during the time of testing with lowest levels occurring at approximately 13-15 days PI in infected animals and highest values at 2-7 days PI in different animals. These values are not considered as absolute due to the enzyme sensitivity of IgG₁ and IgG₂ in intestinal secretions.

Too few animals and observations of variables were included for ideal evaluation for significant factors. However, when the values for immunoglobulins were evaluated with time, total leucocyte, PMN, and lymphocyte numbers and lymphocyte transformation activity for Pearson correlation coefficients it was observed that a highly significant ($P > 0.0001$) positive correlation existed between IgG₁ and IgG₂ while an inverse correlation ($P < 0.05$) existed between IgA and IgG₁ and IgG₂.

The immunoglobulin class of virus neutralizing antibodies was not determined for either serum or fistular secretions.

**Histological studies**

Tissues fixed with glutaraldehyde and subsequently impregnated with paraffin for sectioning gave excellent sections when stained with hematoxylin and eosin.

Sections of biopsies collected prior to inoculation of each animal were used as a basis to which sections of tissue collected following infection were compared. Photomicrographs of tissue from animals #2 and #3 will be used to demonstrate similar findings in all animals.
Sections of tissue collected from #2 at the time of surgery and three days prior to infection appeared normal with an intact epithelium. The epithelial cells in crypts had basal oriented nuclei and a cytoplasm to nucleus ratio of approximately 1:2. The lamina propria contained numerous cells closely apposed without edema or evidence of hyperemia (Figure 12).

The section of biopsy collected on PI day 2 included connective tissue from the submucosa. Capillaries in the submucosa appeared engorged but patent. Edema was evident in both the submucosa and mucosa resulting in attenuation of the connective tissue and dilation of lymphatic ducts (Figure 13). Lacteal ducts appeared bulb like in the villi and contained erythrocytes (RBCs). Villous capillaries were greatly engorged. One villus was noted to have a blood filled pouch extending from the lacteal duct. It was covered by a bilayered squamous-like epithelium (Figure 14). RBCs were evident between cells of the lamina propria and stacked in rouleau formation between columnar epithelium. The epithelial lining of crypts had little cytoplasm in contrast to cells of this area in the preinfection biopsy.

Sections of tissue collected on PI day 4 were much the same as those from PI day 2 but with reduced hyperemia and an apparent increase in goblet cell number and villi contraction (Figure 15). The degree of hyperemia and edema were again increased on PI days 6, 8, 10, and 13. Mononuclear cell infiltration was noted during this time. Numerous PMNs were noted in capillaries on PI day 13 at a time when the blood leucocyte level had increased to the preinoculation level.
Figure 12. Photograph of a tissue section from animal #2 taken at the time of surgery to establish a Thiry-Vella fistula. Note depth of crypts (C), complete epithelium (arrows) patent lacteals (L), and distribution of goblet cells (G). The lamina propria (LP) does not have a high number of infiltrating mononuclear or eosinophilic cells at this stage. Hematoxylin and eosin stain. Magnification: 100X
Figure 13. Photograph of a section taken by blind biopsy from a Thiry-Vella fistula of animal #2, 2 days after intraluminal inoculation with bovine virus diarrhea virus. Note marked hyperemia, with presence of red blood cells between epithelial cells (arrow), and in distended lacteals (L). The nucleus to cytoplasm ratio had decreased at this time especially in crypts (C) and goblet cells (G) were increasing in number compared to preinoculation samples. Hematoxylin and eosin stain.
Magnification: 160X
Figure 14. Photograph of a microscopic blood filled pouch extending from side of intestinal villus in section from animal #2, 2 days postinoculation with bovine virus diarrhea virus. The extended area (A) is covered by bilayered epithelium (arrow) devoid of normal morphology. Epithelium on lower portion of villus lacks normal amount of cytoplasm (double arrow). Hematoxylin and eosin stain. Magnification: 160X
Figure 15. Photograph of a section of biopsy sample from Thiry-Vella fistula of animal #2, 4 days after inoculation with bovine virus diarrhea virus. Villi are contracted and cytoplasm reduced. Hemorrhage evident by presence of erythocytes between epithelial cells (arrow). Hematoxylin and eosin stain. Magnification: 160X.
On PI, day 15 a partially organized clot overlay the villous surface, the epithelium was loose and detached in several areas and many mononuclear and PMN cells were present in the overlaying material. Villi were edematous and had cellular infiltration in the lamina propria.

