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**Studies on the Development of Intestinal
Secretory Immunity to Colibacillosis in
the Piglet**

by

William Dennis Allen, FIMLS, BA(OU)

A Thesis Submitted for the Degree of Doctor of
Philosophy in the Discipline of Biology in
Accordance with the Requirements of the
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Abstract

The interaction between the cellular components of the intestinal mucosa and its luminal environment has been studied in the piglet with the objective of establishing an early onset of secretory antibodies against pathogenic E. coli.

Morphological evaluation of the mucosal architecture during development showed that inflammatory changes occur in apparently healthy suckling piglets as early as 10 days after birth. These changes, which become progressively more severe as the animal ages, are greatly exacerbated by E. coli infection. The proximal small bowel is the area most affected, indicating that protective immune function should be preferentially directed towards this region.

A quantitative evaluation of macromolecular absorption at different levels of small intestine, using both horse-radish peroxidase and E. coli endotoxin indicated that uptake was greatest in the anterior small bowel and that the rate of absorption decreased with age. Uptake of one macromolecule was not affected by the presence of the other suggesting lack of competition for binding sites on the absorptive epithelium.

Immunofluorescent studies on intestinal mucosa of pigs of varying age demonstrated IgM containing cells in duodenal lamina propria of 2 day old animals. IgM cells continued to outnumber IgA and IgG cells until the animals were weaned at 3 weeks old, thereafter IgA cells predominated.

In vitro evaluation of intestinal secretory antibody production in response to oral immunization showed specific antibodies in 14 day old pigs immunized from 4 days old. The greatest response was obtained from the anterior small bowel, again emphasising the importance of this region.

The protection conferred by early oral vaccination was determined, in vivo, in piglets experimentally infected with enteropathogenic E. coli and weaned at 14 days of age. The faecal E. coli count of the vaccinated group was reduced 100 fold compared to the placebo group and their weight gain after 1 week was 15% greater. The implications of these findings are discussed.

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Preface

The experimental work described in this thesis was carried out in the Department of Immunology of the Unilever Research Laboratory, Colworth House, Sharnbrook, Bedfordshire during the period 1976-1980. Section 5 has been published in *Immunology* 32 819 (1977).

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SECTION 1

A CONSIDERATION OF THE ROLE OF INTESTINAL SECRETORY IMMUNE
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Modern farm animal production relies extensively on antibiotics and chemotherapeutics for prophylactic medication and as growth promoters. Gross misuse of these products has led to an alarming decline in their efficacy as therapeutics and caused major concern over the ever increasing spectrum of drug resistant plasmids in the microflora of farm animals. Following the report of an enquiry into animal medication (Swann, 1969), regulations were introduced to severely limit the use of such drugs in British agriculture. Nevertheless despite this legislation, illegal practices still abound and there has been no obvious decline in the number of drug resistant strains of bacteria excreted in marketed animals (Smith, 1975, 1976). There is as much cause for anxiety over the reservoir of "R" factors now as there was ten years ago.

In the search for alternative methods of promoting the health of intensively reared livestock, immunobiological control of disease is receiving increasing attention. So far as enteric conditions are concerned, the secretory immune mechanisms of the gut have been a target for stimulation, due to their potential for convenient large scale vaccination against infectious agents which affect mucous surfaces.

The role of immune mechanisms operating in the intestine for the control of enteric colibacillosis in the young pig will be considered in this introduction and the efficacy of oral vaccination as an immunoprophylaxis will be discussed as a basis for further studies.

ENTERIC COLIBACILLOSIS - ECONOMIC CONSIDERATIONS

Enteric disorders associated with enteropathogenic Escherichia coli affect many mammalian species, including domestic farm animals. They are a particular cause for concern in modern agriculture where intensive livestock practices facilitate the rapid spread of infection throughout herds, with serious economic consequences. The young are especially susceptible to attack. This point is amply demonstrated in a study on the association between gastroenteritis and E. coli in pigs, conducted by the Veterinary Investigation Service (M.A.F.F.) (Anon, 1960) when it was found that of all deaths from disease of the alimentary system in animals up to 4 months of age, 40% were attributable to enteropathogenic coli. In a similar study on calves the incidence of mortality due to pathogenic E. coli was 25% (Anon, 1965). These studies, concentrating as they do on mortality, take no account of the level of morbidity and its impact on the economic performance of a herd. Although they are difficult to quantify, the losses due to morbidity are likely to be far greater than those caused by deaths. Together they constitute a shortfall in potential productivity of the order of several hundred million pounds per annum in the E.E.C. alone.

ESCHERICHIA COLI INFECTION IN PIGS

Colibacillosis in pigs is manifested by two clinical entities: diarrhoea with toxæmia and oedema disease. Although both can occur simultaneously, oedema disease generally affects pigs over 8 weeks of age and whilst specific E. coli serogroups are associated with this condition it is still imperfectly understood. It will not be considered further in this treatise.

The enteric form is the one most commonly seen and primarily affects pigs under 8 weeks of age. E. coli is seldom recognised as a specific cause of diarrhoea in older pigs, although evidence is beginning to emerge suggesting a sub-clinical condition which may give rise to a degree of debilitation leading to a reduction in performance and growth rate.

The incidence of E. coli associated enteric infection in young pigs may be divided into specific periods on the basis of age. These have been variously classified as neonatal diarrhoea or piglet enteritis affecting animals 1-4 days old and weaning diarrhoea usually seen in piglets during the week after weaning (Nielsen, Moon and Roe, 1968). Stevens (1963) classified this latter group as "milk scours" affecting piglets 3 weeks old and considered it to be caused by a sudden change in the composition of sow's milk at that time. In the author's experience this milk scour can occur as early as 10-12 days of age and is not associated with weaning.

Whereas neonatal enteritis is a straightforward enteric infection of newborn piglets by enteropathogenic coli, probably acquired from the sow, the syndrome in older pigs appears to be stress induced, requiring some change in diet or management to stimulate the proliferation of the enteropathogen (Kenworthy & Allen, 1966a) particularly in the duodenum and anterior third of the jejunum (Smith & Jones, 1963; Smith & Linggood, 1971). One feature of importance is the removal of mother's milk antibody at weaning thereby effectively withdrawing a continuous passive control of the gut microflora.

Porcine E. coli serotypes

Escherichia coli exist as normal commensal organisms in the alimentary tract. There are however a number of well defined serotypes which are pathogenic. Generally each animal species is characterized by its own serotypes though some serotypes may be found in more than one species (Orskov & Orskov, 1978). These may be present in small numbers in the apparently normal host without signs of ill effect (Arbuckle, 1968) but under certain conditions they proliferate and are pathogenic.

The serotypes more particularly associated with diarrhoea in the newborn and weanling pig were first defined by Sojka, Lloyd & Sweeney (1960) and represented a major step towards a more precise understanding of diarrhoeal disease in the young animal. There are believed to be at least 164 different E. coli O antigens, 71 K antigens and 56 H antigens recognised, capable of being combined in many different ways to generate over 10,000 different serotypes (Orskov and Orskov, 1978). Despite this, however, the number of serotypes implicated in the majority of porcine colibacillosis outbreaks is surprisingly small (table 1.1). Many carry transmissible plasmids which confer drug resistance, ability to synthesise enterotoxins, or adherent properties for enterocyte membrane receptors. Perhaps the most thoroughly investigated of these is the surface K88 antigen (Orskov, Orskov, Sojka & Leach, 1961) which promotes adherence to small intestinal villi and which was early thought to be a specific virulence determinant for piglets. This is now known not to be an essential characteristic (Isaacsson, Nagy & Moon, 1977) and it is thus possible for enterotoxigenic strains that do not carry K88 or other known fimbrial colonization factors to cause diarrhoea (Orskov & Orskov, 1978).

IMMUNE DEFENSE MECHANISMS IN THE ALIMENTARY TRACT

I) Maternal immunoglobulins and passive immunity in the neonate

The newborn piglet and calf are both entirely dependent upon passively acquired antibodies for protection against neonatal colibacillosis. The young of all mammalian species are initially reliant upon antibodies derived from maternal sources for early defense against infection since, although the capacity to synthesise antibody exists before birth (Binns, 1967; Bognar, 1973; Gay, 1979) the immune mechanisms have not normally been primed to function.

In the primate passive immunity is conferred 'in utero' by transplacental transfer of immunoglobulins from maternal to foetal circulation. However, in the pig and calf, as in many other species, transplacental transfer does not occur and passive immunity is acquired solely by absorption of colostral antibodies across the small intestinal mucosa into the neonatal circulation. To facilitate this process, enterocytes in the small bowel of the newborn are peculiarly adapted (Smith & Jarvis, 1978) to permit the uptake of substantial quantities of unaltered maternal immunoglobulin. This hyperpermeability is short lived however

lasting for only 24 to 36 hours (Lecce & Morgan, 1962) after which time 'closure' occurs. Most passive immunity is acquired during the first few hours of life and the efficiency of absorption decreases rapidly. In the pig it is estimated to have a half life of about 3 hours (Speer, Brown, Lloyd & Catron, 1959). An interesting observation in the calf suggests that the period of permeability differs for each class of immunoglobulin (Penhale, Logan, Selman, Fisher & McEwan, 1973).

A variety of mechanisms have been proposed to explain the closure phenomenon. The absorption of protein (Lecce, Matrone & Morgan, 1961) glucose (Lecce, 1966) and polyvinyl pyrrolidone (Lecce, Matrone & Morgan, 1961, Hardy, 1969) have all been shown to curtail the process of absorption. El Nageh (1970) found termination of absorption in the calf to be associated with a replacement of the epithelial cells present on the small intestine at birth, by a new generation incapable of absorbing macromolecules. This view accords with a recent study in the pig, by Smith & Jarvis (1978) in which they demonstrated that enterocytes lining the small intestine at birth, capable of containing protein filled vacuoles were gradually replaced by cells which never formed such vacuoles. The time taken to complete cell replacement was 19 days, some three times longer

than that proposed by earlier workers. The discrepancy may be accounted for by the greater sensitivity of the techniques used, enabling a more detailed study of cell migration to be made. Nevertheless the majority of cells lining the intestine were non vacuolated by 4 days of age.

Immunochemical studies of post colostrum piglet and calf serum show all three classes of immunoglobulin IgM; IgG and IgA are absorbed from the colostrum with little evidence of selectivity (Porter, 1969a; 1972) although in the pig there is some selectivity against secretory IgA. The antibody function of this molecule is confined principally to the gut lumen, it contributes little to circulating passive immunity (Porter, 1969b). Passively acquired circulating immunoglobulin levels fall rapidly with an almost exponential decline. The mean half lives of IgM; IgA and IgG in the pig have been calculated as 2.8, 2.7 and 9.1 days respectively (Curtis and Bourne, 1973). This short half life of passive protection results in an apparent critical antibody deficiency in the pig developing at about 14 days of age (Miller, Harman, Ullrey, Schmidt, Luecke & Hofer, 1962) which persists until active immunity has developed sufficiently.

II) The ontogeny of the intestinal secretory immune system

Whereas protection against neonatal colibacillosis is conferred by passively acquired antibody, defense against coli-enteritis in older animals depends on the development of an active secretory immune mechanism within the intestine. Most studies, conducted on animals which may be regarded as being immunologically mature, have shown the predominant immunoglobulin in intestinal plasma cells to be of the IgA class (Crabbe, Carbonara & Heremans, 1965; Rubin Fauci Sleisinger, Jefferies & Margolis, 1965; Crandall, Cebra & Crandall, 1967; Crabbe, Bazin, Eyssen & Heremans, 1968; Felsenfeld, Greer, Kirtley & Jiricka, 1968; Vaerman & Heremans, 1970; Atkins, Schofield & Reeders, 1971) and whilst, because of its relative abundance in intestinal secretions, considerable emphasis has been placed on the role of IgA, other immunoglobulin classes have been comparatively neglected.

A local secretory immune system, primarily mediated by IgA, similar to that of other species, has been defined in the pig (Porter & Allen, 1969; Vaerman, 1970; Atkins, Schofield & Reeders, 1971) and the calf (Porter, Noakes & Allen, 1972) but studies on the young of these two species have also emphasized the importance of IgM (Allen & Porter, 1970; 1975). In the

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newborn animal there are few if any, immunoglobulin containing cells in the intestinal lamina propria and lymphoid follicles are poorly defined; a state which persists if the animal remains germ free (Porter, Kenworthy, Noakes & Allen, 1974). However with the establishment of a gut microflora Peyer's patches develop and the lamina propria becomes infiltrated with immunocytes, many of which contain IgM. During the first few weeks of life IgM containing cells may be numerically superior to those containing IgA (Allen & Porter, 1973a, 1975) and the potential of this class of antibody, acting in concert with IgA, is considered important to the local immune defense of the developing neonate (Allen & Porter, 1970).

It is possible that IgM plays an initiating role in the onset of antibody response in the mammalian exocrine system in much the same way it does in the systemic response (Allen & Porter, 1973). The observation of Cebra (1969) lends support to this thesis in that rabbits infected with *Trichinella* showed a relative increase in intestinal IgM cells during the period 7-13 days post infection. Repeated injection of anti-u-chain serum into germ-free mice leads to a decrease in the number of IgM cells and a virtual absence of IgA cells in the intestinal lamina propria (Lawton, Asofsky, Hylton & Cooper, 1972) a finding consistent with the proposal that IgM cells are the precursors of IgA.

The pig and the calf are ideal models for studying the ontogeny of the immune response. Because of the absence of transplacental passage of maternal immunoglobulin to the foetus their tissues are virtually devoid of immunoglobulins at birth. Nevertheless they are immunologically competent and can mount an immune response to antigenic challenge pre-partum (Binns, 1967; Bognar, 1973; Gay, 1979). Sheep also respond to antigens administered into foetal tissue (Silverstein, Thorbecke, Kramer and Lukes, 1963) and it is interesting to note that antigens introduced into the amniotic fluid of pregnant sheep are ingested by the foetus. Here the characteristics of the gut immune response feature the development of IgM cells in the foetal intestine (Husband & McDowell, 1975) exactly as occurs naturally in the neonate.

The mechanism of transport across the epithelium of immunoglobulins synthesised locally in the intestinal lamina propria has recently been described (Allen, Smith & Porter, 1973, 1976; Brown, Isobe & Nakane, 1976); the secretory pathway appears to be the same for both IgA and IgM. Immunoglobulin, secreted by plasma cells located close to the basement membrane of crypt epithelial cells, passes into the interepithelial cell spaces to be absorbed, probably pinocytotically, into epithelial cell cytoplasm. It passes through the cytoplasm to accumulate in the supranuclear region from where it is excreted into the intestinal lumen.

The quantitative distribution throughout the intestinal lamina propria, of immunoglobulin containing cells is of interest.

There are up to ten times more cells in the proximal small gut than at more distal sites. This finding has been made in a number of species (Crabbe, Bazin, Eyssen & Heremans, 1968; Willard, Cooke, Rodkey, Dayton & Anderson, 1978) including the pig (Allen & Porter, 1973a; Brown & Bourne, 1976) and the calf (Allen & Porter, 1975).

Precursors of intestinal secretory antibody cells arise in gut associated lymphoid tissue (GALT) principally Peyer's patches (Craig & Cebra, 1971) from whence they selectively 'home' to the lamina propria via the thoracic duct and blood circulation (Gowans & Knight, 1964; Guy-Grand, Griscelli & Vassali, 1974, Pierce & Gowans, 1975). Although the mechanism of this selective homing is not known, antigen appears to be one factor. The numbers of cells localizing in the laminal tissues of immunized animals are highest in the region of intestine in which antigenic challenge occurs (Pierce & Gowans, 1975; Husband & Gowans, 1978), findings which could account for the presence of high numbers of plasma cells in the proximal small bowel, especially as this region is the major site for protein digestion and absorption (Holdsworth, 1972). Regional blood flow also influences cell distribution along the alimentary tract. Recent studies in mice have shown a positive correlation between the distribution of mesenteric lymph node cells and regional blood flow (Parrott & Ottaway, 1980). Moreover this was not affected by the absence of dietary antigenic challenge implying that the mechanism is independent of antigen.

The presence of large numbers of plasma cells, synthesising secretory antibodies in the anterior small intestine is of importance to the young animal in its resistance to colibacillosis since a feature of the disease is the proliferation of enteropathogens in the upper small bowel (Smith & Jones, 1963; Smith & Linggood, 1971). Thus the resistance to infection will be enhanced provided the antibodies produced are specific for the strain of enteropathogen involved and are able to be secreted in sufficient quantities early in the infection. These conditions can only be fulfilled if the immune mechanism has been adequately primed in advance of infection by immunization, either by natural or artificial means.

IMMUNOPROPHYLAXIS IN THE CONTROL OF COLIBACILLOSIS

I) Neonatal enteritis

Because immune defense against E. coli neonatal diarrhoea depends upon passively acquired maternal antibodies the only practical means of enhancing protection is by ensuring the production of adequate amounts of colostral antibody of the relevant specificity. Although the foetus is capable of mounting an active immune response, pre-partum, to antigen administered to it by either the intra-amniotic or the intra-muscular route

(Conner, Richardson & Carter, 1973; Gay, 1975; Olson & Waxler, 1976; Gay, 1979) the techniques involved are such that they cannot be considered to have practical application in modern intensive animal systems. The dangers of abortion or the prospect of performing 1,000 laparotomies per year, which would be required for the average 500 sow unit, is enough to dissuade even the most dedicated veterinarian and far outweighs the benefits obtained.

Traditionally the production of hyperimmune colostrum is achieved by parenteral immunization of the sow with a vaccine consisting of a polyvalent suspension of inactivated pathogens, usually incorporating an adjuvant. The vaccine is administered by the intramuscular route two doses being given during the first pregnancy and a single booster dose during each pregnancy thereafter. This protocol results in the production of elevated levels of specific colostrum antibodies of the IgG class which do confer a degree of protection to the newborn offspring against neonatal colibacillosis. However there is evidence that hyperimmune colostrum containing IgG antibodies may adversely influence the subsequent capability of the neonate to initiate its own active immunity and that antibodies of the IgM class would be preferred (Porter, 1969b). It is of interest to note that in a study of protective efficiency in neonatal piglets

given a lethal infection of a virulent enteropathogenic coli, the protection obtained from colostrum IgG antibodies was 64% survival whereas that conferred by IgM was 98% compared with a control level of 24% (Porter, Linggood & Chidlow, 1978). Furthermore the antibody profile in colostrum following an E. coli infection of the sow during the last 2 to 3 weeks of pregnancy are predominantly of the IgM class (Porter, Linggood & Chidlow, 1978).

In an attempt to stimulate a natural production of specific antibody a "feedback" system is advocated by some workers in which dung from sows with litters suffering from neonatal diarrhoea is fed to pre-parturient sows. An extension of this practice is the oral dosing of sows with cultures of live enteropathogens some two weeks before parturition (Kohler, Cross & Bohl, 1975). Whilst these procedures can stimulate the production of hyperimmune colostrum containing antibodies of the IgM class (Porter, Linggood & Chidlow, 1978) and mortality from neonatal E. coli enteritis is reduced, the heavy infection load introduced into the environment adversely affects the offspring and although they survive, their ability to thrive is severely impaired (Porter, Allen & Blades, 1979).

Recently a vaccination protocol combining the benefits of both oral and parenteral immunization has been advocated (Chidlow & Porter, 1979a) with the twin objectives of reducing the overall level of enteropathogenic coli in the farm environment and at the same time stimulating the production of hyperimmune colostrum antibody, mediated mainly by IgM. In porcine neonatal enteritis, the sow is a potential source of infection (Arbuckle, 1968); by oral immunization during the last 60 days of gestation this source of environmental contamination can be reduced (Chidlow & Porter, 1979b). At the same time oral immunization has a priming effect such that a single intramuscular injection of the same vaccine antigens, without adjuvant, results in the production of a high level of circulating IgM antibody. By timing the injection, so that the peak of IgM production coincides with the translation of immunoglobulins from the circulation to the colostrum, potent passive protection against neonatal enteritis is assured (Chidlow & Porter, 1977).