The tissue collected from calf #3 at 3 days prior to inoculation was hyperemic and had evidence of edema in the submucosal connective tissue. The epithelium was entire and intact with that of the crypts having a ratio of cytoplasm to nucleus of approximately 1:2. At 3 days PI the villi obtained by biopsy were enclosed in an organized clot in which there were numerous lymphocytes, macrophages and PMN cells (Figure 16). The cytoplasm to nucleus ratio of crypt epithelial cells had reduced to near 1. An increase to greater than 60 eosinophils per 400X field of villous lamina propria was noted compared to 5-10 in comparable areas in the preinoculation sample. The tissue collected at 5 days PI had increased hyperemia and edema with evidence of hydrophic change. The villi were contracted with an apparent increase in goblet cells. The goblet and eosinophil cell numbers were further increased at PI day 7. Villi were very hyperemic and edema was evident within villous lamina propria and the submucosa. The cytoplasm of crypt epithelium was uniformly attenuated and evident as cytoplasmic strands.

By 10 days PI the inflammatory responses had moderated with evidence of less hyperemia and edema. The epithelial monolayer was intact and had increased depth. Subsequent biopsies collected at 13
Figure 16. Tissue biopsy from animal #3, 3 days after inoculation with bovine virus diarrhea virus into the lumen of a Thiry-Vella fistula. Note mononuclear cell infiltration into lamina propria (M) and clot surrounding villi (C) cut in cross section plane of villi. Hematoxylin and eosin stain.
Magnification: 250X
and 15 days PI had still less edema and hyperemia. The epithelium appeared normal with an increase of cytoplasm of crypt epithelial cells but the eosinophil cell concentration remained increased over that of the preinoculation tissue samples.

There was very little evidence of inflammation in the preinoculation tissue biopsy of #35. The epithelial cells had a cytoplasm to nucleus ratio of approximately 2:1. The only notable change observed in the biopsy collected on PI day 3 was a two fold increase in eosinophils in the villous lamina propria. At 5 days PI the villi were contracted and had been infiltrated with leucocytes. The lacteal ducts were greatly enlarged without evidence of erythrocyte accumulation from diapedesis as evident in cases above. Edema was also evident in the submucosal connective tissue. There was less evidence of hyperemia or submucosal edema on PI day 7 although the lacteal ducts were still dilated. The epithelium of the crypts contained little cytoplasm, the villi were contracted and had an increased number of mucus secreting goblet cells. Goblet cell numbers were further increased by PI day 10 and 12. Edema was decreased on PI day 10 but increased again and was accompanied by hyperemia by PI day 12.

Sections obtained from control animal #118 at the time of surgery had no evidence of severe hyperemia or edema. Moderate edema was present in submucosal connective tissue on PI days 3 and 7. Crypt epithelial cell cytoplasm to nucleus ratio was less than 1 in all specimens obtained through PI day 11 and villi had become
contracted with increased numbers of goblet cells on the lower portions.

The appearance of sections from the second control animal, #556, was very similar to that of #118 except that lacteal ducts were dilated and hyperemia was evident on PI day 7. There was no evidence of hyperemia or edema in specimens collected on PI day 11. The number of eosinophils was not as great in tissues from control animals as those from experimentally infected animals.

Sections stained with immunoperoxidase conjugates were first viewed by light microscopy. The intensity of peroxidase labelling varied with depth from the outer borders of the tissue blocks. Therefore, the inner portions showed only light staining due to reduced penetration of the conjugates and Karnovsky's solution. Treatment of tissue with 1% sodium nitroferricyanide did not completely inactivate innate peroxidase. Cells which were peroxidase positive included erythrocytes, eosinophils, mast cells, and macrophages in addition to cells which were labelled with the specific conjugates. Erythrocytes were readily recognizable by their morphology and the lack of nuclei. The other cells which generally contain innate peroxidase store the enzymes in vacuoles thus giving a granular staining appearance while plasma cells possess immunoglobulins within the cisternae of endoplasmic reticula. An enumeration of plasma cells containing each immunoglobulin was therefore made by counting the number of cells with the morphology typical of plasma cells and which had a relatively uniform distribution of peroxidase staining in contrast to the course
granular staining of enzyme secreting cells. The results are tabulated in Table 6. The number of cells staining with each conjugate with specificity for the various Ig fractions varied greatly in both experimentally infected and control animals. The lowest number of IgA positive cells was demonstrated at PI day 8 for animal #2, PI day 15 for animal #3, and PI days 1 and 12 for animal #35. The number of IgG secreting cells tended to be slightly greater than for those secreting IgA but not as great as that for those secreting IgM. Although it was attempted to obtain sections of all tissues in the longitudinal axis of villi the angle and depth contributed variables influencing the location of cells observed. There was no correlation of observed Ig cell types with one another or other variables.

**Electron microscopic examination**

A representative number of glutaraldehyde fixed samples were cut into 40 µm thick sections prior to immunoperoxidase staining. These sections were subsequently pelleted and processed for electron microscopy. Such treatment allowed for more even penetration but resulted in increased fragility and a loss of orientation so that it was impossible to determine the original location of various cells in relation to villous structure.

Electron microscopic examination of tissues stained with the anti-BVD immunoperoxidase was initially conducted without additional staining to avoid obliteration of peroxidase labelling. Peroxidase positive structures in tissue from both virus infected and virus free animals included those cells listed above which contain innate or endogenous peroxidase.
Table 6. Percent of plasma cells observed in biopsies from intestinal fistulae which stained with immunoperoxidase conjugates for the various immunoglobulins

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<th>Animal No.</th>
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<th>% of PC Positive for Ig&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup>Percent of cells which stained with immunoperoxidase stain specific for listed immunoglobulins.

<sup>b</sup>Days in relation to time of inoculation.

<sup>c</sup>Animals were inoculated via Thiry-Vella fistulae with BVD virus (#2, #3 and #35) or virus-free material (#118 and #556).

<sup>d</sup>Sections unsuitable for cell evaluations.
The mucosa of tissues obtained prior to infection and from control animals possessed an intact epithelium (Figure 17). Blood vessels were patent, nonengorged and in close apposition with surrounding cells (Figure 18). Cells of Peyer's patches were intact and in close association with adjacent cells (Figure 19). In addition, the tissue of infected animals contained cells scattered in the lamina propria which stained lightly with the anti-BVD conjugate and by low power magnification resembled plasma cells.

The tissue from infected animals, by contrast had dark staining material overlying the microvilli of the upper portions of villi and within crypts (Figure 20). The peroxidase label was evident without additional staining on these areas as well as scattered in the cytoplasm of epithelial cells. Further staining with uranyl acetate and lead citrate was essential to produce the necessary contrast for detailed evaluation. Due to the heavy concentration of peroxidase label it was difficult to determine if intact viruses were present. Biopsies obtained 24 hours after inoculation contained complete virions in vacuoles of epithelial cells (Figure 21). Vacuoles increased in number, size and number of virions contained in later biopsies. Vacuoles developed initially near the nucleus and were generally associated with one or more mitochondria. Virions were observed which appeared to be budding through limiting membranes of vacuoles. Areas of dark staining granular material which may have been precursor structures were also observed in cells (Figure 21). Capillaries were observed to be engorged and to frequently contain
Figure 17. Electron micrography of microvillous surface of bovine intestinal epithelial cells prior to infection with bovine virus diarrhea virus (BVD). Tissue was treated with immunoperoxidase conjugate specific for BVD virus and stained with uranyl acetate and lead citrate. Magnification: 16,000X
Figure 18. Section of bovine ileal lamina propria prior to infection with bovine virus diarrhea (BVD) virus. Note small vessel (V); endothelial cells, E; leucocyte in lumen LC; collagen fibers (CF) in central portion of field. Fibrocytes are also present. Section stained with immunoperoxidase conjugate for BVD, uranyl acetate and lead citrate. Magnification: 7,800X
Figure 19. Closely packed cells of bovine Peyer's patch in biopsy taken prior to infection with bovine virus diarrhea (BVD) virus. Note young plasma cells (PC) medium sized lymphocytes (ML), macrophage (M) reticular dendritic processes (RD) and sinusoid-like cavities (SC). Section stained with immunoperoxidase conjugate to BVDV, uranyl acetate and lead citrate. Magnification: 5,000X
Figure 20: Microvillous surface of bovine intestinal epithelium 72 hours after intraluminal introduction of bovine virus diarrhea (BVD) virus. Tissue was treated with immunoperoxidase conjugate to BVD without subsequent staining with uranyl acetate lead citrate. Black immunoperoxidase deposits (arrows) on tips of microvilli appears to associate with virus or virus components. Magnification: 20,000X
Figure 21. Tissue from intestinal biopsy of calf 72 hours after intraluminal inoculation with bovine virus diarrhea virus (BVDV). Note numerous vacuoles (arrows) with enclosed virions in area of shallow crypt. Microvilli (M) at this point is not notably labelled with immunoperoxidase.
Magnification: 15,000X