This protocol has not yet been exploited for the prophylaxis of colisepticaemia in the bovine, although oral administration of antigens to this species does produce a similar priming effect on the circulatory antibody response (Allen & Porter, 1975), resulting in elevated levels of serum IgM antibodies (author's unpublished results). Furthermore in the bovine, as in the pig, colostrum IgM antibodies confer the greatest degree of protection to the neonatal calf (Logan & Penhale, 1971).

II) E. coli associated enteritis

During the first few days of life the levels of passively acquired antibody declines (Porter & Hill, 1970) due to increasing blood volume, catabolism and utilization to provide immune protection. Sow milk contains secretory IgA antibodies, the main function of which appears to be the provision of further local defense in the alimentary tract (Porter, 1969a; Hill & Porter, 1974). There is no similar component in any quantity in bovine milk however (Porter & Noakes, 1970). Experience has shown that the protection conferred by maternal antibodies lasts for as little as the first week and the young piglet or calf increasingly depends on the development of its active secretory immunity for survival against later infection by enteropathogens.

The concept of oral vaccination to control enteric microbial infections is not new. It was well described by Besredka (1927) who demonstrated that rabbits could be protected against lethal doses of *Shigella dysenteriae* by being given oral doses of heated cultures of the pathogen beforehand. However, because at that time, the efficacy of immunization protocols was assessed by their ability to produce blood borne antibodies, which is not a characteristic of oral vaccination, the significance of these

and similar earlier studies has only been appreciated during the last 20 years or so. Successful vaccination of children against poliomyelitis by oral administration of inactivated virus (Sabin, Michaelis, Ziring, Krugman & Warren, 1963) contributed much to promoting fresh interest in this area.

Similarly, studies in fistulated baby pigs and calves have shown that the appearance of antibodies in intestinal secretions can be stimulated by local application of heat inactivated E. coli antigens within the first 3 weeks of life, providing the basis for enhancing the competence of the young animal to cope with the challenge from enteropathogens after weaning (Porter, Kenworthy, Noakes & Allen, 1974; Allen & Porter, 1975). In neither species however did the secretion of intestinal antibody provide evidence of an amnestic response. This apparent lack of memory in the secretory antibody system, in contrast to that normally associated with systemic immunity has also been reported by other workers (Freter & Gangarosa, 1963; Ogra & Karzon, 1969; Lee & Foo, 1978).

In practical terms, oral vaccination alone has much to commend it. In the farm situation, inclusion of the vaccine in feed ensures that it is given regularly without either the need for extra labour, or handling of animals. Thus the requirement for repeated dosing is met and more importantly the vaccine is given without causing stress, since this is a major factor in E. coli enteritis. The value of oral vaccination in controlling E. coli disease in the weanling piglet and calf has been extensively tested both at laboratory level and in extended field trials (Porter, Kenworthy, Holme & Horsfield, 1973; Porter, Kenworthy & Allen, 1974; Porter, Kenworthy & Thompson, 1975; Balger, Chorher, Plank, Barstedt, Schels & Meyr, 1976; Cottureau, Ferrando, Tournut & Faugere, 1978). The benefits have clearly been demonstrated in terms of a decrease in the numbers of enteropathogens infecting the gut, lowered mortality, less requirement for medication and improved health resulting in better overall performance.

CONCLUSIONS AND AREAS FOR FURTHER STUDY

Colibacillosis is an ever present problem in modern livestock management, one which, with increasing intensification, is gaining in severity to the point which forces farm units to cease operating because they are no longer profitable. In many

instances massive use of drugs is failing to control the problem, and indeed is exacerbating it by creating even greater problems of drug resistance. Against this background the prophylactic use of oral vaccines for young pigs and calves constitutes a radically new concept in herd health management, particularly when combined with the oral-parenteral protocol for vaccination of the dam to confer enhanced passive protection to the neonate.

Increasing intensification has brought a demand for earlier weaning. More and more, pig herds previously weaning at 5-6 weeks of age are changing to 3-4 week weaning and there is an increasing number of units weaning earlier than 3 weeks (Ridgeon, 1979). Calves are regularly removed from their dams soon after birth and after passing through several markets, where they are subjected to severe stress and risk continual infection, arrive on rearing units barely 1 week old. In these situations the requirement for procedures which will permit the stimulation of active secretory immunity at the earliest possible moment assumes even greater importance.

No one has yet studied macromolecular uptake in the intestine of the very young animal following closure to maternal immunoglobulin. The question whether the passage of antigen across the mucosa in the younger animal is sufficient to stimulate an ultra-early gut secretory immune response, capable of coping with the challenge created by modern intensive management, remains to be determined. Indeed the effects of coli infection on the developing neonate have not been fully investigated. This could be a significant factor in both the ability to stimulate early secretory immunity and on the animals overall performance.

Table 1.1

Escherichia coli serotypes associated with enteric conditions
in pigs (Sojka, 1973, Modified Linggood, 1978)

I. Enterotoxigenic types.

A. Strains frequently encountered in colibacillary diarrhoea
particularly in new born piglets.

08 : K87, K88ab

08 : K87, K88ac

045ac : K'E65', K88ac

0138 : K81, K88ac

0141 : K85ab, K88ab

0147 : K89, K88ac

0149 : K91, K88ac (Abbotstown)

0157 : K'V17', K88ac

B. Strains less frequently found in colibacillary diarrhoea

0157 : K'V17'
09 : K'P16'
010 : K'V50'
035 : K'V79'
0108 : K'V189'
0115 : K'V165'
0119 : K'V113'
064 : K'V142'
0101 : K'613'

C. Strains often found in both oedema disease and colibacillary diarrhoea particularly in weaned pigs

0141 : K85ab - K85ac
0141 : K85ac
0138 : K81

II. Nonenterotoxigenic groups associated with oedema disease.

0139 : K82
045ac : K'E65'

SECTION 2

OBSERVATIONS ON THE MORPHOLOGY OF THE SMALL INTESTINAL
MUCOSA OF THE DEVELOPING PIGLET

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BACKGROUND AND OBJECTIVES

Toxic molecules and infectious agents most frequently gain access to the body across mucosal membranes lining those organs which are in direct contact with the external environment. Of these, the intestinal tract encounters the greatest level of challenge and is most likely to suffer impaired functional efficiency in consequence. Toxic dietary and microbial metabolites within the lumen can have a profound effect on both mucosal architecture and its functional efficiency. Indeed the mucosa may become so deranged that it is no longer able to absorb sufficient nutrient to support life.

Intestinal malabsorption is frequently associated with alteration in the mucosal morphology (review: Jeffries, Weser & Sleisenger, 1964) and numerous studies have been made of factors which may bring about such changes. The microbial flora has been shown to have a considerable effect on a variety of tissues in the gastro-intestinal tract. In studies of intestinal tissues, germ-free animals compared to their conventional counterparts, showed lower values in terms of weight (Glimstedt, 1936; Gordon, 1959) lymphoid tissue (Glimstedt, 1936; Gustafsson, 1948; Miyakawa, Iijima, Kobajashi & Tajima, 1957; Gordon, 1959; Phillips Wolfe & Gordon, 1959) and development of the lamina propria (Gordon, 1959; Gordon & Bruckner-Kardoss, 1961; Kenworthy & Allen, 1966b).

A malabsorptive syndrome associated with the stress of weaning has been demonstrated in the young conventional pig (Kenworthy & Allen, 1966a). Conversely a marked hyperpermeability to macromolecules, by the mucosal lining, may result from the release of vaso-active compounds through mast cell degranulation as part of the inflammatory response (Murray, Jarrett & Jennings, 1971) permitting the passage into the lamina propria of toxic molecules which would otherwise be excluded.

Defects of mucosal epithelium could have a profound effect on the development and efficiency of intestinal secretory immune mechanisms. These may vary from either a reduction or stimulation in the production of antibodies to an interference with their secretion across the epithelial lining.

The early development of an effective secretory immune defense in the intestine is crucial to the survival of the young animal, particularly in intensively reared farm livestock, and the condition of the gut mucosal architecture is clearly relevant to its ontogeny. Therefore a morphological study of the small intestine of young pigs was undertaken covering the first few weeks of life through weaning. These studies were an essential background for a fuller understanding of events occurring in

the developing gut mucosa during this critical period. In addition a comparative evaluation was made of the effect of postweaning E. coli infection on the absorptive epithelium and a sequential study of the changes occurring in the intestinal mucosa of piglets of varying age performed.

MATERIALS AND METHODS

Tissues

Intestinal tissues used for these investigations were derived from a variety of sources. Those used for the sequential morphological studies were taken from the two litters of piglets varying in age from 8 to 28 days and included both animals suckling on the dam, and those which had been weaned for a varying period of time (table 2.1). This group of animals was also used for the studies on the influence of age on macromolecular uptake (*vide infra* - Section 4).

Material used in the comparative investigations included specimens from a germ free pig obtained from laboratory files as well as tissues taken from conventional piglets at post mortem (*vide infra* - page 32)

Observations were made on tissues from 3 levels of small intestine, the duodenum, taken immediately distal to the pyloric junction; the mid jejunum and the terminal ileum immediately proximal to the ileocaecal junction. All tissues were obtained within 5 minutes of death and fixed in buffered formol saline pH 7.2.

Macroscopy

For macroscopic observation the formol fixed tissues were opened along the line of mesenteric attachment and reflected to enable the luminal surface to be viewed under a Projectina projection microscope. The specimens were immersed in buffer and illuminated by oblique light to avoid reflections. Macro-photographs were taken on Ilford FP4 cut film using optics which gave a final magnification of x30.

Scanning Electron Microscopy

Selected specimens of the formol fixed tissues were dehydrated through graded alcohols and subjected to critical point drying by a Polaron drier E3000. They were then coated with platinum in a Polaron Sputter coater E5100 for 3 minutes at 20 m.a. at 2.4kv. in an Argon atmosphere of 0.05 torr. Scanning studies were conducted on a JEOL JSM35 scanning electron microscope with an accelerating voltage of 5-15KV.

Microscopy

Haematoxylin and Eosin stained sections of paraffin embedded blocks of the formolin fixed tissues were prepared by standard techniques. The sections were examined on a Reichert Diapan microscope and photographs taken on Ilford FP4 film using a Reichert "Photo automat" camera.

Measurement of Surface Area

Comparative measurements of the intestinal epithelial surface area were made on haemotoxylin and eosin stained sections of tissues from three 5 week old pigs. One was a germ free pig maintained on a bovine milk based diet supplemented with vitamins and minerals (Kenworthy & Allen, 1966b), the remaining two animals were conventionally reared animals which had been weaned at 21 days of age onto standard early weaning pellets, one was suffering from a moderately severe Escherichia coli associated enteritis, the other had recently recovered from the condition. All three animals were involved in experiments not otherwise associated with the present study.

Five segments of tissue from each of three areas of small intestine, duodenum, jejunum and ileum per animal were examined, five serial transverse sections, 5µ thick were prepared from each segment, a total of 75 sections per animal, 25 from each area.

Each section was systematically scanned on a Quantimet 720 B computerised microscope using a x2.5 objective with a x10 eyepiece. The computer was programmed to measure the perimeter of the epithelial surface, all other extraneous edges were edited using a light pen (Plate 2.1). Measurement was in picture points

which were converted to μm using a calibration of 0.206 picture points per micron. Each field was measured 5 times and the mean recorded. The relative differences in surface areas were calculated by comparing the means of the lengths of the epithelial cell perimeters of the 25 sections from each area.

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RESULTS

Gross Morphology

At post mortem the small intestine of the unweaned piglets appeared normal. However 2 of the animals weaned for 1 and 3 days respectively (pigs nos. 41 and 38) showed a slight reddening of the duodenal mucosa whilst in the pig weaned for 7 days (no. 40) the whole of the small intestine was inflamed.

Macroscopy

Macroscopic examination of tissues from the duodenum, jejunum and ileum of piglets of varying ages showed a progressive change in the morphological appearance of villi at all 3 levels, as the animals matured.

Despite individual variation between animals a general pattern emerged. In the younger animals, villi tended to be long, slender, fingerlike processes with intact epithelium (Plate 2.2 and 2.3). As the animals aged they became shorter in length and a varying degree of epithelial desquamation was present. Under the low power microscope desquamated villi gave the appearance of having had their tips shaved off (Plate 2.4) but

scanning electron microscopy clearly demonstrated villi with tips denuded of epithelium leaving the underlying lamina propria exposed (Plate 2.5). This finding was particularly well demonstrated in the duodenum of the 19 day old pig (no. 39).

The bases of villi in the older animals were thickened and villous fusion occurred giving rise to leaflike forms (Plate 2.6). These were particularly prevalent in the weaned pigs.

Whilst the pattern of change was similar at all three levels of intestine, some differences were noted. The duodenum was the region most affected. In the young animal the villi are longer in this area than at more distal sites and consequently stunting was more severe, also epithelial desquamation was most extensive at this site. Very little epithelial desquamation was observed in the ileum although stunted villi occurred at an earlier stage in this tissue than elsewhere.

Microscopy

Microscopic examination confirmed and extended the macroscopic observations. Sections from the younger animals showed long slender villi covered by apparently healthy columnar epithelium having a good nuclear to cytoplasmic ratio and a clearly defined microvillous border. There were, however, early degenerative

changes in the underlying lamina propria. In the duodenum of the 10 day old piglet, for example, some interstitial micro-oedema was observed, particularly towards the tips of villi (Plate 2.7). Similar changes were not found in the jejunum but were occasionally present in ileum. The lamina propria at all levels showed a small degree of infiltration by mononuclear cells, mainly lymphocytes and macrophages.

In older animals, i.e. from about 15 days of age, degeneration of the epithelium was present, pallisading, cytoplasmic vacuolation and pyknotic nuclei were seen. The nuclear/cytoplasmic ratio declined. In the more severely affected animals (e.g. no. 26) epithelial cells became either cuboidal or even flattened (Plate 2.8). Inter-epithelial theliolymphocytes were more numerous in the older animals compared with the younger ones, in which they were comparatively few.

The sparsity of goblet cells on duodenal and jejunal villous epithelium was particularly noticeable. Whereas the numbers of goblet cells in the crypts appeared normal, relatively few cells were containing mucin were seen above the crypt villous junction. This was most marked in pigs which had been weaned.

The underlying lamina propria showed more extensive oedema, leading to separation of the epithelium from the villous core and eventual desquamation (Plate 2.9). This occurred in the duodenum as early as 12 days of age. Increased infiltration by lymphocytes plasma cells and macrophages and to a lesser extent polymorphonuclear leucocytes occurred. The plasma cells were mainly confined to intercryptal lamina.

There was an age related association in the severity of the degenerative changes. In general, the older the animal the more severe the change although there were occasional exceptions, mainly in unweaned piglets. In the weaned pigs the severity of the changes were clearly influenced by the length of time the animals had been weaned and were most marked in pig 40 which had been weaned for 7 days.

Surface Area Measurement

The effect which changes in morphology of the small intestine had on the epithelial surface area was evaluated by comparative measurements of a series of sections by the Quantimet image analyser. The measurements were made on tissues from three 5 week old pigs - one a germ free animal, the other two conventionally reared. One of the conventional animals was suffering from E. coli post-weaning enteritis whilst the other had recently recovered from this condition.

The tissues of the germ free pig morphologically resembled those of a new born animal. The villi were long slender fingerlike processes with an intact epithelium. The underlying lamina propria showed a minimum of cellular infiltration (Plate 2.10). In contrast the two conventional animals showed a considerable alteration in morphology. The diarrhoeic animal had extensive epithelial desquamation accompanied by infiltration of the lamina propria by mononuclear cells, lymphocytes and macrophages (Plate 2.11). Whilst, the recovered animal showed extensive villous fusion and the presence of leaf-like structures. In this animal also, cellular infiltration of the lamina propria was marked (Plate 2.12).

Table 2.2 shows the means of the measurements of the epithelial cell surfaces of each of 3 regions of the small intestine of the 3 pigs. Figure I expresses the change in surface area of the two diseased animals relative to that of the germ free pig which is taken as 100%.

There was a decrease in surface area at all 3 levels of the gut in both pigs. The loss of area being most marked in the duodenum. In the recovered pig, the duodenal surface area was only 20% of that of the germ free animal. Interestingly the surface area of the recovered pig was less than that of the pig suffering from enteritis, this is clearly shown by comparing sections of the two animals.

DISCUSSION

The mucosal architecture of the small intestine may be influenced by a variety of factors. These include dietary components (Rubin, Brandborg, Flick, Phelps, Parmentier & Van Niel, 1962) and bacterial metabolites (Pearson, McNulty & Logan, 1978); changes may occur both in the epithelium lining the villi and in the underlying lamina propria. Epithelial desquamation of villi appears to be a general phenomenon of the gut response to injury (Takeuchi, Sprinz, LaBrec & Formal, 1965), whilst changes in the underlying lamina propria which include interstitial oedema, cellular infiltration by polymorphs, lymphocytes and other inflammatory cells, (Kenworthy & Allen 1966b) are reactions to the presence of deleterious substances in the tissues.

The finding in this study that inflammatory changes can occur in the intestinal mucosa of apparently healthy pigs from a very early age is of importance for it suggests the possibility of a deficiency in the immune defense at this time. In contrast to primates which acquire passive protection via maternal antibodies transferred from the maternal to foetal circulation during the third trimester of pregnancy (Hyvarinen, Zelter, Oh & Stiehm, 1973) neonatal pigs, calves and lambs rely on the absorption of colostrum antibodies immediately after birth (Brambell, 1970). These antibodies are absorbed, intact, across the intestinal epithelium and provide the new born animal with a

Level of serum antibody (Porter, Noakes & Allen, 1970; Porter & Hill, 1970). Some of this absorbed antibody may be resecreted into the intestine to provide a measure of early protection (Allen & Porter, 1973b). However the protective efficacy of passively derived antibody rapidly declines in the pig lasting for just over 1 week (Porter, Parry & Allen, 1977).

The fall in the level of passive immunity gives rise to a deficiency in systemic antibody during the first 2-4 weeks of the pig's life which may be of crucial importance (Miller, Harman, Ullrey, Schmidt, Leucke & Hoefer, 1962). To some extent this loss of protection is ameliorated by the secretory IgA antibody present in sow's milk, which, since it is not absorbed by the piglet, appears to function by providing a measure of local defense in the lumen of the alimentary tract (Porter, 1969b; Hill & Porter, 1974). The effectiveness of milk IgA in protecting against infection by enteropathogens has not been evaluated although orally administered antibodies have been shown to afford some degree of benefit (Kohler & Bohl, 1966; Miniats, Mitchell & Barmum, 1970; Brandenburg & Wilson, 1973).

The piglets used in the present studies were derived from multiparous sows maintained in a closed herd and whilst levels of specific antibody in the sows' milk were not determined it is unlikely that they would be subnormal. Nor was there any evidence to suggest impaired absorption by the piglets, of milk components. The lacteals were clearly discernible in the mesentry, at post-mortem, by their milk fat contents.

The evidence of early inflammatory change seen in animals only 10 days old may be associated with the clinical appearance of "milk scour" in pigs from 12 days of age. Moreover if, as seems likely, these morphological changes are accompanied by impaired functional efficiency, animals reared in an environment heavily infected with enteropathogens are subjected to even greater risk. This would explain why the 'milk scour' condition often becomes complicated by infection with pathogenic E. coli. It highlights a possible deficiency in the degree of protection conferred by sow milk antibodies at a time when the piglets own active response has not yet developed sufficiently to generate a significant level of defense. Furthermore the current trend towards weaning as early as 14 days of age appears to be fraught with danger. It will only be successful if active secretory immunity can be stimulated at a very early age affording better protection of the mucosal surface. Injury to the intestinal mucosa at this critical time may not only delay the onset of active synthesis by immunocytes populating the lamina but could interfere with the transfer of secretory antibody across the epithelium.