Vacuoles in center boxed area photographed at higher magnification (120,000X) and inset in lower right hand corner. Note viruses which appear to be budding into vacuole (arrows) and particles which appear to be heavily labelled with immunoperoxidase conjugate (double arrows). At upper left is inset enlargement of area similar to that in circled area. Such ribosome or ribosome-like aggregates were observed in infected cells and may represent precursors for or replication zones for virus.
Magnification: 94,500X
marginated leucocytes which may have been in an early stage of migration into the damaged tissues (Figure 22). Areas of edematous fluid and collagen fibers separated the capillaries from the surrounding cells. Although viral replication was not observed in endothelial cells, the presence of capillaries with necrotic changes including pycnotic nuclei and loss of cellular structure were noted. Mononuclear cells in Peyer's patches possessed vacuoles containing viruses (Figure 23). These cells were presumptively identified as lymphocytes on the basis of morphology and internal structure (Olah et al. 1975; Azar 1979). In addition, cells which were observed at low magnification to be peroxidase positive and to resemble plasma cells were noted by higher magnification to be cells in the early stages of degeneration and therefore the peroxidase observed may have been nonspecifically attached or of endogenous origin which had been released by disruption of membrane systems.
Figure 22. Blood vessel in ileal biopsy taken 72 hours after intraintestinal inoculation of calf with bovine virus diarrhea (BVD) virus. Note lymphocytes (L) on each side of basement membrane. Vessel is surrounded by wide space filled only with edematous fluid and collagen microfibers (CM). Endothelial cells contain vacuoles of various sizes. Section stained with anti-BVDV immunoperoxidase, uranyl acetate and lead citrate. Magnification: 10,000X
Figure 23. Lymphocytes (L) in ovine Peyer's patch 72 hours after inoculation with bovine virus diarrhea (BVD) virus. Vacuole (arrow) in upper portion of central cell contains particles approximately 40 nm in diameter. Bordering the vacuole are three spheres which may represent degenerate mitochondria. Disruption of cell membrane has occurred at left and bottom (double arrows) and particles 50-100 nm appear to have been released. Tissue treated with anti-BVDV immunoperoxidase and stained with uranyl acetate and lead citrate. 
Magnification: 25,000X
DISCUSSION

The factors involved in prevention of and recovery from infections of the intestine vary with the infectious agent and the route by which infection occurs. Animals in this study were infected with BVD virus by inoculation into the lumen of intestinal fistulae and ligated portions of the ileum. The appearance of viral particles within epithelial cells at 24 hours following inoculation was strong evidence that infection was initiated at the luminal border of the epithelium. Infections with this virus have been initiated by various routes including intravenous, intranasal, and oral routes by a number of workers (Fernelius and Lambert 1959; Mills and Luginbuhl 1968; Tyler and Ramsey 1965). Similarities between the results of their studies and findings from intraintestinal infection were observed.

Mills and Luginbuhl (1968) and Tyler and Ramsey (1965) noted febrile responses at 2-4 days and again at 7-8 days PI dependent upon the virus strain given. Leucopenia commonly occurred between febrile responses and tended to be nonselective with both granulocytes and agranulocytes being reduced. Comparable observations were made on calves in this study infected via the intestinal lumen with leucocytic depression preceding febrile responses by approximately 12 hours in animals #2, #3, #4, and #5. The comparatively small depression of leucocytes in animal #35 may have been due to other predisposing factors such as other viral infections and/or innate immunity. This animal had been purchased with animals #118, #556 and
two other animals not included in this study from a commercial herd where the servicing veterinarian had concluded there were no active BVD virus infections. The conversion of the latter four of these animals from seronegative to positive for BVD virus neutralizing antibodies between the time of initial testing and transfer to the university facilities would indicate that BVD virus was present in the herd. Although #35 was seronegative at the time of inoculation, natural infection with BVD cannot be ruled out and could account for the occurrence of detectable neutralizing antibodies in fistular secretions at 6 days PI while animals #2 and #3 did not have appreciable virus neutralizing antibodies until PI days 12 and 10, respectively. Classick (1970) detected febrile responses and leucocyte depressions in preimmunized animals when subsequently challenged with BVD virus. However, the period between the immunization and challenge would have been much greater in his work (>100 days after immunization) than between a possible natural infection within days of the experimental infection of animal #35 in this study. A febrile response would have been more probable in Classick's work than in animals in the initial stages of infection since in the latter the immune system would have been actively involved in limiting the infection. A comparable time period was noted in this animal and in other animals without possible prior exposure (#2-#5) from the time of experimental infection and resultant febrile responses and represents strong evidence that animal #35 was not previously infected. Although seroconversion was noted for animal #556 following transfer to the university facilities
subsequent tests failed to demonstrate neutralizing antibodies in either serum or fistular secretions. Furthermore there was no evidence of BVD infection as indicated by isolation or demonstration of virus by light or electron microscopy. However, Malmquist (1968) and Coria and McClurkin (1978) have found that some animals may be infected for years with BVD virus without the production of detectable levels of neutralizing antibodies.

Severe leucopenia occurred in all animals following inoculation with lymphocytes being most consistently suppressed. A similar sequela has been noted following infection with other viruses including canine distemper virus (McCullough et al. 1974), feline leukemia virus (Anderson et al. 1971) and avian laryngotracheitis virus (Chang et al. 1977). The general leukopenic depression associated with BVD infection may have resulted from various factors including chemotactic attraction of PMNs into areas of tissue damage and the accumulation of lymphocytes in various tissues due to unknown stimuli. PMN cells accumulate in areas of edema and tissue damage and were observed in increasing numbers in experimentally BVD infected animals following infection. The abnormal condition in fistulated animals would have accounted for nonspecific accumulation in addition to the response which would have occurred due to virus infection.

Trapp (1960), Thomson (1972) and Mills et al. (1968) have reported that lymphocyte cuffing of blood vessels occurs following infection with BVD virus. This was not observed in this study nor was it reported by Peter et al. (1967) or Peter et al. (1968).
The biopsies obtained in this study were not obtained with the advantage of those observed in other studies where the intestine was opened longitudinally and selected lesions processed for microscopy. Therefore, the observations made here from blind biopsies were not necessarily of comparably effected tissue. In the event of lymphocyte cuffing being the normal sequela in BVD infections this factor could account to a degree for the depression of lymphocytes. Bovine lymphocytes also appear to be quite susceptible to corticosteroids (Muscoplat et al. 1975) which are released from the adrenal gland as a result of various stimuli including stimuli occurring during the course of diarrhea in cattle (Lopez et al. 1975). It could be speculated that lymphocytopenia might also result from BVD virus infection and destruction either as a result of virus induced lysis or cell-mediated responses resulting in selective elimination of virus infected cells. Particles identified as BVD viruses were observed in closed and externally ruptured vacuoles of lymphocytes in this study. The involved lymphocytes were located in Peyer's patches which are characteristically depleted of lymphocytes in BVD infections (Peter et al. 1957; Thomson 1972; Ramsey and Chivers 1953; Carlson et al. 1957). Truitt and Shechmeister (1973) have demonstrated BVD virus replication in both lymphocytes and macrophages without apparent lytic results. However, cell mediated lysis has been noted by numerous workers as discussed by Zinkernagel and Doherty (1979) and may therefore be involved in lymphocyte depletion. Circulating lymphocytes which remain at the time of greatest leucopenia have been shown in
this study and by others (Pospisil et al. 1975b; Muscoplat 1973a; Reggiardo 1975) to have a marked depressed transformation response when exposed to PHA. This response is viewed as a representation of depressed CMI response (Blomgren and Svedmyr 1971; Janossy and Greaves 1971) and has concurrently been associated with a transient depressed sentinel effect which may result in bacteremia.

Reggiardo (1975) found that 27/47 blood samples from experimentally BVD infected cattle and 2/36 from control animals contained a variety of bacteria. Similar cultures in this study resulted in the isolation of bacteria from 4/15 blood cultures from infected animals and 1/4 from control animals. **Bacillus** and alpha hemolytic **Streptococcus** were the most commonly isolated bacteria in each case. The development of bacteremia could result from a number of factors including damage of the epithelium which would allow bacteria to enter the vascular system more readily. This would appear to be true particularly at Peyer's patches where the penetration of bacteria and other foreign material is fairly common in the normal state. Lymphocyte depletion and ulceration of Peyer's patches which result from BVD infection would allow ready access to the vascular system by bacteria common in the gut lumen. Normally bacteria gaining access to the deeper tissues would be phagocytized and eliminated. However, due to the depression of leucocyte number and depressed phagocytic activity of PMNs and macrophages, bacteria may remain relatively unchecked in the system and contribute to varied and complex secondary bacterial infections.
The evidence of depressed T cell function as reported by Muscoplat (1973a), Reggiardo (1975) and in this study appears conclusive that T cells and therefore CMI responses are also reduced with BVD infection. Recovery from numerous viral infections appear to be largely dependent upon T cell recognition and responses (Merigan and Stevens 1971). These cells may also be involved in the recognition of invading bacteria and subsequent activation of macrophages for enhanced phagocytosis and elimination. Therefore, suppression of T cell function would also increase the chances for bacteremia to develop.

IgA levels in intestinal fistular fluids were noted to decrease following BVD virus inoculation. The lowest values observed for two animals on PI day 2 preceded by 1 day decreased lymphocyte numbers in peripheral blood and suppressed lymphocyte transformation activity. A similar correlation was observed on PI day 10 and 11 in one infected animal. Similarly a third animal (#35) which had low IgA levels through PI day 10 had moderately reduced leucocyte numbers but a marked reduction of lymphocyte transformation activity through PI day 13. This finding suggests that B cell and plasma cell activities are depressed in addition to T cell function. BVD virus isolation from lymphocytes is common therefore indicating that they are infected. Depression of IgA secretion could therefore occur by alteration of the role of IgA plasma cell precursor lymphocytes raised in the Peyer's patches or by direct infection and alteration either by suppression or lysis of IgA producing plasma cells already
in residence in lamina propria. Although a reduction of IgA bearing
cells in the lamina propria was not conclusively observed in this
study, the elimination of plasma cells and/or their precursors would
be the most logical mode of action leading to immunotolerance for
this virus as reported by Muscoplat (1973b) and Coria and McClurkin
(1978).