Alteration of the morphological structure of intestinal mucosa has considerable bearing on its ability to function (Zamcheck, 1960). The effects of injury to surface epithelium is not confined to the absorptive ability of individual cells but may have far reaching long term consequences for the host.

Epithelial cells are generated by the division of relatively undifferentiated cells in the crypts of Lieberkuhn, they migrate from the crypts upwards towards the tips of the villi from where they are normally extruded into the gut lumen (Leblond & Stevens, 1948; Leblond & Meissier, 1958). As they migrate the cells mature, attaining an enhanced absorptive ability as they ascend the villus (Kinter, 1961). An increase in the rate of cell loss results initially in an increase in the rate of migration of cells from the crypt without, however, any increase in the rate of maturation. There is in consequence a rise in the proportion of immature cells clothing the villi.

Changes in the rate of epithelial cell turnover may also affect the functional capability of the cryptal cells. In the pig these cells are responsible for transporting secretory antibodies, synthesised locally in plasma cells populating the lamina propria underlying the crypt (Allen & Porter, 1973a; Allen, Smith & Porter, 1973; 1976). They are also responsible for the synthesis of secretory component (Brandtzaeg, 1974; Allen & Porter, 1973b). It is interesting to note that in studies

on weanling pigs, whereas there was no apparent lack of secretory component in cryptal epithelium, absence of secretory immunoglobulin was observed in some crypts even though the underlying plasma cells appeared to be synthesising normally (Allen & Porter, 1973b).

As the level of inflammation increases and the rate of epithelial desquamation rises and villous atrophy occurs, adjacent villi fuse and assume a variety of bizarre forms ultimately resulting in a reduction of the surface area of the absorptive lining of the gut (Kenworthy, 1967). These changes occur to a varying degree at all levels of the affected small intestine. Previous attempts to quantify the extent of surface area loss have relied on counts of epithelial cells, or planimetric measurement of projected images. Such methods are both time consuming and tedious and are fraught with inaccuracies. The advent of image analysis has enabled the quantification of a variety of parameters in histological preparations, with a hitherto unattainable accuracy. Comparison of surface area loss by image analysis, in the present study, not only demonstrated the extent of the damage throughout the gut but more particularly highlighted the fact that the duodenum was affected to a far greater extent than more distal tissues. Since the proximal gut is the major site for nutrient digestion and absorption (Holdsworth, 1972) the severe loss of surface area and functional

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efficiency occurring in this region as a result of enteropathogenic coli infection results in nutrient malabsorption (Kenworthy & Allen, 1966a) and a failure of the affected animal to thrive. The contribution of oral immunization towards minimising the effect of impaired nutrition has been shown in studies on older pigs orally vaccinated with polysaccharide from pathogenic E. coli. Vaccinated animals showed an average improvement in weight gain of 0.7 Kg over the first 4 weeks after weaning, compared with unvaccinated controls (Porter, Kenworthy & Allen, 1974).

Table 2.1

Age and number of days weaned of pigs used for sequential study

<u>Pig No.</u>	<u>Litter No.</u>	<u>Age</u>	<u>Days Weaned</u>
30	1	8	0
32	1	10	0
34	1	12	0
31	2	15	0
36	1	16	0
33	2	17	0
39	1	19	0
35	2	21	0
41	1	23	1
37	2	23	2
42	1	24	2
38	2	24	3
43	1	26	4
40	2	28	7

Table 2.2

Linear surface measurement of small intestine of 3 five week old piglets of varying health status

<u>Pig No.</u>	<u>Health status</u>	<u>Linear surface u</u>		
		<u>Duodenum (SD)</u>	<u>Jejunum (SD)</u>	<u>Ileum (SD)</u>
151	Germ free	547,842 (<u>+115,191</u>)	392,074 (<u>+85,000</u>)	601,168 (<u>+104,391</u>)
117	Scouring	188,663 (<u>+45,144</u>)	213,190 (<u>+60,304</u>)	283,330 (<u>+91,763</u>)
79	Recovered	99,056 (<u>+32,317</u>)	122,126 (<u>+17,557</u>)	142,459 (<u>+20,934</u>)

Results are expressed as means of measurement on 25 transverse sections at each intestinal level. (SD) = Standard Deviation.

Relative Surface Area of Piglet Small Intestine

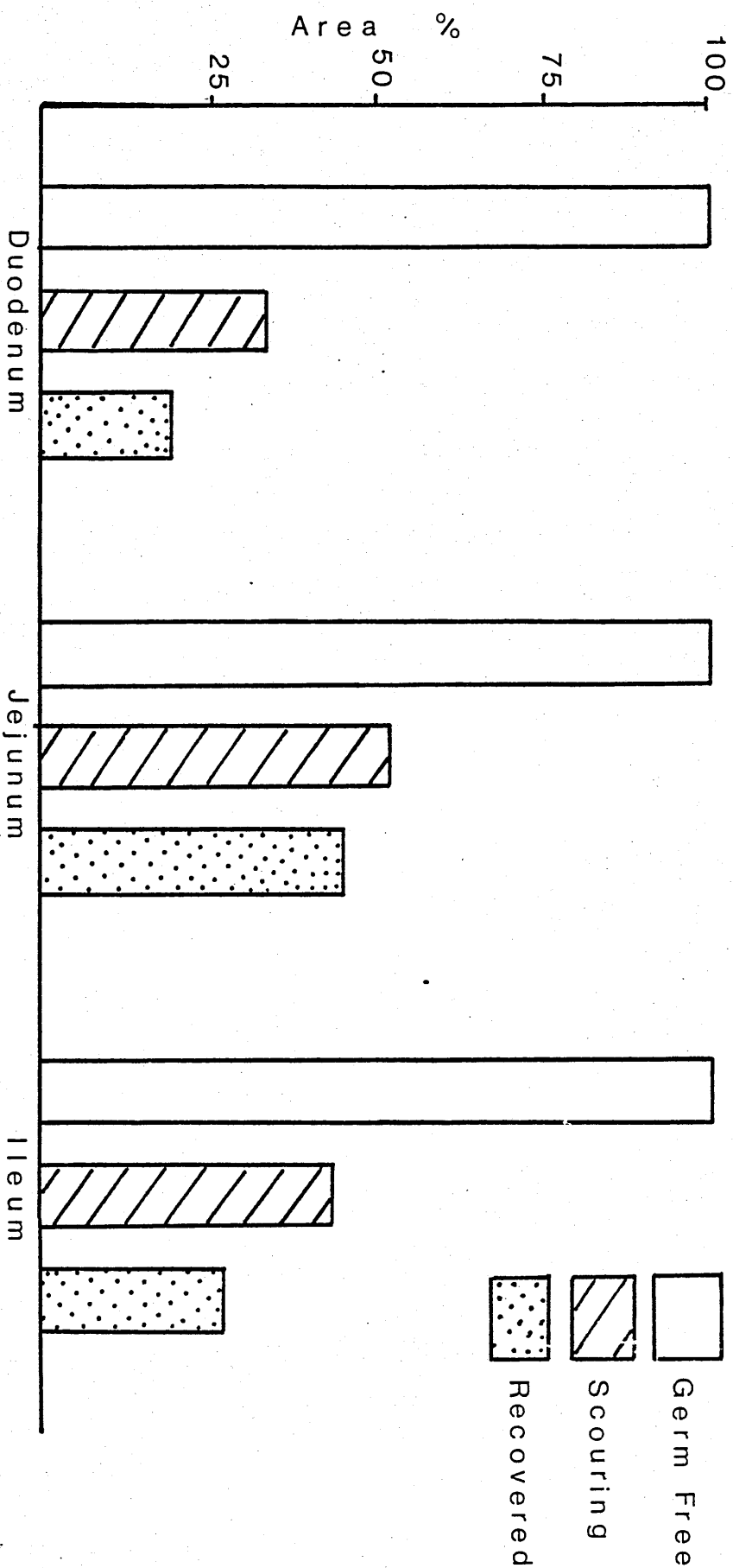


Figure 2.1

Legends

Plate 2.1 Photographs of the display on the viewing screen of the Quantimet 720 B computerised microscope.

- a) An area of transverse section of intestine stained by haematoxylin and eosin. The mode is set to give a normal microscopic image.
- b) The same field as in (a) with the mode set to define all tissue edges.
- c) The same field as (b), only the perimeter of the epithelial surface is now displayed. All other extraneous edges have been removed by editing.

Plate 2.2 Macrophotograph of the anterior small intestine of an 8 day old piglet (No. 30) showing long slender fingerlike villi (x 30).

Plate 2.3 Scanning electron micrograph of the anterior small intestine of an 8 day old piglet (No. 30) showing the intact epithelial covering of the villi (x 200).

Plate 2.4 Macrophotograph of the anterior small intestine of a 19 day old unweaned piglet (No. 39). The tips of many villi show erosion of the epithelium exposing the underlying lamina propria (x 30).

Plate 2.5 Scanning electromicrograph of the anterior small intestine of a 19 day old unweaned piglet (No. 39) showing desquamation of the epithelium, exposing the underlying villous cores. Note also the presence of intact villi (x 200).

Plate 2.6 Macrophotograph of the anterior small intestine of a 28 day old piglet, weaned for 7 days (No. 40). There is extensive shortening and fusion of the villi resulting in bizarre, leaflike structures (x 30).

Plate 2.7 Microphotograph of the duodenum of a 10 day old piglet (No. 32). Although many villi are long and slender, sub-epithelial oedema is clearly shown. There are signs of early cellular infiltration of the lamina propria. (H+E x 500).

Plate 2.8

Microphotograph of the duodenum of a 16 day old piglet (No. 36). There is a reduction of the nucleus/cytoplasm ratio of the epithelial cells resulting in many appearing cuboidal rather than columnar, pallisading of epithelial cells is also seen. The lamina propria is infiltrated by mononuclear cells (H+E x 850).

Plate 2.9

Microphotograph of the duodenum of a 21 day old piglet (No. 35). Sub-epithelial oedema is clearly shown causing separation of the epithelium from the villous cores and epithelial desquamation (H+E x 500).

Plate 2.10

Microphotograph of the anterior jejunum of a 5 week old germ free piglet. The villi are long and slender with intact epithelium. The ratio of cytoplasm/nucleus of the cells is high and there is a minimum of cellularity of the lamina propria (H+E x 320).

Plate 2.11 Microphotograph of the anterior jejunum of a 5 week old conventional weaned piglet, suffering from enteropathogenic coli associated enteritis. The villi are shortened and there is epithelial desquamation of the tips. Some cellular infiltration of the lamina propria is also apparent (H+E x 320).

Plate 2.12 Microphotograph of the anterior jejunum of a 5 week old conventional weaned piglet which suffered enteropathogenic coli associated enteritis at 4 weeks of age. The villi are almost non-existent, the epithelial cells show evidence of marked degeneration and derangement, there is considerable infiltration of the lamina propria by mononuclear cells (H+E 320).

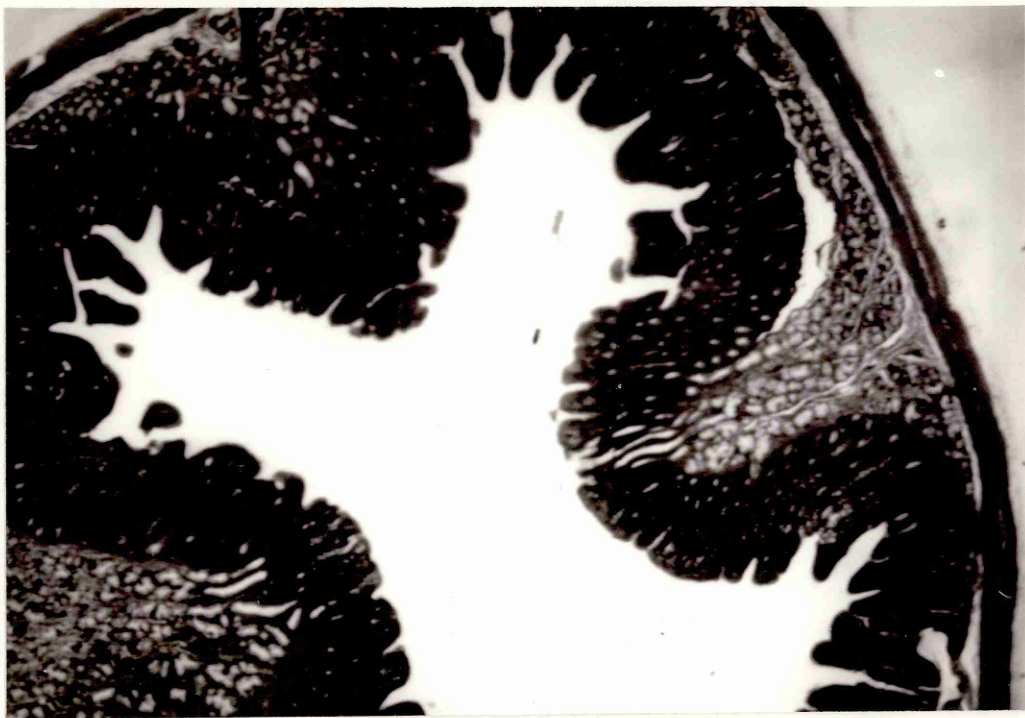


Plate 2.1(a)



Plate 2.1(b)

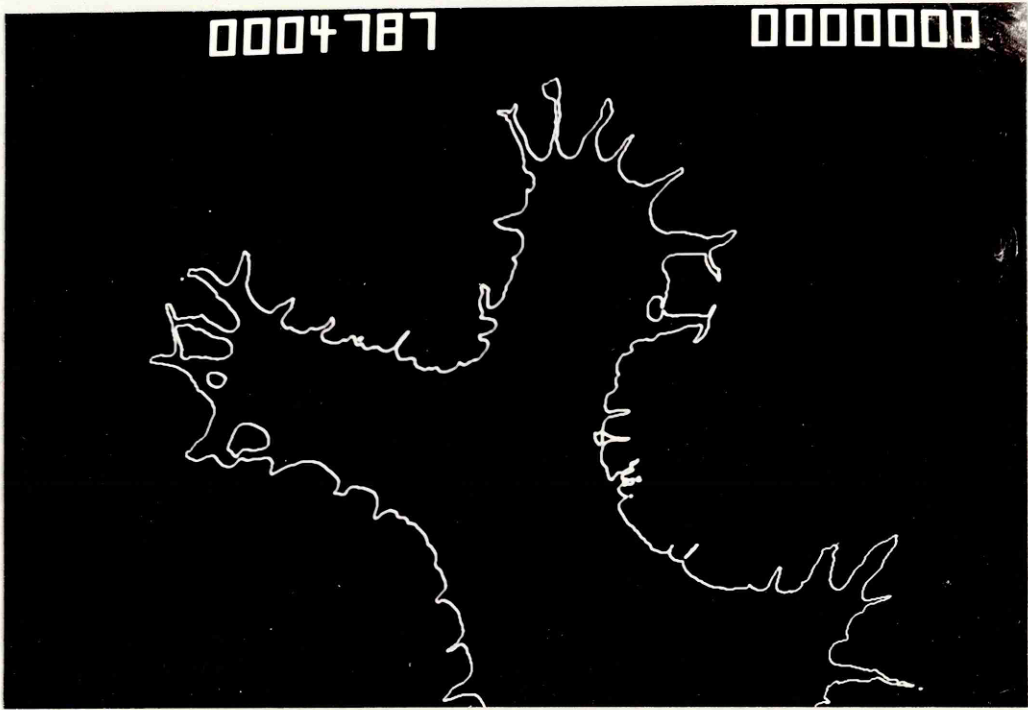


Plate 2.1 (c)