IgM levels in fistular secretions remained relatively stable in
contrast to IgA and IgG levels. A moderate increase at 7 days PI
in calf #2 coincided with an increase of greater magnitude in IgA
levels and may reflect an initiation of a specific immune response,
although virus neutralizing antibodies were not detected in fistular
secretions or serum until PI days 12 and 11, respectively. Greater in­
creases in IgM levels in fistulae were noted for animals #3 and #35
on PI days 10-13 and 15, respectively. This coincided directly with
detectable levels of neutralizing antibodies in fistular secretions
of animals #3, although neutralizing antibodies were not detected in
the serum of this animal through PI day 11. Neutralizing antibodies
were detected in fistular secretions of animal #35 at 6 days PI when
IgM levels were low but not in serum until 15 days PI. The evalua­
tion of IgM levels in fistular secretions is complicated by the
presence of two types of IgM i.e. that containing a secretory com­
ponent, secretory IgM, and a more enzyme sensitive IgM which does
not have the secretory component (Brandtzaeg and Baklien 1977).

It is generally accepted that immunoglobulins bearing the sec­
retory component, SIgA and SIgM, are actively transported across
secretory membranes while IgG molecules are passively transported with increased loss during times of edematous responses. The significant (P < 0.05) inverse correlation of IgA and IgG in fistular secretions may result from an increased IgG flow but less efficient IgA transport when the tissue was edematous. IgA levels decreased in animals #2 and #3 as virus neutralizing antibodies appeared in the secretions at a time when hyperemia and edema were decreasing. However, IgG levels were at their highest when neutralizing antibodies were first detected in animal #35, a fact which may reflect an anamnestic response.

Shope et al. (1976) concluded from limited studies of calves with depressed and normal T cell functions in the presence or absence of passively acquired anti BVD antibodies that it is the presence of specific antibodies and not T cell capabilities which protects animals from disease due to infection with this virus. If B lymphocyte activity is depressed, as suggested by reduced IgA secretion as well as that of T lymphocytes, factors involving other portions of the immune response would be involved to a greater degree than normally anticipated for recovery from the initial phase of infection. At this time it can only be speculated that additional factors would include nonspecific processes of interferon release, beta lipid, phagocytosis, enzyme release from degranulating phagocytic cells and damaged epithelium as well as activation of the alternate complement system due to release of activator and increased mucous secretion substances. The action of such nonspecific factors could then minimize viral replication and damage while the weakened
populations of T and B lymphocytes were able to replicate and develop both specific and further nonspecific responses. Antibodies whether actively or passively acquired appear capable of preventing disease due to BVD infection (Shope et al. 1976; Kendrick and Franti 1974). As long as passively acquired maternal antibodies remain in the blood of calves these animals are immune. However, the presence of antibodies does not appear to prevent infection and possible transmission of virus. Nor does the immune response, as demonstrated by production of neutralizing antibodies, prevent penetration and replication of viruses in the lymphocytes and macrophages once these cells are freed of specific antibodies (Truitt and Shechmeister 1973).

A low virus neutralizing capacity in fistular secretions obtained from noninfected animals in this study was noted. Such neutralizing activity could have resulted from action of enzymes or other nonspecific factors such as lipids or lipoproteins as noted by Nash et al. (1971), Falkler et al. (1975), and substances resulting from various stimuli discussed by Halder et al. (1977), and Veitri and Kirk (1971). The neutralizing activity rose rapidly to a TCPR\textsubscript{50} of 512 in the three animals tested and was on the decline at the time of the last determination in two of these. The rapid development and decline is compatible with reports of IgA responses which according to Svennerholm et al. (1978) may be demonstrated early in mucous secretions and decline rapidly until a subsequent exposure appears to stimulate another phase of antibody production. If stimulation of deeper lymphoid tissues occurs such a decline would
be followed by a primarily IgG response which may or may not have been expressed in intestinal secretions. Concurrent separation of the intestinal secretions was not attempted due to inadequate volumes but had such volumes been available would have added to the understanding of the type of response which occurred. A greater quantity of IgG than normally secreted into the intestine would have been expected in these fistulae due to the edema resulting from the altered state of that portion of intestine and the periodic biopsy procedures.

Although neutralizing antibodies were detected in fistular secretions, it was possible to demonstrate the presence of viruses in 11/14 fistular samples. This is a higher recovery rate than from buffy coat samples. It has been demonstrated that this virus is released in the various secretions for prolonged periods (Mills and Luginbuhl 1968). However, the viremic state appears to be of shorter duration and some evidence exists which suggests a spasmodic type of release into the blood. Additional problems of isolation from the white blood cell population have been attributed to inadequate removal of neutralizing serum antibodies. This could have contributed to the low recovery rate of viruses from the blood of animals in this study.