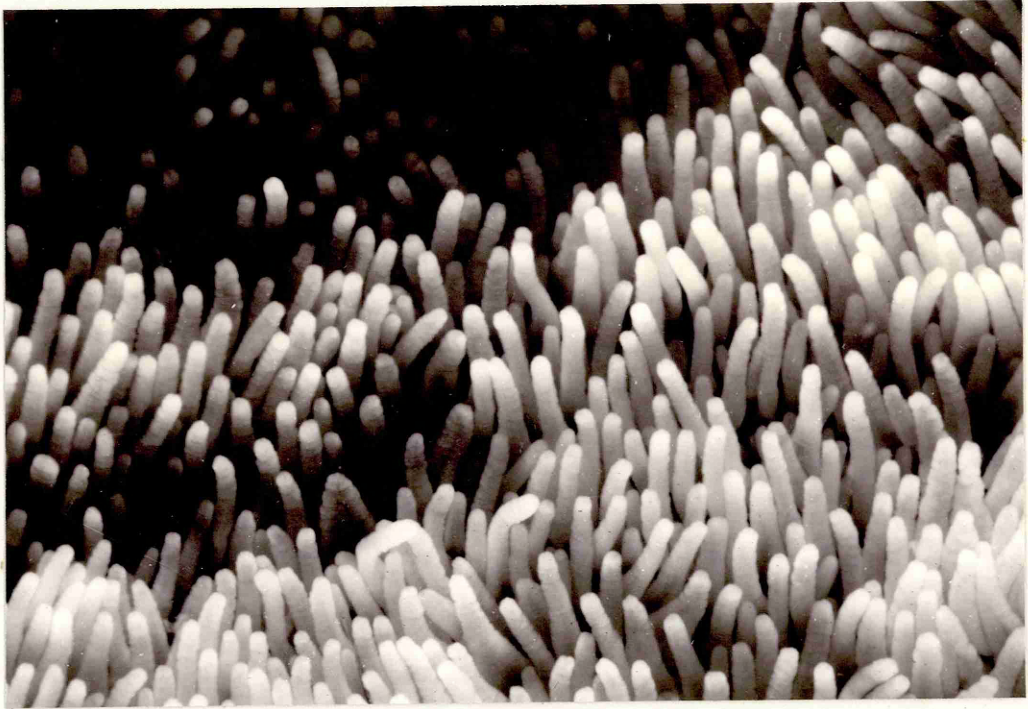


Plate 2.2



Plate 2.3



Plate 2.4



Plate 2.5



Plate 2.6

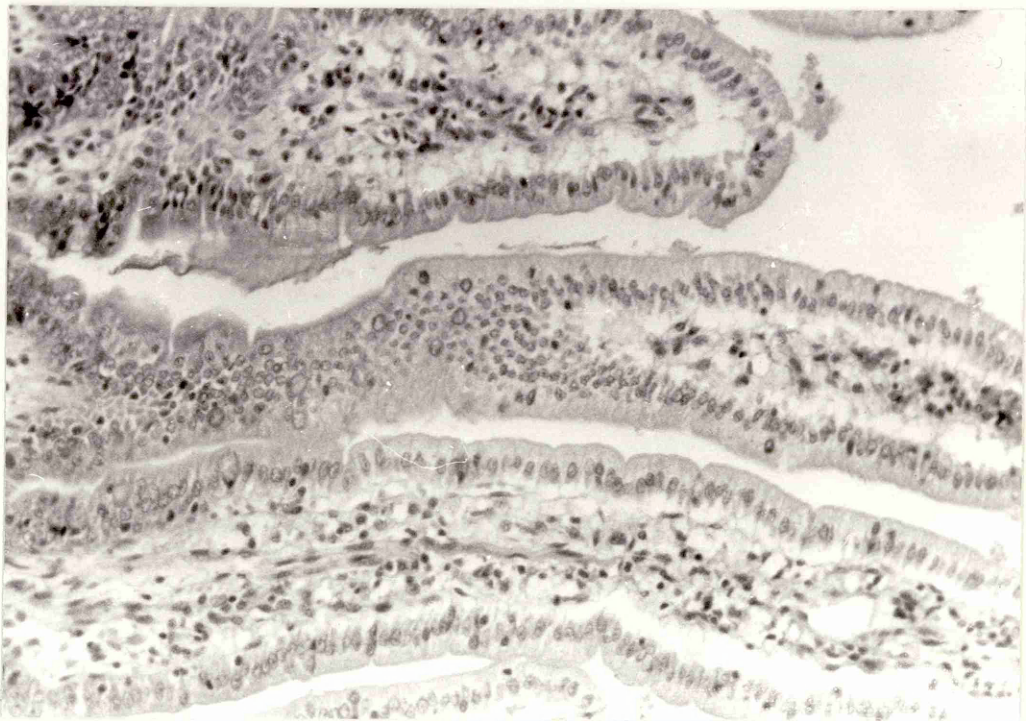


Plate 2.7

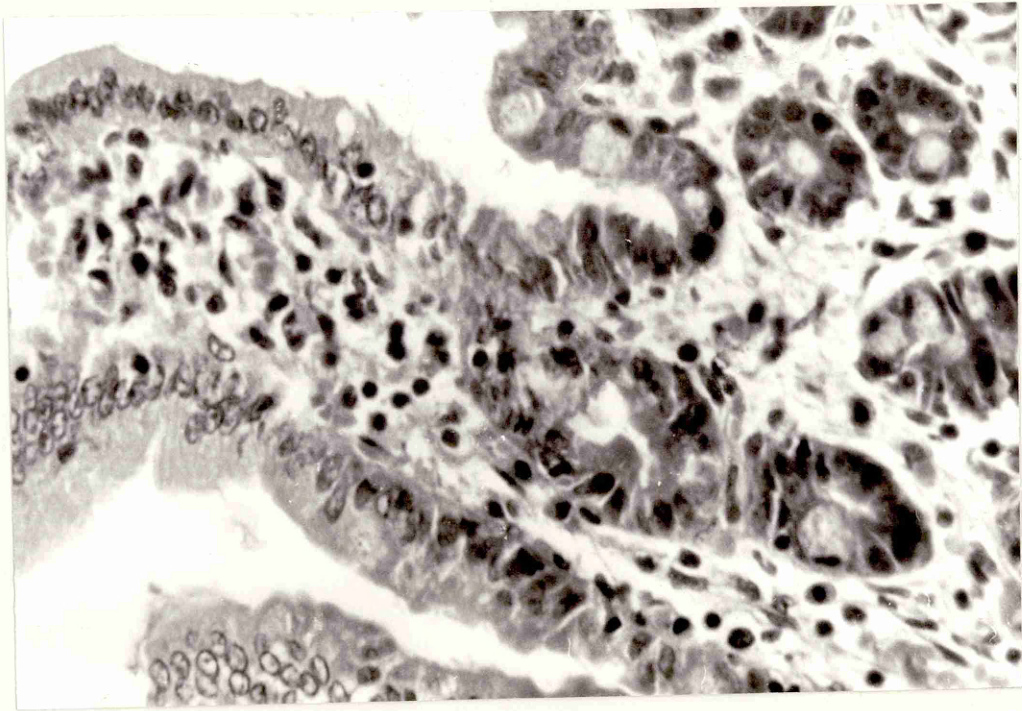


Plate 2.8



Plate 2.9

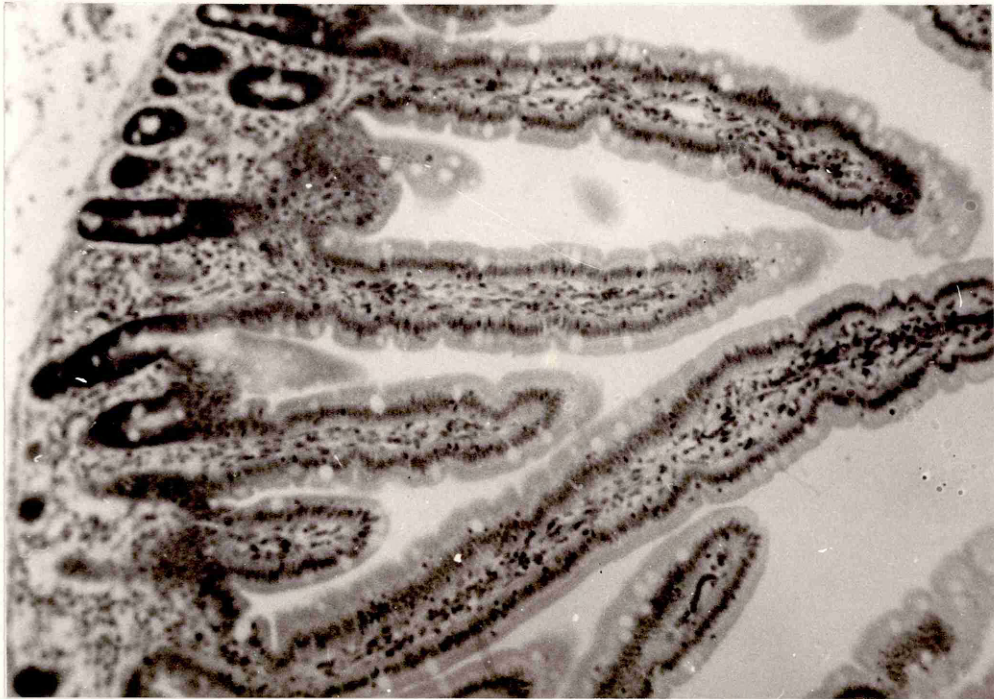


Plate 2.10

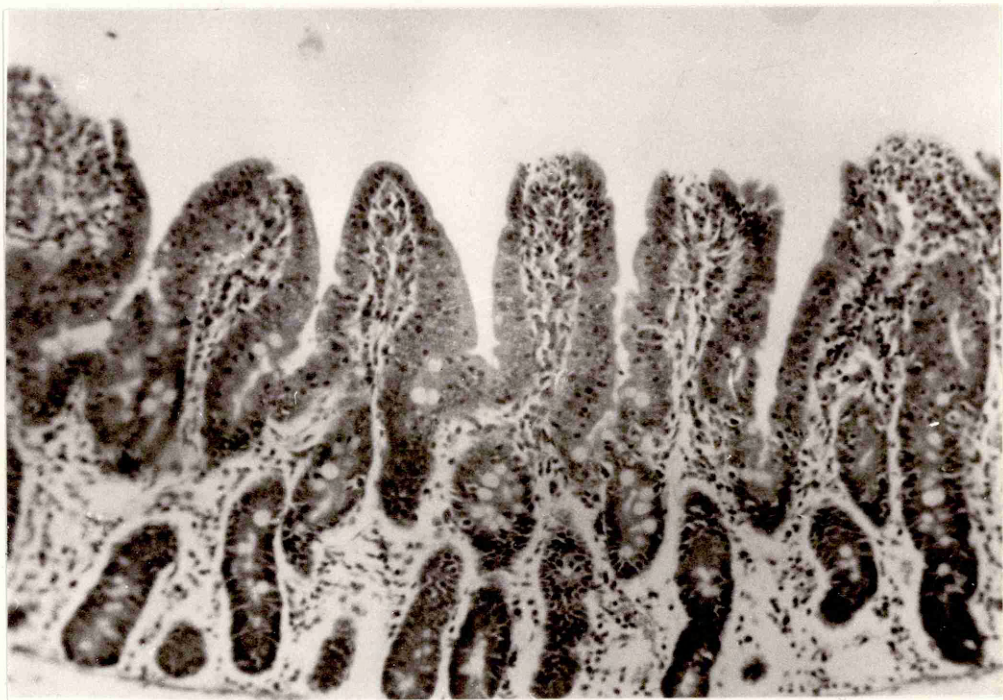


Plate 2.11

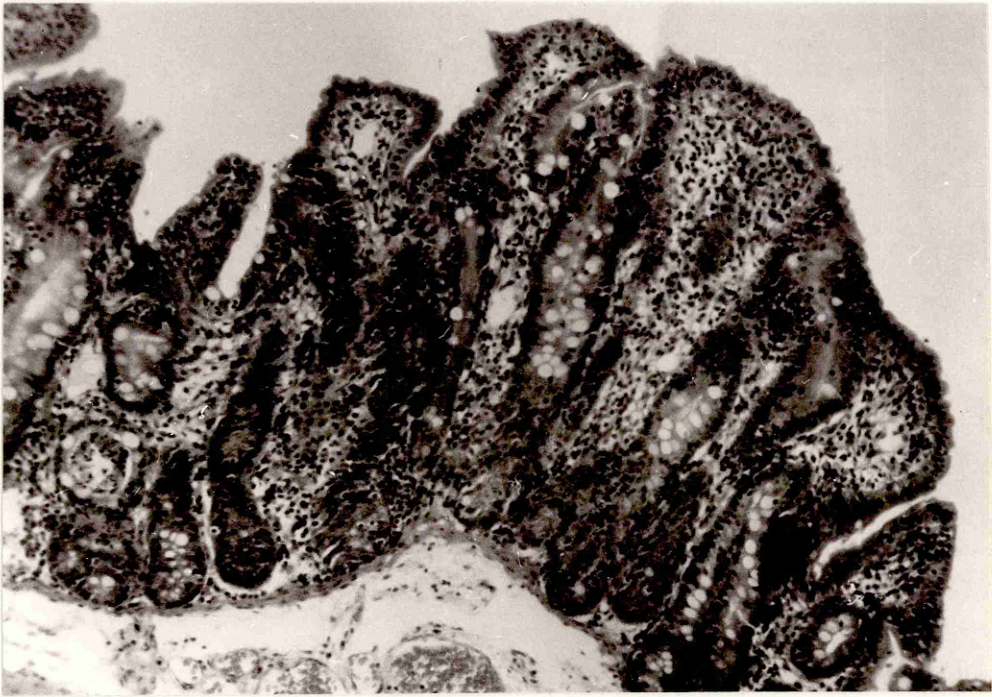


Plate 2.12

SECTION 3

A COMPARATIVE STUDY OF THE RELATIVE ABILITY OF
DIFFERENT LEVELS OF PORCINE SMALL INTESTINE TO
ABSORB MACROMOLECULES

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BACKGROUND & OBJECTIVES

Whilst there is considerable evidence to show that intact proteins can be absorbed by epithelial cells lining the small intestinal tract of the neonate of many species, uptake of antigenic macromolecules by adult intestinal epithelium has been less well defined. Whereas the quantity of maternally derived globulin absorbed by neonatal enterocytes is considerable, once gut closure has occurred amounts fall to nutritionally insignificant levels. Indeed the failure of earlier workers to demonstrate the uptake of macromolecules in adult animals led to the conclusion that macromolecular absorption ceased when the epithelial cell membrane matured. (Clark, 1959; Payne & Marsh, 1962).

However, there is evidence which clearly demonstrates that macromolecules can be absorbed, in quantities sufficient to elicit a response in the host. (Dayton, Small, Channock, Kaufmann & Tomasi, 1969). Ferritin, when administered to germ free mice in the drinking water gave rise to specific antibody containing cells in the intestinal lamina propria (Crabbe, Nash, Bazin, Eyssen & Heremans, 1969). Studies in both fistulated piglets and calves have shown that the introduction into the lumen of the gut, of heat inactivated E. coli antigens will stimulate both a cellular and secretory response in the local

intestinal secretory immune system (Porter, Kenworthy, Noakes & Allen, 1974; Allen & Porter, 1975). Oral immunization of young pigs and calves with heat inactivated pathogenic E. coli antigens stimulates a secretory immune response which confers protection against subsequent challenge by the enteropathogen (Porter, Kenworthy & Allen, 1974; Balger, Chorherr, Plank, Bostedt, Schels & Mayr, 1976). Furthermore oral immunization followed by parenteral immunization has been shown to produce increased levels of circulating antibody (Allen & Porter, 1975; Rowley 1977; Chidlow & Porter, 1978).

Uptake of macromolecules by intestinal epithelium is an energy dependent process (Walker, Cornell, Davenport & Isselbacher, 1972) but it is also influenced by the presence of secretory antibodies. Oral immunization of rats with either human serum albumin (Andre, Lambert, Bazin & Heremans, 1974) bovine serum albumin or horse radish peroxidase (Walker, Isselbacher & Bloch, 1972) results in a significant decrease in the uptake of the immunizing macromolecule on subsequent administration, although the absorption of unrelated macromolecules remains unaffected (Walker, Isselbacher & Bloch, 1972b).

Antigenic stimulation via the gastro-intestinal tract does not invariably result in immunization however. There have been a number of reports of oral administration of antigen giving rise to a specific hyporesponsiveness in the subject when subsequently challenged by the same antigen, whether administered locally or parenterally (Chase 1946, Thomas & Parrott, 1974; Andre, Heremans, Vaerman & Cambiaso 1975, Kenrick & Cooper, 1978). Although the mechanisms whereby this phenomenon occurs are not fully understood they are clearly of importance to the host especially when considering the prospects for enhanced resistance against enteropathogens. However it is thought unlikely to be a general phenomenon and the invariable accompaniment of oral immunization (Rowley, 1977).

The relative capabilities of different regions of the alimentary tract to absorb antigenic macromolecules appears unresolved. In this respect the distribution of immunoglobulin producing cells along the gut mucosa is of interest. The source of intestinal immunocytes is thought to be the gut associated lymphoid tissues (G.A.L.T.) principally Peyer's patches (Craig & Cebra, 1971). This tissue is predominantly located in the distal portion of the intestine. It has an epithelial covering which includes a specialized 'M' cell facilitating the uptake of antigen into the lymphoid follicles (Owen & Jones, 1974). In contrast studies on the distribution of immunoglobulin containing cells at different levels of porcine small intestine

show that the numbers of cells in the lamina propria of the duodenum are more than 10 fold greater than those of more distal sites whilst the numbers of cells in the jejunum is about twice that in the ileum. (Allen & Porter, 1973a.) Similar results have been observed in other species (Crabbe, Bazin, Eysen & Heremans, 1968). The distribution of immunocytes might be expected to reflect the magnitude of antigenic challenge which would suggest that more antigen is absorbed in the duodenum than at more distal sites. It is interesting to note that uptake of horse-radish peroxidase in the rat, was greater in the jejunum than the ileum (Walker, Cornell, Davenport & Isselbacher, 1972).

In the previous section inflammatory change was found to be most severe in the anterior small intestine and in the older animals, particularly post-weaning. The question arose whether these changes were such as to prevent antigen gaining access to the mucosal tissues in sufficient quantity to stimulate a secretory immune response and whether any particular level of the small bowel was more affected than another. The present study was undertaken to examine these points.

MATERIALS AND METHODS

The absorption of horse radish peroxidase (H.R.P.) by small intestine was studied in vitro by methods similar to those described by Walker, Cornell, Davenport & Isselbacher (1972). Tissue was obtained at post mortem from healthy 3-4 week old pigs which had been weaned when 14 days of age. They were killed by intracardiac administration of Euthatal (May & Baker Ltd) and the entire small intestine was quickly removed and placed in Krebs-Ringer solution, previously gassed with a mixture of 95% Oxygen, 5% Carbon dioxide. Care was taken not to damage the tissue during removal, particularly when detaching the duodenum from the adjacent pancreas and colon, lest the serosal layer was stripped away.

Everted gut sacs were prepared according to the methods of Wilson & Wiseman (1954) and Crane & Wilson (1958). The latter preparations enabled samples of serosal fluid to be obtained during incubation. Lengths of intestine of approximately 30cms, were taken from the duodenum, (immediately below the pylorus) the mid jejunum (the mid point was established by doubling the whole of the small bowel) and the ileum (immediately preceding the ileo-caecal junction) and gently everted over a glass rod having a smooth end. Sacs 5cm long were prepared and a measured

volume of Krebs-Ringer introduced into the serosal cavity. Four replicate sacs were prepared from each length of intestine. They were incubated at 37°C for 3 hours in 25cms tubes containing either Krebs-Ringer (controls) or Krebs-Ringer plus 10uM Crude Horse radish peroxidase 50 units/mg (Sigma Chemical Co). The incubation medium was oxygenated with a mixture of 95% O₂/5% CO₂ delivered into the medium through a fine polythene canula and the gas flow adjusted to provide a continuous fine stream of bubbles, sufficient to ensure the circulation of the medium without damage to the tissue.

Samples of the incubation medium were removed at intervals of 1, 60, 120 and 180 minutes and samples of the serosal fluid were removed from the Crane & Wilson preparations at the same times. At the conclusion of the experiments the sacs were washed in running water, gently blotted dry and the serosal fluid removed with a syringe and measured. Any specimen showing evidence of a marked discrepancy between the volumes of serosal fluid at the beginning and end of the experiment were presumed to have leaked and discarded.

Assay of Horse Radish Peroxidase

Horse radish peroxidase was assayed by the modified method of the Worthington Biochemical Corp. described by Walker, Cornell, Davenport & Isselbacher (1972). The rate of increase in optical density at 460mu was determined on a Cecil recording spectrophotometer (Cecil Instruments Ltd, Cambridge, England) enzyme concentrations determined from a standard curve relating enzyme activity to wt of H.R.P. The protein concentration of H.R.P. was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) modified as described by Walker, Cornell, Davenport & Isselbacher (1972). Total protein contents of the gut sacs were determined by an automated Kjeldahl technique (Montag, 1974).

Histochemistry

Histochemical studies were performed on tissues from 3 animals similar to those used for the in vitro absorption studies. Everted gut sacs were prepared and incubated in Krebs-Ringer buffer containing H.R.P. In two animals crude H.R.P. (Sigma) was used and incubation continued for 3 hours, in the third H.R.P. factor VI (Sigma) was substituted and the incubation time shortened to 1 hour. In addition 4mm thick rings of everted gut were also prepared from the third animal and incubated in H.R.P. factor VI. Replicate control tissues were incubated in Krebs-Ringer alone, under identical conditions.

After incubation transverse sections 4mm thick were taken from the sacs and these together with the rings, were fixed for 2 hours in 3% glutaraldehyde in 0.1M cacodylate buffer pH 7.4 and 4°C. They were washed for 18 hours in several changes of cacodylate buffer 4°C.

Peroxidase activity was visualized by incubating the tissues for 2 hours at room temperature in 0.1M tris HCl buffer containing 0.05% 3,3,di amino benzidine tetrahydrochloride and 0.03% H₂O₂ (30 vol). After incubation the tissues were washed for 18 hours in cacodylate buffer at room temperature and paraffin embedded sections prepared by standard techniques.

RESULTS

The rates of uptake and release across the serosa of H.R.P. by duodenum, mid jejunum and terminal ileum was studied in tissues taken from seven 3-4 week old pigs weaned when 14 days of age.

(a) Adsorption of H.R.P.

Uptake of H.R.P. from the incubation medium was measured at intervals over a period of 3 hours. It was found that the rate of uptake during the first minute was extremely high as much as 90% of the total adsorption onto the tissue occurring during this time. This was especially true for the more distal regions of intestine. As incubation continued, the rate of adsorption decreased. The amount of enzyme taken up from the incubation medium reached a peak and then declined presumably due to H.R.P. adsorbed on to mucin becoming detached from the tissue and released back in to the medium. Because the onset of this release was random the results are expressed as the means of four readings taken over the 3 hour period.

Table 3.1 and Fig. 3.1 shows the relative adsorption for the 3 levels of small intestine. As some workers have reported the results of their uptake studies as H.R.P. protein adsorbed per unit tissue protein (Walker, Cornell, Davenport & Isselbacher, 1972). The results of the present study are similarly expressed. They are also shown in terms of wt of HRP/wt of tissue. The findings are virtually identical. The results are expressed as percentage of the total amount of enzyme adsorbed by the three levels of gut.

Adsorption of H.R.P. was slightly higher for the duodenum than for jejunum, when the results were expressed on a weight for weight basis they become marginally significant. Uptake by both duodenum and jejunum was significantly greater than for ileum ($P = 0.05$).

(b) Absorption of H.R.P.

Transport of macromolecules across the mucosal surface is a two part process, adsorption onto the epithelial cell surface followed by absorption into the tissue (Walker, Isselbacher and Block, 1974). Adsorption is not a reliable indicator of transport, release into the serosal fluid is considered to reflect this process more accurately (Walker, Cornell, Davenport & Isselbacher, 1972).

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Table 3.2 and Fig. 3.2 show the relative amounts of H.R.P. released into the serosa at the various levels of intestine. The results are expressed as a percentage of the total enzyme released into the serosal fluid by the three blocks of tissue tested in each experiment. Again there is little difference in findings between the results whether expressed in terms of H.R.P. protein/tissue protein or on a weight to weight basis. Significantly more of the enzyme adsorbed onto the epithelium reached the serosal fluid in the duodenum than in either the jejunum or ileum whilst the absorption by the jejunum was significantly greater than that of ileum. ($P = 0.05.$)

This increased rate of transport by duodenal mucosa was well demonstrated in the assays of serosal fluid taken at intervals from the sacs prepared by the method of Crane & Wilson (1958). Samples were taken at hourly intervals and showed a gradual increase in the level of peroxidase during the period of incubation. The levels of peroxidase found in the serosal fluids of the duodenum were consistently greater than those of either jejunum or ileum, whilst the level in the jejunum was generally higher than ileum (Table 3.3).

(c) Histochemistry

Histochemical analysis was performed on 3 pigs. Microscopical examination revealed that whilst the intestinal mucosae of two of the animals was normal, the third showed signs of moderate inflammation of the mucosae with epithelial cell desquamation particularly of the anterior region of the intestine. Whereas the tissues from the healthy animals withstood the incubation procedures without sustaining any gross damage, those from the animal with inflammation showed evidence of further epithelial desquamation of the villi, reflecting the fragile nature of the tissues in this condition.

Tissues incubated in crude H.R.P. showed very weak reactive product staining, rendering detailed interpretation unreliable. Blocks incubated in H.R.P. factor VI whilst demonstrating clear evidence of uptake across the villous epithelium failed to show any evidence of H.R.P. in the lamina propria below the crypt villous junction, nor was there any reaction of endogenous peroxidase in this region either, indicating that an incubation time of 2 hours for the 3,3 di amino benzidine tetrahydrochloride was too short to permit penetration much beyond the epithelium.

Strong peroxidase activity was found in the mucin layer covering the villi. There was also staining of villous epithelium.

This was most pronounced towards the tips of villi and was of two kinds. Senescent cells, in the process of being extruded into the lumen showed a general uniform staining of cytoplasm (Plate 3.1). Other epithelial cells showed a weaker reaction principally in the apical cytoplasm. Very little staining was seen in the subnuclear region. Not all cells showed the same level of activity and some cells appeared to be unstained, suggesting that they were not involved in uptake during the incubation period.

There was strong staining of the intercellular spaces and of the basement membrane beneath cells showing uptake.

In the underlying lamina propria where uptake of peroxidase by epithelial cells had occurred, there was a general staining of the tissues. The endothelium of the subepithelial capillaries was stained but because of the intense endogenous reaction of the red cells it was not possible to determine whether the substrate had passed into the capillary network. No reactive product was seen in the central lacteals although, like the capillaries the endothelium showed evidence of staining.

An observation of particular importance was the finding that where epithelial desquamation had occurred, considerable amounts of reactive product was seen diffusing in to the laminal tissue (Plate 3.2). This was particularly noticeable in areas where the laminal tissue protruded through a gap in the epithelial lining, and where presumably desquamation had occurred in vivo. In these areas the epithelial cells at the edge of the break were rounded and often fused into the protruding lamina. In other areas the discontinuity of the epithelial sheet was abrupt, the cells at the edge ragged and the underlying lamina did not protrude. These areas, which were considered to be desquamated as a result of the incubation procedure, did not show such marked peroxidase uptake.

There was little variation of the pattern of staining either of epithelial cells or in the underlying lamina propria in the different levels of intestine. However in the ileum, although reaction product was seen in the epithelial cells overlying the papillae above Peyers Patches very little staining of the underlying lymphocytic tissue occurred compared with that of the lamina propria in the adjacent villi despite the fact that endogeneous peroxidase in red cells was equally well stained in both areas.

No reaction product, apart from that associated with endogenous peroxidase in red cells and eosinophil granules was seen in control tissues incubated in buffer alone.

DISCUSSION

Uptake of horse radish peroxidase was shown by Walker and his colleagues (Walker, Cornell, Davenport & Isselbacher, 1972) to be significantly greater in adult rat jejunum than in ileum. In the present study similar results were obtained for the absorption of H.R.P. by porcine intestine. In addition comparative studies between duodenum and more distal small intestine showed that duodenal uptake was either equal to or greater than that of jejunum. Furthermore release of the absorbed material into the serosal fluid was significantly greater ($P = 0.05$) in the duodenum than in either the jejunum or ileum.

Although in the present study, the proportion of H.R.P. which was actively transported was not determined, histochemical examination revealed that where epithelial desquamation occurred quite large amounts of antigen were able to enter the lamina propria. Nevertheless in those areas in which the epithelium was intact, the presence of peroxidase in the apical cytoplasm as well as in the intraepithelial cell spaces indicates that the macromolecule can be absorbed via the epithelial cell and accords with the findings of other workers (Cornell, Walker & Isselbacher, 1971).

The proximal gut is a major site for protein digestion and absorption (Holdsworth, 1972), and is therefore an area likely to be subjected to considerable challenge from dietary derived antigens. Local immunization has been shown to interfere with the uptake of macromolecular antigens in jejunal tissue (Walker, Isselbacher & Bloch, 1972), probably by the formation of immune complexes at the mucosal surface which are then degraded by proteolytic pancreatic enzymes (Walker, Wu, Isselbacher & Bloch, 1975), thereby reducing the amount of antigen available for absorption. It is perhaps significant that in pigs of the age used in the present studies the pancreatic duct emptied in to the duodenum some 6 cms below the pylorus. Presumably proteolytic digestion is likely to be lower in this proximal segment and hence the challenge by undegraded antigen more severe.

The immunocytes populating the gut lamina propria are thought to be derived from precursor immunoblasts originating in gut associated lymphoid tissues (GALT) principally Peyer's patches (Craig & Cebra, 1971) in response to antigens in the intestinal lumen. These precursors are large lymphocytes which enter the blood stream via the thoracic duct lymph and then 'home' back to the lamina propria of the bowel where they transform into active immunocytes (Gowans & Knight, 1964; Griscelli, Vassalli & McCluskey, 1969; Hall & Smith, 1970; Hall, Parry & Smith, 1972; Pierce & Gowans, 1975). The mechanism of the homing

process is not known but it would appear to be under multifactorial control, in particular the role of antigen in determining the distribution of specific antibody producing cells in the lamina propria is unclear.

There is a selective pressure for GALT derived immunoblasts to re-locate in intestinal mucosal tissues since lymphoid cells derived from peripheral lymph nodes do not infiltrate the intestinal mucosa in contrast to GALT derived cells which home specifically in these tissues (Guy-Grand, Griscelli & Vassalli, 1974; Hall, Hopkins & Orlans, 1977). The rate of infiltration of intestinal lamina propria is the same in antigen free grafts of foetal small intestine as it is for normal intact gut (Parrott & Ferguson, 1974; Guy-Grand, Griscelli & Vassalli, 1974).

Infection of mice with *Trichinella spiralis* cause non-specific accumulation of lymphoid cells in the gut, cells from normal donors migrating to the tissues as readily as those from infected donors (Rose, Parrott & Bruce, 1976). These findings indicate that homing of GALT derived cells to the intestinal mucosa is an intrinsic process and not one which is 'antigen driven'.

Antigen does seem to influence the subsequent fate of immune cells infiltrating the gut, however. Immunization of an isolated segment of human colon with polio vaccine stimulates the production of specific antibody which is entirely confined to the immunized segment of the bowel. (Ogra & Karzon, 1969). The numbers of specific immunocytes infiltrating the lamina propria of Thiry-Vella loops in rats immunized with cholera toxoid was consistently higher in a loop challenged with the toxoid compared with an unchallenged control loop. Cells only appeared transiently in the control tissues (Husband & Gowans, 1978). Topical application of the toxoid at different levels of the intact gut resulted in the concentration of specific immunocytes being greatest at or distal to the site of application but not proximally (Pierce & Gowans, 1975). In contrast specific immunoblasts showed no tendency to localise in the gut wall at a site where killed bacteria were injected into the tissue (Hall, Hopkins & Orlans, 1977).

It would appear that whilst homing of specific GALT derived immunoblasts to the gut is not antigen dependent, the presence of antigen within the lumen can influence their accumulation and proliferation in the lamina propria once they have entered the tissues. The timing of the homing event also appears to be important. Immunoblasts from mesenteric lymph nodes accumulate in the small intestine of mice during the period 2-4 days after infection with *Trichinella spiralis* but not at later times (Rose, Parrott & Bruce, 1976). This finding together with the recent demonstration that lymphoblast migration to the small intestine is connected with blood flow (Parrott & Ottaway, 1980) suggests that infiltration of mucosal tissues may be associated with the early inflammatory response enabling an increase in the numbers of precursor immunoblasts to infiltrate the challenged tissue where they are immobilised and proliferate following contact with antigen. The observations are of particular relevance when considering the early protection of the young animal against enteric infection. It offers the prospect of mounting a rapid and effective defense providing the host is equipped with sufficient precursor cells through previous immunization.

With regard to this observation it is interesting that immunofluorescent studies of the distribution of plasma cells synthesising secretory immunoglobulins show that the number of cells in porcine duodenum is more than 10 fold greater than in jejunum (Allen & Porter, 1973a). It seems possible that this higher population in the anterior site may be a response to a greater antigenic challenge especially as macromolecular uptake appears to be related to the amount of material available for absorption (Warshaw, Walker, Cornell & Isselbacher, 1971). It would be of interest to compare the numbers of immunocytes in the lamina propria of duodenum above and below the pancreatic duct.

Table 3.1 Relative uptake of HRP by different levels of Porcine Small Intestine.

a) Comparison of uptake calculated by Protein/Protein and Wt/Wt basis

Pig No.	Percentage of total uptake of ug HRP Protein/mg Tissue Protein			Percentage of total uptake of ug HRP/g Tissue		
	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
1	59.5	30.5	10	57.5	30	12
2	40.5	36.5	22.5	46.5	34	19.5
3	34.5	40.5	24.5	38	37	24.5
4	37.5	42	20	40	37	23
8	45	42	13	49	36	15
9	39	39	22	37	37	26
10	46	38	16	46	30	24
Mean	43.14	38.35	18.29	44.86	34.43	20.57

Results calculated from means of hourly readings from 3 replicate sacs from each level per animal - incubated for 3 hours.

b) Statistical analysis of results

	% of total uptake ug HRP Protein/mg Tissue Protein		% of total uptake ug HRP/g Tissue
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Means of 21 observations per organ

Duodenum	43.1429	Duodenum	44.8571
Jejunum	38.3571	Jejunum	34.4286
Ileum	18.2857	Ileum	20.5714

Average significant

difference	8.7764	7.8357
Standard deviation	7.5360	6.7278
Standard error	2.8483	2.5429

(P = 0.05)

Table 3.2 Relative transfer of HRP to serosal fluid of different levels of Porcine Small Intestine

a) Comparison of transfer calculated by Protein/Protein and Wt/Wt basis.

Pig No.	Percentage of total transfer ug HRP Protein/mg Tissue Protein			Percentage of total transfer ug/HRP/g Tissue		
	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
1	47	43	10	43	48	9
2	62.5	33	6.5	51	41	8
3	69	31	0	60	40	0
4	54	38	8	46	47	7
8	86	6	8	85	8	7
9	66	12	22	69	12	18
10	72	28	0	67	33	0
Mean	64.95	27.29	7.76	60.06	32.71	7.09

Results calculated from mean values on 3 replicate sacs at each level per animal after 3 hours incubation.

b) Statistical analysis of results

% of total transfer ug HRP Protein/mg Tissue Protein	% of total transfer ug HRP/g Tissue
--	--

Means of 21 observations per organ

Duodenum	64.9500	Duodenum	60.0557
Jejunum	27.2857	Jejunum	32.7086
Ileum	7.7571	Ileum	7.0929

Average significant

difference	16.4588	18.8678
Standard deviation	14.1327	16.2012
Standard error	5.3417	6.1235

(P = 0.05)

Table 3.3 Horse radish peroxidase levels in serosal fluid of intestinal sacs prepared by the method of Crane and Wilson (1958).

Pig No.	Organ	Incubation Time (minutes)		
		60	120	180
2	Duodenum	1.05 ⁽¹⁾	2.75	5.6
	Jejunum	0.23	2.47	3.59
	Ileum	0.18	0.70	1.35
3	Duodenum	0.35	1.81	ND ⁽²⁾
	Jejunum	0.15	0.43	0.60
	Ileum	0	0	0
4	Duodenum	3.33	9.1	ND
	Jejunum	0.61	1.63	3.25
	Ileum	0.15	0.31	0.46
5	Duodenum	0.28	0.31	0.45
	Jejunum	0	0.06	0.08
	Ileum	ND	0.03	0.03

(1) ug HRP/g tissue

(2) ND = Not determined

Legend

Figure 3.1. Adsorption of H.R.P. by porcine small intestine

(a) ug HRP protein/mg tissue protein

(b) ug HRP/g tissue

Figure 3.2. Transmission of HRP to serosal fluid

(a) ug HRP protein/mg tissue protein

(b) ug HRP/g tissue

Results derived from the means of 21 gut sacs from each of 3 levels of intestines of 7 animals and expressed as the proportion of the total adsorption or transmission of all 3 levels.

(D - duodenum J - jejunum I - ileum

Bar = standard error)

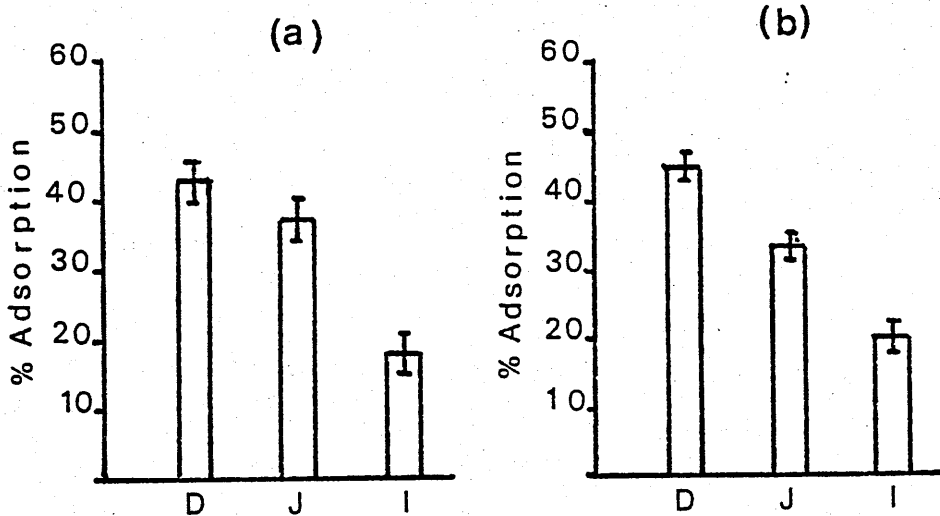


Figure 3.1

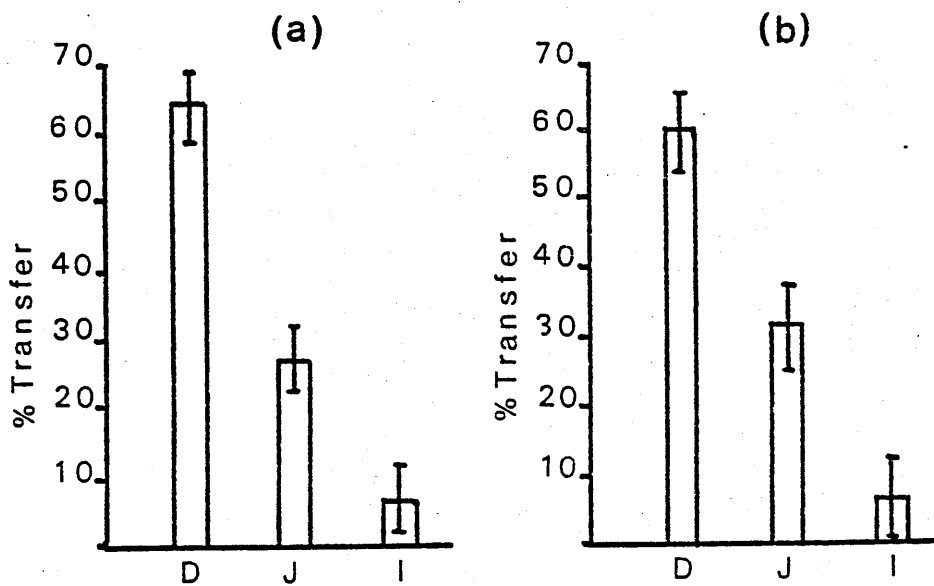


Figure 3.2

Plate 3.1. Porcine jejunum stained with 3,3'-di amino benzidine tetrahydrochloride to show the localization of horse radish peroxidase.

The section shows the tip of a villus in which a general cytoplasmic staining of a senescent cell being extruded into the lumen can be seen. There is also a small area of more extensive epithelial cell desquamation where peroxidase uptake is intensified, staining of the interepithelial cell spaces and basement membrane can also be seen.

(original magnification x 200).

Plate 3.2. Porcine jejunum stained with 3,3'-di amino benzidine tetrahydrochloride to show the localization of horse radish peroxidase.

This section of the tip of a villus with extensive epithelial cell desquamation.

Note:- the intense staining of the villous lamina propria where it protrudes into the lumen.

(original magnification x 200).