Demonstration of virus infection in tissue culture monolayers was comparable by immunofluorescent (IF) and immunoperoxidase (IP) methods. Each technique has advantages with those of IF including requirement of a shorter staining period than required for IP. This procedure however requires a greater expenditure for microscopic
equipment including fluorescent attachments and filters while the IP procedure utilizes normal light microscopy. In addition the IP stained slides give a more permanent preparation which can be observed over a greater period of time than can IF stained slides and a more defined localization of labelled material in monolayers. The application of this procedure to electron microscopy adds to its desirability. Increasing use of immunoperoxidase staining for various viral (Shimizu et al. 1978; Leduc et al. 1968; Kurstak et al. 1971; Wicker and Avrameas 1969; Johnson and Swoveland 1977; Pospisil et al. 1975b) bacterial (Cizar et al. 1978; Dahlen et al. 1978), and subcellular components (Guillouzo et al. 1976) are evidence of a growing preference in comparison to IF preparations.

Edema and hyperemia were the most common findings in tissues from animals in this study. Although these findings were most consistent in samples following infection, they were not confined to such samples but were also observed in post surgical biopsies obtained from fistulae prior to infection. In one animal (#2), hyperemia decreased between the second and sixth PI days, a time coinciding with the time between febrile responses, and suppressed lymphocyte number and transformation activity.

The observation of decreased villous height probably resulted from reduced mitosis of crypt epithelial cells. The corresponding increase in goblet cell numbers may have occurred due to the stimulating effects of antigen-antibody complexes as reported by Jubb and Kennedy (1970) for this disease and Kent and Moon (1973) for
other diseases affecting the intestine.

Although vascular cuffing was not evident, mononuclear cell infiltration into the lamina propria did occur and was followed closely by a sharp increase in the number of eosinophils as reported elsewhere (Tyler and Ramsey 1955). The migration of mononuclear cells into the lamina propria could have accounted for some degree of lymphocytopenia as noted by reduced numbers in peripheral blood. The majority of mononuclear cells in tissue were morphologically consistent with the appearance of lymphocytes. The localization of these cells in the lamina propria would position them for immunological responses of either the humoral or cell-mediated types.

As an increase of PMNs was noted in peripheral blood following leucocyte depression, PMNs were noted to increase in the blood vessels supplying the lamina propria with a consequential increase of PMN numbers in the intestinal lumen. Evidence that these cells may be immunologically induced to migrate into the intestinal lumen (Bellamy 1978, Bellamy and Nielsen 1974) without injury to the mucosa and remain active would suggest that their presence in the later stages of BVD virus infection was in response to either the BVD virus or secondary bacterial infection. The time sequence observed was essentially what would be expected following immunological recognition of the BVD virus and antibody production. However, due to the apparent immunological suppression, as indicated by reduced lymphocyte transformation, and subsequent bacterial invasion it is impossible to separate the eliciting roles of viruses and bacteria.
The tissue blocks which were stained with immunoperoxidase conjugates for bovine IgA, IgG, and IgM and BVD virus provided structurally comparable tissue sections. However, penetration of tissue blocks by the conjugates was not satisfactory, a fact which may have been due to a number of factors. Dahlen et al. (1978) reported that fixation with glutaraldehyde results in a marked reduction of immunoperoxidase labelling of surface materials of bacteria. They found conjugate treatment prior to fixation gave optimum results although paraformaldehyde fixation preceding exposure to conjugates gave intermediate results. Paraformaldehyde-fixed tissue from animals #4 and #5 of this study resulted in relatively coarse granular coagulations of tissue as viewed by electron microscopy. Therefore, such tissues were not satisfactory for observation of minute structures such as viruses. For that reason, glutaraldehyde-fixed tissues were used throughout the remainder of the project.

Most of the cells which contained peroxidase contained the enzyme in large aggregates as expected in eosinophils, mast cells, and macrophages. Cells with a more diffuse and lighter stain were concluded to be immunologically active cells; however, the results of this study did not correlate well with those of Brown et al. (1974) and Allen et al. (1973) who used immunoperoxidase conjugates to demonstrate immunoglobulin containing cells in pigs. The number of cells reported by those workers to contain specific immunoglobulins were greater in number and distribution than observed in this study. Their reports included cells with a greater degree of granularity than was accepted.
in my experimentation. The major reason for excluding such cells was that such cells were also observed in control sections treated only with Karnovsky's solution and in control sections taken from animals prior to infection with BVD and stained with the anti-BVD conjugate. Although Brown et al. (1974) and Allen et al. (1973) did recognize eosinophils as carriers of endogenous peroxidase, no mention was made of the fact that macrophages may also contain innate peroxidase. Light staining of apical portions of epithelial cells and the brush border with IP conjugates for IgA and IgM corresponded to the findings of Allen et al. for IgA.

In addition to the staining noted at epithelial brush borders, cells resembling plasmacytes in the lamina propria by light microscopy were also observed to be peroxidase positive. However, the identity of these cells remained questionable since examination by electron microscopy revealed evidence of degenerative changes.