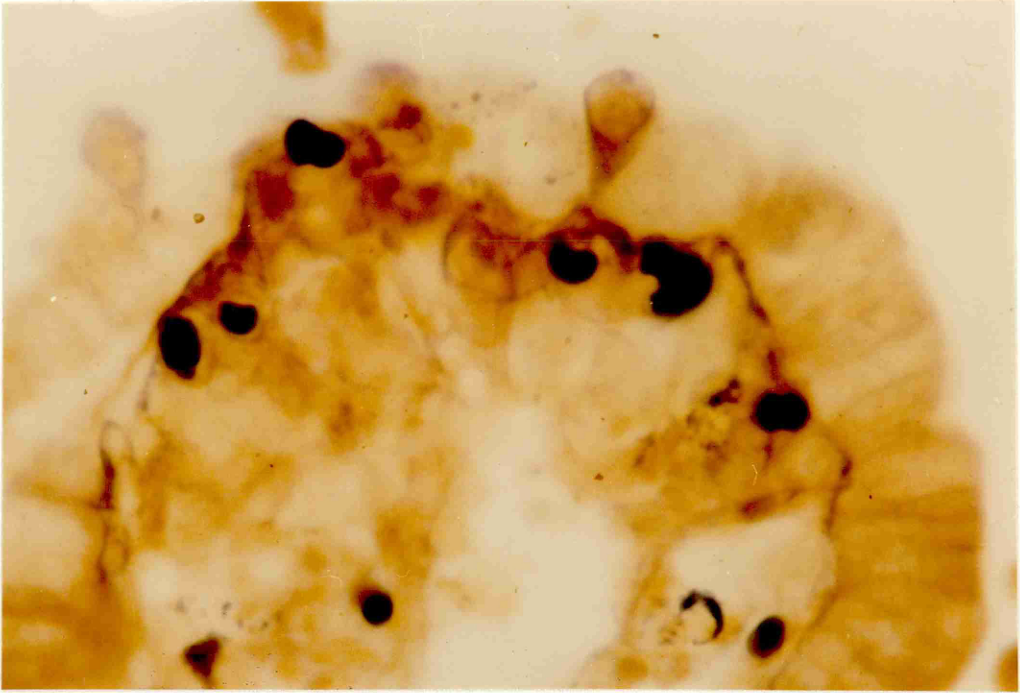


Plate 3.1

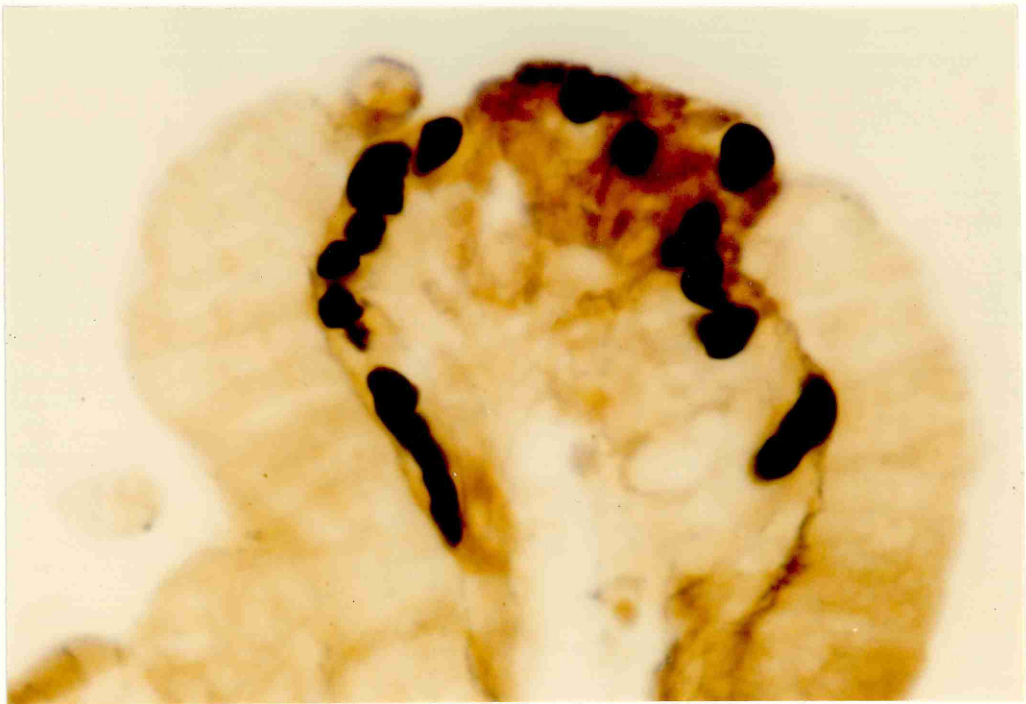


Plate 3.2

SECTION 4

A COMPARATIVE STUDY OF THE RELATIVE ABILITY OF SMALL INTESTINE
FROM PIGS OF VARYING AGE TO ABSORB HORSE RADISH PEROXIDASE AND
BACTERIAL ANTIGEN.

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BACKGROUND & OBJECTIVES

In the first series of experiments performed on 4 week old weaned pigs, there was evidence of damage to the intestinal epithelium, probably as a consequence of post weaning enteritis.

Histochemical analysis revealed that in addition to antigen being absorbed across intact epithelium quite large amounts were entering the lamina propria in areas where epithelial desquamation had occurred, a finding which could have significantly influenced the results.

The studies were conducted using horse radish peroxidase as a marker antigen and there was no evidence to indicate whether similar results would be obtained with other antigens. In the young pig, E. coli associated enteritis occurs at specific periods in the animals life, at birth, at around 12 days of age and again when the animal is weaned. It is caused by a limited number of known strains of enteropathogen (Sojka 1971) a feature which makes immunization with a polyvalent vaccine a real possibility. Protection against the neonatal phase of the disease rests entirely on passively acquired maternal immunity conferred by the ingestion, by the newborn piglet, of hyperimmune colostrum. Survival from infection at the later stages of life depends, however, largely on the early onset of an active intestinal secretory immunity (Porter, Parry & Allen, 1977).

The ability of epithelial cells of porcine small intestine to absorb maternal immunoglobulins is extremely short lived and lasts for only 24-36 hours (Lecce & Morgan, 1962). After this time closure occurs and uptake of macromolecules in any quantity ceases. Nothing is known of the effect closure exerts on the absorption of antigen across the gut of pig during the post closure phase. In view of the potential for oral immunization of the very young animal against enteropathogens, it was particularly important to determine whether bacterial antigens would display uptake characteristics similar to those obtained with H.R.P.

The present series of experiments was undertaken to clarify these and related questions. The objective was to compare the ability of intestinal mucosa from young pigs to absorb H.R.P. and bacterial antigen and evaluate the influence of age upon antigenic uptake. The results show that endotoxin from enteropathogenic Escherichia coli is taken up in a manner essentially similar to that of H.R.P., and again there was a superior absorption in the proximal compared with the distal gut of pigs. Furthermore, there was a variation in antigen uptake with age, as the animals mature the amount of antigen absorbed was reduced, a factor which may have to be taken into account when considering the practical approach to immunisation via the gut mucosa.

MATERIALS AND METHODS

Uptake of antigen by porcine small intestine was studied in vitro by the everted sac technique described previously (Vide Supra - Section 3). Two antigens were used, horse-radish peroxidase (HRP) and radiolabelled coli endotoxin.

Preparation of Radiolabelled Endotoxin

The radiolabelled endotoxin was prepared from a porcine enteropathogenic Escherichia coli serotype 08. K87, K88 a b. The organism was isolated on blood agar and cultured in a synthetic medium based on a modification by Braude, Carey, Sutherland & Zalesky (1955) of Gladstones medium (1937).

The medium comprised 112.5g KH_2PO_4 : 12.5g $(\text{NH}_4)_2\text{SO}_4$ and 12.5g NH_4Cl dissolved in 12 litres distilled water. 700mls of N. NaOH was added and the volume made up to 15 litres with distilled water before the pH was adjusted to 7.6 with N. NaOH and the volume made up to 25 litres.

The medium was distributed into 4 litre amounts in 6 litre conical flasks and autoclaved at 115° for 20 minutes. It was then stored until required.

Immediately before use 40ml of sterile $\frac{M}{6}$ $MgSO_4 \cdot 7H_2O$;
20ml of sterile 50% glucose solution and 10ml of sterile M.
 $NaHCO_3$ was added aseptically to each flask.

A starter culture of the organism was prepared by growing a purified isolate in 10mls of synthetic medium at $37^{\circ}C$ for 6 hours, after which time the whole culture was transferred to 600mls of medium and reincubated at $37^{\circ}C$ for a further 18 hours. 100mls of the starter culture was inoculated into each 4 litre aliquot of medium and the flasks incubated for 24 hours in a shaking incubator at 37° .

After incubation the cultures were rapidly cooled to 0° by immersion in an ice-salt mixture and the bacteria recovered by centrifugation at 2° . The bacterial deposit was resuspended in 95% ethanol and washed three times in 95% ethanol followed by two washings in acetone before being dried at 37° for 2 days.

A Boivin endotoxin was prepared from the dried bacteria by the method of Mesonbrau, Mesonbrau and Mitrica (1961). The dried organisms were suspended in distilled water at a concentration of 50mg/ml and 30% chloroform added. The suspension was allowed to stand at room temperature for 3 days, with constant stirring to effect bacterial lysis. It was then centrifuged and the clear

supernatant retained. This was cooled to 4° and the pH adjusted to 3.5 with chilled trichloroacetic acid. The mixture was recentrifuged and the precipitate redissolved in 1/10 of the initial volume of physiological saline. The pH was adjusted to 8.5 with N. NaOH and the extract lyophilised and stored.

Radiolabelling of the endotoxin was achieved by the method of Braude, Carey, Sutherland & Zalesky (1955). The dried endotoxin was resuspended in distilled water at a concentration of 5mg/ml. The suspension was incubated for 24-48 hours at 37°C with ⁵¹Cr Cl₃ at a concentration of 9mg endotoxin per 0.1 m.ci. After incubation it was dialysed against successive large volumes of distilled water until all free isotope had been removed. The labelled endotoxin was stored at -20°C until required for use.

Counts in excess of 1×10^6 CPM/ml were routinely obtained.

Intestinal tissues were obtained from pigs varying in age from 8-28 days. They were all reared on the sow until 21 days old when they were abruptly weaned onto standard early weaning pellets.

Antigen Uptake Studies

Antigen uptake studies were performed on three litters of pigs totalling 24 animals. Four replicate everted sacs were prepared from each of three areas of small intestine, duodenum, mid-jejunum and ileum.

Tissues were also taken from the three areas of the intestines of pigs of two of the litters for routine histological examination.

Three of the sacs per area were incubated in Krebs Ringer solution containing antigen, either H.R.P., radiolabelled endotoxin, or both. The remaining sac was incubated in Krebs Ringer alone, as a control.

Tissues from one litter were incubated in ^{51}Cr labelled endotoxin 5ug/ml, those from a second litter in H.R.P. factor II (Sigma) 0.4g/l only, and from a third in H.R.P. factor II 0.4g/l plus ^{51}Cr labelled endotoxin 300 ug/ml.

Incubation time was reduced from 3 to 2 hours compared with the previous studies, samples of luminal incubation medium being removed at intervals of 5, 60 and 120 minutes. At the conclusion of the experiments the serosal fluid was collected.

Assay of Antigen

Horse radish peroxidase was assayed by the modified method of the Worthington Biochemical Corp described by Walker, Cornell, Davenport & Isselbacher (1972). Radiolabelled endotoxin was assayed by determining the activity of ^{51}Cr in the samples on either a Wilj 2001 Gamma counter or on a Philips automatic liquid scintillation analyser model PW 4510/01.

As the previous studies had shown that results of antigen uptake were virtually identical irrespective of whether they were calculated on the basis of H.R.P. protein/tissue protein or H.R.P. wt/tissue wt, it was decided to use a wt/wt basis for the calculations in this series of experiments.

Morphological Studies

The morphological appearance of the mucosa of the tissues from which the everted sacs were prepared was determined by microscopic examination of paraffin embedded sections stained by haematoxylin and eosin. The sections were prepared from representative blocks of tissue taken from portions of intestine immediately adjoining those used for the preparation of the sacs.

Immunohistochemistry

The distribution of immunocytes synthesising immunoglobulins in the lamina propria of the duodenum was studied in 4 four week old pigs which had been weaned at 21 days onto standard early weaning pellets.

Ten blocks of tissue 0.5cm thick were taken from the duodenum of each animal at intervals of 3.0cms commencing as near to the pylorus as possible and fixed in ethanol at 4⁰C. Five μ thick sections were prepared by the method of St. Marie (1962) and cell synthesising IgA and IgM were enumerated by the immunofluorescent technique described earlier (Allen & Porter, 1973a).

The point at which the pancreatic duct emptied into the duodenum was located at post mortem.

RESULTS

Uptake studies

The effect of gut maturation on the uptake and transmission of antigen by intestinal epithelium was studied on gut sacs prepared from tissues taken from three litters of piglets of varying ages.

The sacs from the first litter were incubated in medium containing radiolabelled 08 antigen at a concentration of 5ug/ml. Analysis of the results showed this to be too low to yield reliable findings of absorption although sufficient labelled antigen was detectable in the serosal fluid for these results to be of value. However the results from this experiment are not considered in detail although the serosal findings were in general agreement with those from the later experiments (Table 4.1).

Sacs from a second litter (litter I) were incubated in medium containing H.R.P. at a concentration of 0.4g/l and those from the third litter (litter II) in medium containing H.R.P. 0.4g/l plus radiolabelled 08 at a level of 300ug/ml. The results from these two experiments are examined in detail.

It must be noted however, that whilst a higher concentration of radiolabelled O8 antigen was used in this second series of experiments a few doubtful results still occurred, in which serosal content readings suggested a high rate of antigen absorption, incompatible with the uptake readings. Since the H.R.P. studies on the sacs in question did not suggest that leakage had occurred these findings cannot be explained. These readings have been excluded from the O8 antigen results.

As in an earlier series of experiments the rate of adsorption of antigen in the present studies was found to be highest during the earlier stages of incubation, declining as the experiment proceeded. Therefore, results of antigen adsorption are expressed as the means of readings obtained during the course of incubation.

Figure 4.1 shows the relative adsorption and transfer values of both H.R.P. and O8 antigens in sacs from duodenum, jejunum and ileum incubated in medium containing both antigens (litter II). Similar results were obtained from sacs incubated in H.R.P. alone (litter I).

No changes in the relative rates of adsorption or transfer of either antigen attributable to the effects of maturation were observed. In general the rates of both adsorption and transfer of antigens were consistently higher in the duodenum and jejunum than in the ileum although the rate of adsorption of O8 antigen by the ileum was greater than that for H.R.P.

Comparison of the rates of uptake of antigen per gram of tissue shows a distinctly age related trend. Adsorption of both H.R.P. and 08 antigens was generally higher in tissues from young animals compared to older ones, (Tables 4.2 and 4.3), although a fairly wide variation of adsorption rate between animals was observed. However the variation in the rate of transfer of antigen to serosal fluid between animals was much less and a similar age related reduction in the amounts of antigen, both H.R.P. and 08 found in serosal fluid was clearly apparent. There was a higher rate of transfer in tissues from the younger animals compared to the older ones (Tables 4.4 and 4.5).

The rates of decline of antigen uptake as animals grew older appeared to be similar in the duodenum and jejunum but was less marked in the ileum. This finding would account for the lack of any age related change in the relative rates of adsorption and transfer.

As the relative rates of antigen uptake were not affected by gut maturation it was possible to group the results of tissues incubated in different antigens and examine the effects of litter to litter variation and antigen competition. Figures 4.2 and 4.3 show the relative adsorption and transmission of H.R.P. in the duodenum, jejunum and ileum of pigs from the two litters, one incubated in H.R.P. alone (litter I) and the other (litter II) in H.R.P. plus 08. Although some variation between the

two litters was apparent, the relative rates of uptake and transmission of H.R.P. did not appear to be influenced by the presence of O8 antigen. Again the consistent feature emerged that the rates of adsorption and transmission were lower in the ileum than in either duodenum or jejunum (Figure 4.4).

The proportion of antigen transmitted to the serosa was greatest in the duodenal tissue irrespective of the nature of antigen. Again the rate of transmission in the ileum was lower than elsewhere (Figure 4.5).

The amount of adsorbed antigen which was transmitted to the serosal fluid did show a variation which appeared to be related to its nature. Although the proportions of antigen transported across the mucosal tissues were similar for both antigens, only about 2.5% of the H.R.P. adsorbed from the luminal medium was found in serosal fluid whereas nearly 50% of adsorbed O8 appeared in serosal fluid (Figure 4.6).

Morphology

Microscopic examination of the representative blocks of tissue from duodenum, jejunum and ileum from which the sacs were prepared showed a progressive, age related change in morphological appearance of the villi.

In general the villi of the younger animals tended to be long slender finger-like processes with intact epithelium having a good nuclear/cytoplasmic ratio. As the animals aged, the villi became increasingly stunted and an increasing amount of epithelial desquamation was observed. This was accompanied by inflammatory changes in the villous lamina propria. These findings have been reported in more detail earlier (Section 2)

Immunohistology

The distribution of immunocytes synthesising immunoglobulins at different levels of the duodenum above and below the ligament of Treitz was examined in 4 four week old pigs which had been weaned for one week. The results in table 4.6 show that there is little or no variation in the number of cells per unit volume of tissue either above or below the pancreatic duct. In the four animals studied this duct emptied into the duodenum approximately 10 cms caudal to the pylorus.

DISCUSSION

Transport of macromolecules across cell membranes has been the subject of considerable study, although much of the attention has been focussed on maternofetal transmission of immunoglobulins, especially in rodents. One of the significant findings of this research has been the highly selective nature of protein uptake by enterocytes (Brambell, 1970). Studies made on mucosal tissues from young rodents suggest that selectivity is effected by the existence of specific receptors probably on the enterocyte microvilli since the phenomenon is exhibited by absorption as well as transmission (Jones & Waldman 1972; Gitlin & Gitlin, 1976; Waldman & Jones, 1976). A similar selectivity of immunoglobulin uptake has been demonstrated in the neonatal pig, (Witty, Brown & Smith, 1969). Furthermore, when mixtures of immunoglobulins, derived from different species are used in the experiments, competition for attachment to the receptors occurs (Jones and Waldman, 1972). Also high levels of immunoglobulin will saturate the mechanism (Jones & Waldman, 1972; Gitlin & Gitlin, 1976). A similar saturation of the macromolecular uptake mechanism has been demonstrated by Nolan, Hare, McDevitt & Vilayat (1977) using endotoxin.

In this respect the observations in the present series of experiments that uptake of H.R.P. was uninfluenced by the presence of O8 antigen is worthy of comment, since it raises the possibility that different receptors are required for each antigen. Alternatively it may be that although the same receptors are involved, the levels of antigen used were insufficient to saturate the system. This seems unlikely as uptake of both antigens were higher in the anterior gut compared to ileum suggesting that in ileum at least an excess of antigen would be present. But even in this tissue evidence of antigen competition was lacking.

The demonstration in the present study that uptake of E. coli endotoxin by porcine small intestine was similar to that of H.R.P. suggests the possibility of a wide variety of bacterial antigens being absorbed, unchanged, across intestinal mucosa, at least in quantities sufficient to be of immunological or pharmacological significance. Until recently it was thought that following gut closure to maternal immunoglobulin, macromolecular uptake was totally and permanently inhibited to intact mucosa.

The ability of the intestinal epithelium to absorb intact protein molecules has been extensively studied in neonatal farm animals since, in these species no transplacental passage of maternal antibody occurs and absorption of colostral antibody is of fundamental importance to survival. However permeability of intestinal mucosa to large molecules in any quantity is extremely short-lived, lasting in the pig for only 24-36 hours (Lecce & Morgan, 1962) after which gut closure occurs.

Early studies in suckling rodents suggested that uptake of large protein molecules was confined to the distal small intestine (Clark, 1959). Similar findings were obtained with radio labelled P.V.P. which was readily taken up by enterocytes from terminal ileum but not by cells from proximal small intestine (Clark & Hardy, 1969). In contrast, more recent work has shown that maternally derived antibody is preferentially transported by epithelial cells from proximal small intestine compared with those from more distal sites (Rodewald, 1973). Similar results have been reported from studies on the transmission of maternally derived IgG antibodies by other workers (Jones & Waldman, 1972, Jones, 1976; Morris & Morris, 1974; 1975). Studies in the neonatal pig have demonstrated uptake of maternally derived IgA by epithelial cells in the anterior small intestine (Allen & Porter, 1973b).

Following closure the proximal small intestine also appears to play an equally important role in the uptake of macromolecules in more mature animals. Walker and his colleagues (Walker, Cornell, Davenport & Isselbacher, 1972) showed that uptake of H.R.P. was significantly greater in adult rat jejunum than in ileum. The demonstration in the present study that both H.R.P. and O8 antigens were absorbed to a greater degree in proximal rather than distal small bowel confirms and extends these findings and from these observations certain features of functional significance deserve consideration.

The greater ability of the anterior small bowel to absorb bacterial toxins is of relevance both to the development of immunity and to the pathological changes occurring in intestinal mucosa in response to infection by enteropathogenic E. coli. Clinical symptoms of E. coli associated enteritis only become evident in young pigs following the colonization of the upper gut by substantial numbers of pathogens (Smith & Jones, 1963, Smith & Linggood, 1971). Significantly this is also the region most susceptible to enterotoxin (Smith & Halls, 1967). It may well be that this greater susceptibility is due, in part to the comparatively large amount of toxin able to penetrate the epithelium and underlying lamina propria.

Uptake of macromolecules by enterocytes is related to the amounts of material available for absorption (Warshaw, Walker, Cornell and Isselbacher, 1971). Antibody synthesising immunocytes localise in intestinal lamina propria distal to the site of topical antigen application (Pierce & Gowans, 1975). In porcine small intestinal mucosa the number of immunocytes synthesising immunoglobulins is more than 10 fold greater in the duodenum than in either jejunum or ileum. Since there would be more undegraded antigenic material in the upper gut, this laminal cell component appears to reflect the increased antigenic uptake which occurs at this site. In the young pig, the duodenum is the region of the gastrointestinal tract most susceptible to infection by enteropathogenic E. coli. It would appear that the efficacy of oral immunization by E. coli vaccines for this condition (Porter, Kenworthy & Allen, 1974) is due to the preferential absorption of antigen in this region stimulating active secretory immunity especially as bacterial endotoxins are not only effective antigens but have powerful mitogenic (Melchers, Braun & Galones, 1975) and adjuvant effects on macrophages and T lymphocytes (Allison, Davies and Page, 1973).

Earlier histochemical studies showed considerable amounts of antigen entering the lamina propria in areas where epithelial cell desquamation had occurred. The present findings suggest that the amount involved is inconsequential compared with that actively transported. Macromolecular uptake was greatest in animals showing least mucosal damage. The results may, to some extent, reflect the reduction in absorptive capability which can arise due to inflammatory changes in the mucosa.

The influence of age on macromolecular uptake, especially in the post neonatal period has not been previously studied. The demonstration in these experiments that macromolecular uptake is greatest in the younger animals is of particular relevance to the ontogeny of secretory immunity. To be most effective antigenic stimulation of the lymphoid cells in the lamina propria underlying intestinal epithelium should occur early in the post-natal phase, thereby ensuring the development of an active secretory immune defense before passive maternal protection declines. It follows that the earlier an oral vaccine is administered the more effective in stimulating active secretory immunity, it is likely to be.

Table 4.1

Antigen levels in serosal fluid of sacs incubated in medium containing 5ug/ml ⁵¹Cr labelled 0.8 endotoxin

<u>Pig No.</u>	<u>Age (days)</u>	<u>Serosal uptake/g tissue (%)</u>		
		<u>Duodenum</u>	<u>Jejunum</u>	<u>Ileum</u>
7	12	46	44	9
12	10	42	42	16
13	11	50	41	8
14	12	31	36	33
15	18	45	26	29
16	19	15	63	20
17	20	46	38	16
18	25	49	38	13
19	27	25	44	31
Mean		38.7	41.3	19.4
S.E.		4.08	9.81	9.44

Results are calculated from means of readings from 3 replicate sacs from each level per animal - incubated for 2 hours.

Table 4.2

Relative uptake of H.R.P. by different levels of small intestine from pigs of varying age.

<u>Pig No.</u>	<u>Age (days)</u>	<u>Days</u>	<u>Uptake of H.R.P. (ug H.R.P./g tissue)</u>		
			<u>weaned</u>	<u>Duodenum</u>	<u>Jejunum</u>
30	8	0	25.86	19.8	12.37
32	10	0	28.33	55.22	26.53
34	12	0	20.47	23.9	17.1
31	15	0	36.75	24.67	10.76
36	16	0	19.84	17.66	2.78
33	17	0	15.69	35.52	25.39
39	19	0	13.62	27.33	8.51
35	21	0	ND	ND	ND
41	23	1	9.11	5.01	6.0
37	23	2	9.88	16.61	5.83
42	24	2	21.84	19.32	12.85
38	24	3	8.27	11.21	8.76
43	26	4	4.90	4.19	5.45
40	28	7	16.78	20.45	13.19

ND = Not Determined

Results are calculated from means of hourly readings from 3 replicate sacs from each level per animal - incubated for 2 hours.

Table 4.3

Relative uptake of ⁵¹Cr labelled O8 antigen by different levels of small intestine from pigs of varying age.

<u>Pig No.</u>	<u>Age (days)</u>	<u>Days</u>	<u>Uptake of 0.8 antigen</u>		
			<u>(CPM/g tissue)</u>		
		<u>weaned</u>	<u>Duodenum</u>	<u>Jejunum</u>	<u>Ileum</u>
30	8	0	2098	-	3199
33	10	0	3704	1577	2225
36	16	0	1494	1647	1127
39	19	0	848	-	731
41	23	1	414	796	465
42	24	2	1046	1267	995
43	26	4	-	309	398

Results are calculated from means of hourly readings from 3 replicate sacs from each level per animal - incubated for 2 hours.

Table 4.4

Relative transfer of H.R.P. to serosal fluid by different levels of small intestine from pigs of varying age.

<u>Pig No.</u>	<u>Age (days)</u>	<u>Days</u>	<u>Serosal H.R.P. content</u>		
			<u>(ug/g tissue)</u>		
		<u>weaned</u>	<u>Duodenum</u>	<u>Jejunum</u>	<u>Ileum</u>
30	8	0	1.88	1.19	0.05
32	10	0	0.66	1.25	0.20
34	12	0	1.47	1.05	1.48
31	15	0	0.43	0.50	0.09
36	16	0	0.17	0.29	0.01
33	17	0	0.23	0.68	0.05
39	19	0	0.45	0.68	0.08
35	21	0	0.22	0.29	0.04
41	23	1	0.02	0.06	0.05
37	23	2	0.17	0.17	0.09
42	24	2	0.34	0.24	0.05
38	24	3	0.09	0.05	0.03
43	26	4	0.04	0.11	0.05
40	28	7	0.04	0.04	0.01

Results are calculated from means of readings from 3 replicate sacs from each level per animal - after 2 hours incubation.

Table 4.5

Relative transfer of ^{51}Cr labelled O8 antigen by different levels of small intestine from pigs of varying age.

<u>Pig No.</u>	<u>Age (Days)</u>	<u>Days weaned</u>	<u>Serosal O8 antigen content</u>		
			<u>(CPM/g tissue)</u>		
			<u>Duodenum</u>	<u>Jejunum</u>	<u>Ileum</u>
30	8	0	1760	-	614
33	10	0	799	-	202
36	16	0	455	913	78
39	19	0	451	-	195
41	23	1	241	224	102
42	24	2	173	295	123
43	26	4		177	66

Results are calculated from mean values on 3 replicate sacs at each level per animal after 2 hours incubtion.

Table 4.6

Distribution of immunocytes synthesising immunoglobulins A and M
in the lamina propria of duodenum of 3 week old weaned pigs.

Distance of sample

caudal to Pyloric junction (cms)	Immunoglobulin	Cell count ⁽¹⁾	S.E.
0	A	287	22.25
	M	337	57
3	A	340	25.25
	M	206	40
6	A	335	10
	M	486	40
9	A	424	56
	M	348	54
12	A	370	35
	M	304	46
15	A	362	36
	M	293	73
18	A	298	27
	M	227	19
21	A	233	14
	M	320	37
24	A	385	45
	M	236	34
27	A	329	29
	M	294	30

(1) Cell count is the number of cells per twenty fields (x40 objective) and is expressed as the mean counts from 4 pigs.

Legends

Figure 4.1

Relative adsorption and transfer to serosal fluid of H.R.P. and E. coli 08 antigens by porcine small intestine from animals of different ages.

- A) Adsorption of H.R.P.
- B) Adsorption of 08.
- C) Transmission of H.R.P.
- D) Transmission of 08.

Results derived from the means of values obtained from 3 sacs from each of 3 levels of intestine per animal incubated in medium containing both antigens and expressed as the proportion of the total adsorption or transmission of all 3 levels (duodenum, jejunum, ileum).

Figures 4.2 and 4.3

Relative adsorption (Figure 4-2) and transmission to serosal fluid (Figure 4-3) of H.R.P. by porcine small intestine incubated in medium containing H.R.P. antigen alone or H.R.P. plus E. coli 08 antigen.

Litter I H.R.P. alone

Litter II H.R.P. + 08

Results derived from the means of gut sacs (No. in column) from each of 3 levels of intestine and expressed as the proportion of the total adsorption or transmission of all 3 levels.

D - duodenum J - jejunum I - ileum

Bar = standard error

Figure 4.4 and 4.5

Relative adsorption (Figure 4-4) and transmission to serosal fluid (Figure 4-5) of H.R.P. and E. coli 08 antigen by porcine small intestine incubated in medium containing both antigens. Results derived from the means of gut sacs (No. in column) from each of 3 levels of intestine and expressed as the proportion of the total adsorption or transmission of all 3 levels.

D - duodenum J - jejunum I - Ileum

Bar = standard error

Figure 4.6

Relative proportion of adsorbed antigen transmitted to serosal fluid by porcine small intestine incubated in medium containing both H.R.P. and E. coli 08 antigen.

Results derived from the means of gut sacs (No. in column) from each of 3 levels of intestine.