Heavily peroxidase-labelled viral components detected at the luminal surface of the epithelial cells could not be positively identified as BVD virus by morphological characteristics observed by electron microscopy. The absence of comparable staining in control tissues gives support to the conclusion that the presence of these particles in infected animals is due to the presence of BVD virus. A granular-like distribution of peroxidase-positive material in the outer portion of epithelial cells which had been treated with immunoperoxidase but not stained with uranyl acetate or lead citrate was suggestive evidence of viral penetration and/or replication. However,
it was not possible to demonstrate intact viral particles in such sections due to low contrast. Inadequate penetration and binding of labelled antibodies to viral antigens necessary for identification of viral components may have been due to a variety of factors. These would include: 1) large molecular size of the immunoperoxidase conjugate, which would have resulted in poor penetration 2) fixation effects on viral antigens resulting in low reactivity similar to that observed by Dahlen et al. (1978) for bacterial antigens, and 3) limited exposure of viral specific antigens in complete virions. Enveloped viruses incorporate host cellular components and therefore may not be antigenically recognized (Dulbecco and Ginsberg 1973). While this may be the case with BVD virus, Pritchett and Zee (1975) were unable to conclude from their studies if host cell polypeptides were or were not intrinsic components of the virus.

A variability of stained viral particles was noted following uranyl acetate and lead citrate treatment. Some particles contained a heavy diffuse stain as viewed by electron microscopy. Such staining which obscured detail of the virus outline is suggestive of immunoperoxidase staining. Reasons for the observation of this type of staining on some viruses while not on others in the same vacuole or extracellular area remain obscure. Possible causes include: 1) the portion of the stained virus in view may contain surface antigen 2) the envelope may have been penetrated thereby exposing the nucleocapsid to the conjugate or 3) a variability in the maturity of the viral particles present. The structure of particles observed in tissue
and identified as BVD virus was very similar to that of equine arteritis virus (Magnusson et al. 1970). The similarities included marginal staining of the nuclear core, an indistinct envelope, and the mode of budding into the cisternae of cytoplasmic vacuoles.

Observations of viruses in epithelial cells and lymphocytes indicated that these are target cells of BVD virus. While it was expected that endothelial cells might also serve as host, and capillaries were observed by electron microscopy to be occluded with RBCs virus replication was not demonstrated in these cells. In those cells supporting virus replication, viruses were observed to bud into vacuoles, which were initially near the cell nucleus. Virus filled vacuoles would presumably migrate to the cell periphery as noted in virus infected lymphocytes. The observation of viruses in epithelial cells and lymphocytes would lend itself to the observed effect on the intestinal and lymphoid response.
SUMMARY

Cattle were infected with bovine viral diarrhea (BVD) virus by intraluminal inoculation of ligated and fistulated segments of the ileum. Diphasic febrile responses occurred at 2-4 and 7-8 days post-inoculation (PI). The number of leucocytes present in peripheral blood commonly decreased following infection with the greatest reduction coinciding with febrile responses. Lymphocytes and polymorphonuclear cells were observed to drop to levels as low as 59 and 9% of preinoculation levels respectively.

Lymphocyte transformation activity was monitored in three animals and found to be suppressed in all animals but markedly suppressed in one. This response was variable, however, with one animal having lymphocyte suppression coinciding with the first febrile response and an enhanced response at the time of the second febrile response.

Viruses were routinely recovered from fistular secretions and the occurrence of viremia was confirmed by periodic isolation of viruses from blood buffy coats. Virus neutralizing antibodies were detected in fistular secretions and blood serum. This activity was generally detected earlier and at higher levels in the fistular secretions.

IgA, IgG₁, IgG₂, and IgM were quantitated in fistular secretions. While the amount of collectable secretions varied markedly, the values detected indicated that IgA values decreased following BVD infection in an inverse relationship with IgG₁ and IgG₂. IgA and IgM values tended to increase in the secretions during the recovery period. This
observation appeared to correlate with the detection of BVD virus
neutralizing antibodies.

Observations of histological sections obtained by biopsies of
the intestine revealed edema and hyperemia as the most common altera-
tions early in the infectious process. Mononuclear and eosinophilic
leucocytic infiltration was a common finding during the later portion
of the study.

Virus antigen was detected by immunoperoxidase conjugate staining
and electron microscopy. This antigen was initially observed at the
tips of microvilli as nondistinct, heavily staining material. Virions
were detected in lymphocytes in Peyer's patches and budding into vacuoles
of the epithelial cells. Although vacuolation of endothelial cells was
observed, viruses were not detected in these cells.

The significant findings of this study include: 1) lymphocytes
of Peyer's patches and epithelial cells serve as target cells for BVD
virus; 2) the detection of viral replication in the above cells re-
veals mechanisms by which the observed lesions and immunological sup-
pression are initiated and; 3) local immune responses occur early in
intestinal secretions coincident with increasing IgA and IgM levels.


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