D - duodenum J - jejunum I - Ileum

Bar = standard error

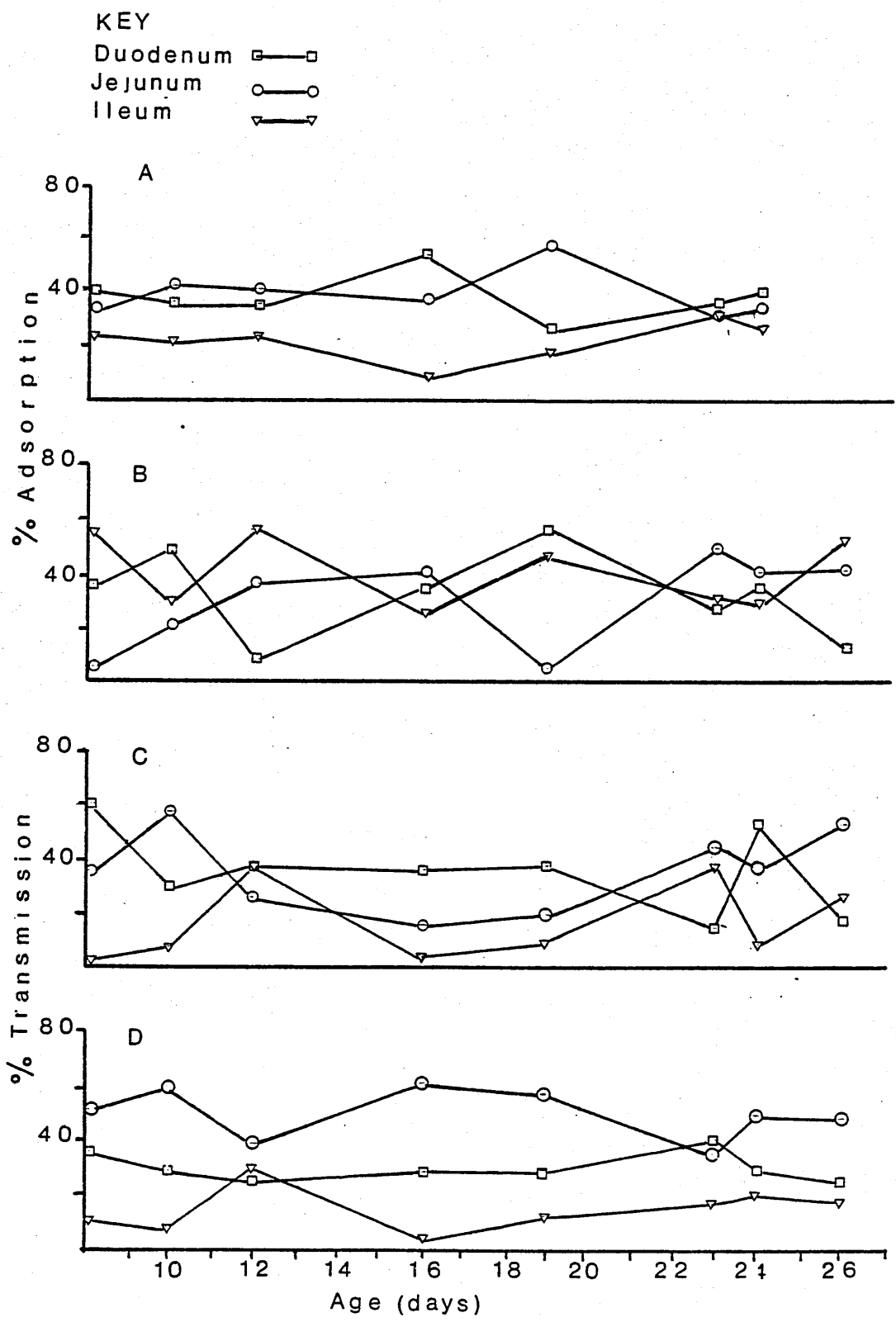


Figure 4.1

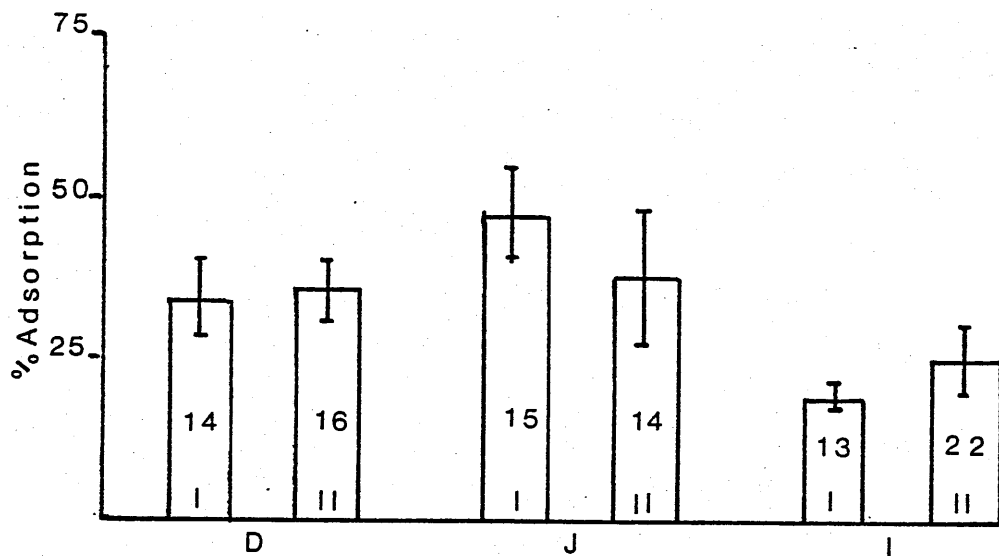


Figure 4.2

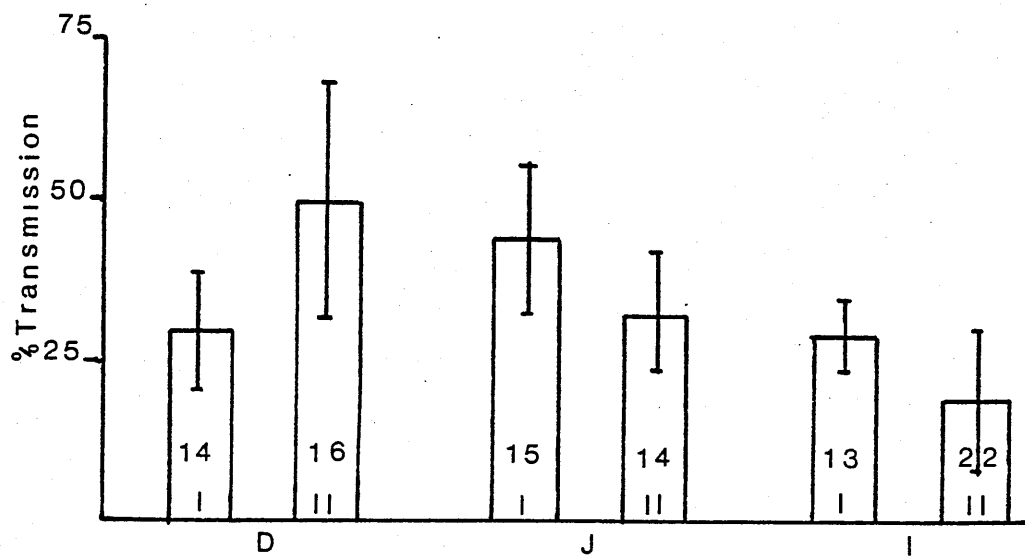


Figure 4.3

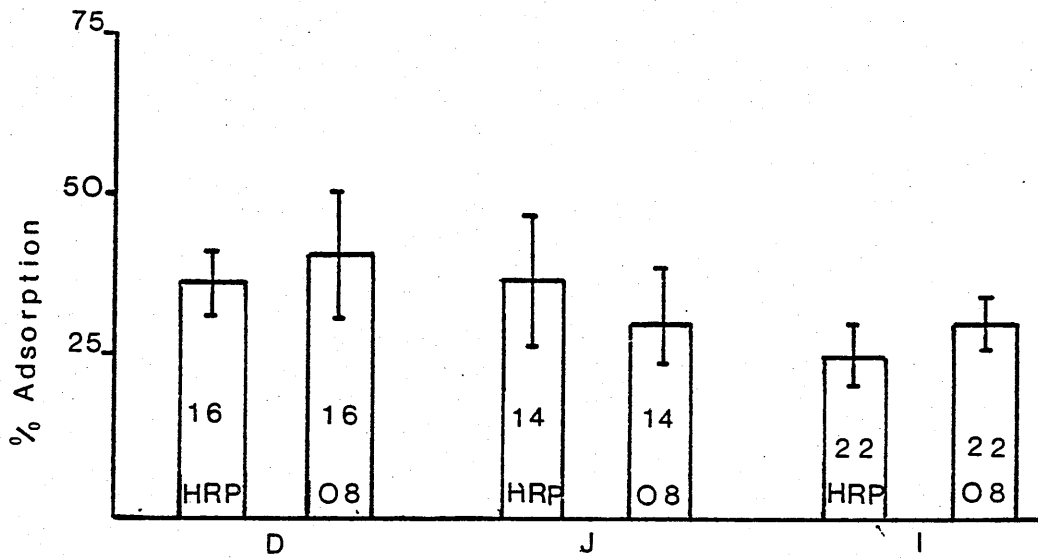


Figure 4.4

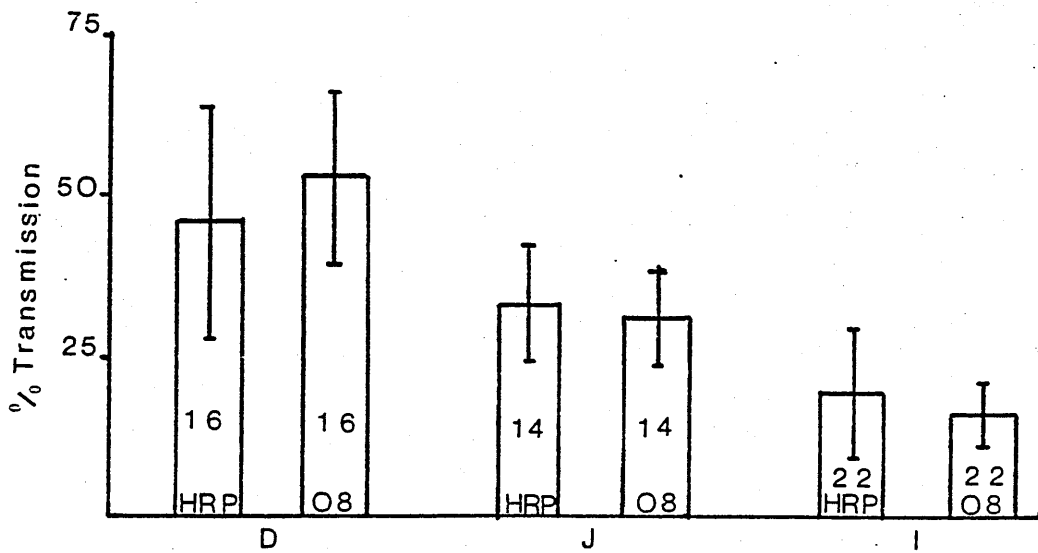


Figure 4.5

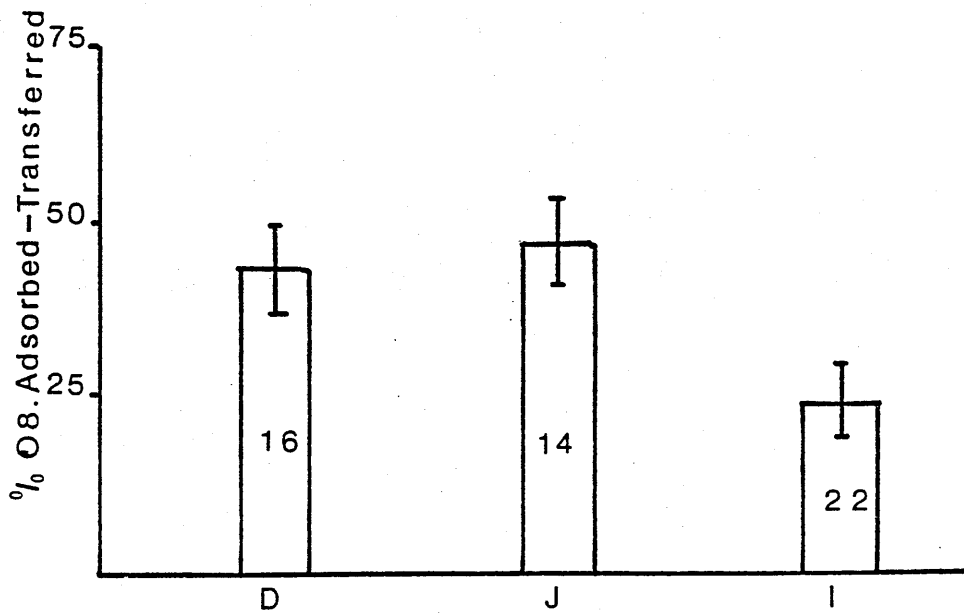
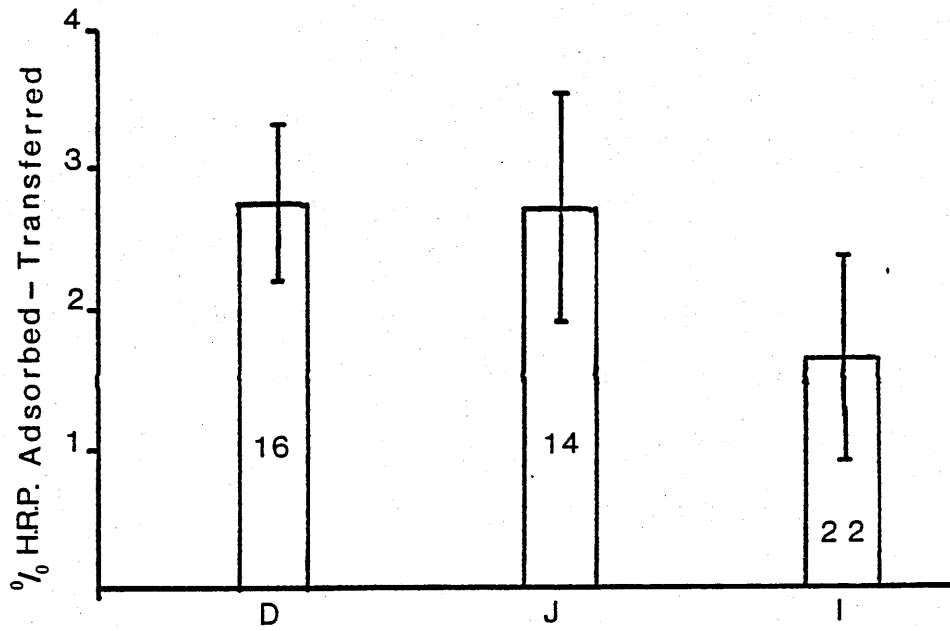


Figure 4.6

SECTION 5

THE FREQUENCY AND DISTRIBUTION OF IMMUNOGLOBULIN BEARING CELLS
IN THE INTESTINAL MUCOSA OF NEONATAL AND WEANED PIGS

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BACKGROUND AND OBJECTIVES

The young animal is subject, in early life, to infection by pathogenic enterobacteria which is frequently fatal. It is generally recognised that resistance to such infections increases with age and since the enteropathogens are generally non-invasive but operate by the production of toxins when in close association with the intestinal mucosa, local immune mechanisms appear to provide the most relevant means of control. The prospect that a satisfactory inter-relationship between declining levels of passive maternal antibody and onset of active synthesis of local antibody against enteric pathogens can be successfully attained by oral immunization of the weanling (Porter 1973), has been successfully tested in pigs (Porter, Kenworthy, Holme and Horsefield 1973; Porter, Kenworthy, Noakes and Allen, 1974) and Calves (Allen and Porter, 1975; Porter, Kenworthy and Thompson, 1975). However, optimisation of early natural resistance mechanisms may only be attained through a thorough understanding of the changes that take place in the maturing animal and it is in this context that the current investigations were initiated.

Studies of intestinal tissue from a number of species have shown that cells bearing IgA greatly outnumber those carrying other immunoglobulins (Crabbe, Carbonara and Heremans, 1965; Crabbe, Bazin, Eyssen and Heremans, 1968; Vaerman and Heremans, 1969).

The intestinal secretory immune system of the pig is analogous to that of the human and other mammals (Porter and Allen, 1970; Porter, Noakes and Allen, 1970) and it too has been shown to have a predominance of IgA cells in the lamina propria (Vaerman, 1970). However, a comparative study of the cells populating the intestinal lamina of 4 week old suckling pigs showed that the number of IgM containing cells far exceeded those bearing IgA (Allen and Porter, 1973a) suggesting that in the early stages of secretory immunity IgM may be of an importance at least equal to that of IgA.

In view of the preceding observations on antigen uptake, it is clear that there are opportunities for oral immunisation in the pig from a very early age. Therefore it will be important to delineate the normal character of the gut immune response between birth and weaning.

The present study compares the populations of different immunoglobulin containing cells occurring in the intestinal lamina propria of pigs of various ages. The significance of the findings for the natural development of secretory immunity is discussed and their relevance to oral immunization at a very early age considered in preparation for further examination of means of enhancing protective antibody function against E. coli disease in the weanling.

MATERIALS AND METHODS

Isolation of specific immunoglobulins and conjugation of specific antisera with fluorescent dyes.

The technique for the isolation of specific immunoglobulins and preparation of antisera conjugated with fluorescein isothiocyanate (FITC) have been described previously (Allen and Porter, 1970). Conjugation of antisera with rhodamine isothiocyanate (Baltimore Biological Laboratory Limited) was achieved by a similar method to that used for FITC.

Animals

Tissues were obtained at post mortem from pigs of different ages, ranging from 2 days to 3 months. They included both unweaned and weaned animals. In all a total of 33 pigs were examined.

Preparation of Tissues

Blocks of tissue were obtained from duodenum, jejunum, ileum and spiral colon. They were either fixed immediately in chilled ethanol or soaked overnight in buffer prior to fixation to remove interstitial immunoglobulin (Brandtzaeg, 1974). Paraffin blocks were prepared by the method of St. Marie (1962).

Staining procedure

Following the removal of wax with chilled xylol and rehydration in chilled ethanol the sections were rinsed in cold phosphate buffered saline (PBS) pH 7.1 and stained overnight at 4° in a humidity chamber to prevent drying. After staining they were washed three times (20 minutes each wash) in PBS with continuous gentle agitation, dried in air and stored unmounted in the dark until required. They were mounted in buffered glycerin pH 8.5 (Nairn, Herzog, Ward and De Boer, 1969) immediately prior to microscopical examination.

Controls

The tests used to control the specificity of the reactions have been described previously (Allen and Porter, 1970).

Microscopy

The FITC stained preparations were examined on a Reichert Zetopan microscope equipped with an HBO 200 light source (Osram). They were viewed either by dark ground using a UGI exciter filter and either a GG 13 plus a Wratten 2B or a GG 9 barrier filter, or by incident light using a 2 FITC 3 and 2.5 BG 38 exciter filter and a 1 GG 9 and 2 OG 515 barrier filter in conjunction

with an interference beam splitter reflecting up to approximately 490 nm. The rhodamine stained preparations were examined either by the same dark ground equipment or by incident light using a green exciter filter 546nm with a 3 OG 590 barrier filter and an interference beam splitter reflecting up to approximately 560 nm.

Counts were made of stained cells present in 20 fields selected at random using a X40 0.9 NA objective in conjunction with a X8 eyepiece.

RESULTS

The number of cells containing the three immunoglobulins IgM, IgA and IgG, infiltrating the lamina propria at different levels of the alimentary tract in animals of various ages are shown in tables 5.1 and 5.2. Table 5.1 details the development of cell populations in the neonate with respect to specific immunoglobulin classes and sites of distribution. Table 5.2 shows the effect of weaning on the intestinal cell populations of the maturing pig.

The first class of immunoglobulin cells to appear in the intestinal mucosa of the suckling neonate were those containing IgM; a small number of which were seen in the duodenum of 2 day old animals. The duodenum appeared to be the preferred site of infiltration since more distal regions of the intestinal tract contained very few IgM cells during the first week of life. Furthermore even in animals 2 weeks old the numbers of cells occurring in the duodenum exceeded those of other sites examined by more than 10 fold.

The development of the population of cells containing IgA was similar in characteristics to that of IgM but occurred at a somewhat later stage. Small numbers of IgA cells were detected in duodenal mucosa of 4-5 day old animals. In contrast IgG containing cells were not found in significant numbers in pigs under 9 days of age.

Comparison of the relative numbers of immunoglobulin containing cells occurring at different levels of intestine show that in all animals examined the numbers of cells occurring in the duodenal mucosa was consistently higher than at more distant sites, irrespective of the class of immunoglobulin (Table 5.2).

In suckling pigs up to 4 weeks of age, the majority of cells in the lamina propria of the small bowel were those containing IgM. Indeed in the first week of life IgA cells accounted for less than 10 per cent of total immunoglobulin cells.

Weaning appears to have had some influence on the change in relative populations of cells. This is particularly evident in the duodenum. Thus, whereas in the 4 week old suckling animal IgM cells outnumbered those containing IgA, the proportions were reversed in pigs which were of the same age but which had been weaned for 1 week (Table 5.3). As the weaned animals matured the proportion of IgM cells declined until by the time they were 12 weeks old nearly 90% of cells contained IgA (Table 5.4).

The total number of immunoglobulin containing cells, irrespective of class, infiltrating the lamina propria increased continually throughout the age period covered by this study. Initially in the youngest animals this was mainly due to increasing numbers of IgM cells but as the animals matured, and especially after weaning it was principally due to IgA cells. The numbers of cells containing IgM or IgG remained constant or showed a slight decline (Table 5.2; fig 5.1).

DISCUSSION

In an earlier comparative study on 4 week old suckling piglets a preponderance of IgM containing cells over those bearing IgA in the intestinal lamina was noted (Allen and Porter, 1973a). This finding was contrary to those of other workers who have demonstrated in a number of species that the majority of immunocytes in secretory mucosa were IgA containing cells. (Crabbe, Carbonara & Heremans, 1965; Rubin, Fauci, Sleisinger, Jeffries and Margolis, 1965; Crandall, Cebra and Crandall, 1967; Crabbe, Bazin, Eyssen & Heremans, 1968; Felsenfeld, Greer, Kirtly and Jiricka, 1968; Vaerman and Heremans, 1969) and it was suggested that the discrepancy may be related to the difference in ages of the animals under study. Those used by other authors could be regarded as being immunologically mature whilst the animals used in this study were in the earliest stages of immune development.

The data presented here confirms this hypothesis. Whereas in the young animal IgM containing cells were the dominant immunocyte, in the intestinal lamina propria, in the immunologically mature pig, as in other species, IgA cells predominated. Moreover, the early appearance of IgM containing cells, preceding that of IgA cells emphasises the important role of this immunoglobulin in the ontogeny of secretory immunity of the alimentary tract.

The initial infiltration of immunocytes into the intestinal lamina propria of the young pig is a response to the challenge of the luminal environment, and in particular bacterial metabolites. Oral administration of gnotobiotic pigs with Escherichia coli antigen resulted in an infiltration of the intestinal lamina by immunocytes initially cells of the IgM class, whereas very few immunoglobulin containing cells occurred in germ free animals of the same age (Porter, Kenworthy, Noakes and Allen, 1974). It is significant that lipopolysaccharide from gram negative organisms is not only a mitogenic stimulator but also an antigen which promotes the preferential synthesis of IgM (Friedman, 1973; Melchers, Braun and Galanos, 1975).

It is interesting to note that in studies of a 6 day old dog (Vaerman & Heremans, 1969), the only immunoglobulin containing cells found in the intestinal mucosa were of the IgM type, although a 3 week old litter-mate possessed numerous IgA containing cells as well. IgM containing cells also constitute a larger proportion of the immunocytes in the intestinal lamina of the young calf than they do in older animals (Allen & Porter, 1975). A similar lack of IgA containing cells has been observed in the alimentary tract of human infants. (Mougenot, Fontaine, Mougenot & Polonovski, 1975, Brandtzaeg & Baklien, 1976). Furthermore, in studies of infants with enteric colibacillosis, IgM antibodies formed an important part of the early response, especially in the youngest children of 4 or 5 months of age. (McNeish, Evans, Gaze & Rogers, 1975).

An animal is likely to experience challenges by new antigens more frequently during the early stage of its development than later on. The establishment of a gut microflora, together with a wide range of new dietary components all contribute to this challenge. As the animal matures, the microflora becomes stabilised and the number of previously unencountered components of lumenal origin diminishes to a low level.

The relationship between the cellular component of the secretory immune system of the alimentary tract and the antigenic load in the lumen may be considered as one of dynamic equilibrium. Whereas in the very young animals the antigenic load placed upon the cellular compartment of the gut is consistently changing, in the mature animal it becomes virtually stable and the cellular component of the lamina is able to equilibrate with the lumenal environment.

Thus challenge by a previously unexperienced antigen results in an initial proliferation by IgM producing cells, followed by a possible greater proliferation of IgA containing cells. In the older animal also, the initial response to an antigen which the host has not previously experienced is likely to be a proliferation of IgM cells. Crandall and his co-workers (Crandall, Cebra & Crandall, 1967) showed a relative increase in

the number of IgM containing cells in the intestinal mucosa of adult rabbits soon after infection with *Trichinella*. A biphasic increase of IgM producing cells has been shown to occur in the intestines of animals orally immunized with sheep red blood cells (Werner, Lefevre & Raettig, 1971).

The magnitude of the immunocyte proliferation is likely to be related to the severity of the antigenic challenge, since for the animal to survive, the level of antibody produced must be sufficient to counteract the potentially harmful effects of the antigen. Conversely, since the absence of long term memory has been demonstrated in the intestinal secretory immune system (Freter & Gangarosa, 1963; Porter, Kenworthy, Noakes & Allen, 1974) it is to be expected that the removal of an antigen from the luminal environment will result in a compensatory decline in the numbers of immunocytes in the lamina propria. In this respect it is interesting to note that a reduction in the cellularity of the lamina propria occurs in animals fed antibiotics (Gordon & Bruckner-Kardoss, 1961).

Studies on IgA producing plasma cells in human intestine indicate that they are short lived mature cells which do not divide and therefore play no part in establishing long term memory. Thus the persistence of antibody production to further antigenic stimulation depends upon the recruitment of new immuno-competent

cells (Mattioli & Tomasi, 1973). There is a general agreement that IgM forming cells are the precursors of cells synthesising both IgG and IgA. The administration of anti-u serum results in the suppression of the production of both these immunoglobulins in addition to that of IgM (Lawton, Asofsky, Hylton & Cooper, 1972). Cooper & Turner, (1969) suggested that IgM memory cells may migrate from a Peyers patch source to other lymphoid tissues, and Craig & Cebra, (1971) have shown such cells to have the potential to proliferate and differentiate into IgA producing immunocytes in the lamina of the small intestine.

Although the intestinal secretory immune system has been the focus of attention in numerous species much of the research has been conducted on mature animals in which IgA is the predominant secretory immunoglobulin. There is relatively little information on the development of the secretory system in the young mammal. The results of the present study indicate that at the earliest stages of secretory immune development IgM is probably of greater significance. Moreover the secretion of IgM across the intestinal epithelium does not depend upon the local availability of serum derived IgM but relates to local synthesis by immunocytes in the lamina propria. It is then actively transported across the secretory epithelium by a route and mechanism similar to that of IgA. (Allen, Smith & Porter, 1976).

The role of IgM in the protection of the newborn deserves serious consideration. It possesses potent antibacterial characteristics and is ideally suited to the provision of early protection, for the neonate, against enteropathogenic challenge. It would appear therefore that the dependence of the very young animal for its secretory immune defence on this class of antibody may be more than fortuitous. Whereas in sow colostrum, approximately 80 per cent of the total immunoglobulin is of the IgG class, the naturally occurring antibody to E. coli pathogens is almost entirely associated with IgM or IgA (Porter, 1969b).

It is worth noting that until recently vaccination protocols aimed at enhancing maternally derived colostrum protection of the newborn against neonatal enteritis involved parenteral immunization resulting in enhanced levels of colostrum IgG. Whilst this may confer effective short term protection the continuing development of active immunity in the neonate may be impaired. The mechanisms involved are not presently understood, but recent work on the pig (Muscoplatt, Setcavage & Kim, 1977) indicates that maternal antibody obtained through colostrum serves to regulate the development of active immune response by inhibiting background development of antibody producing cells. Furthermore it is specific antibody of the IgG class which exerts these suppressive effects. In contrast there is good evidence that IgM does not suppress the stimulation of synthesising immunocytes (Henry & Jerne, 1968) but may even contribute to the enhancement of a primary immune response.

In this respect the effectiveness of a vaccination protocol, aimed at producing hyperimmune colostral antibodies of the IgM class by a combination of oral and parenteral immunization, in conferring protection on newborn piglets against acute neonatal enteritis (Chidlow & Porter, 1979 a, b) is particularly relevant. It presents the best possible conditions in the newborn pig intestine for the early stimulation of active secretory immunity and it would appear that immunization protocols should be designed with the express intention of producing secretory antibodies of the IgM class.

Table 5.1 Numbers of cells containing IgM, IgA & IgG in the intestinal mucosa of suckling pigs of different ages

PM No.		640	641	655	656	642	643	647	648	650	651	652
Age (days)		2	2	4	4	5	5	9	9	12	12	15
Organ	Ig											
Duodenum	M	1	9	32	12	78	171	401	351	833	366	811
	A	0	1	1	5	10	4	461	126	222	155	613
	G	0	0	0	0	2	0	73	54	176	14	40
Jejunum	M	1	0	5	1	4	1	25	28	25	132	71
	A	0	0	2	0	0	1	12	14	192	38	38
	G	0	0	0	0	0	0	0	4	12	2	2
Ileum	M	0	1	2	2	0	6	5	1	95	22	17
	A	0	0	1	3	0	0	21	5	223	45	47
	G	0	0	0	0	0	3	0	0	4	0	2
Colon	M	0	0	0	0	0	0	14	4	96	34	29
	A	0	0	0	0	0	0	35	11	120	33	17
	G	0	0	0	0	0	0	5	1	2	4	12

Counts expressed as the numbers of cells per twenty fields (X40 objective)

Table 5.2 Numbers of cells containing IgM, IgA & IgG in the intestinal lamina propria of pigs of different ages

Age		<u>DAYS</u>					<u>WEEKS</u>				
		2	4	5	9	12	15	4	4	9	12
No. animals		2	2	2	2	2	1	9	3	7	3
Suckling or Weaned		S	S	S	S	S	S	S	W	W	W
Organ Ig											
Duodenum	M	5	22	125	376	585	811	1542	1117	632	546
	A	1	3	7	298	188	613	1039	1433	2992	4747
	G	0	0	0	1	95	40	95	329	269	ND
Jejunum	M	1	3	3	27	101	71	208	353	183	318
	A	0	1	1	13	115	38	145	255	834	2727
	G	0	0	0	2	7	2	60	23	112	ND
Ileum	M	1	2	3	3	59	17	110	73	22	155
	A	0	2	0	13	134	47	91	212	38	1005
	G	0	0	2	0	2	2	21	7	26	ND
Colon	M	0	0	0	9	65	29	ND	316	150	137
	A	0	0	0	23	77	17	ND	280	1027	1562
	G	0	0	0	3	3	12	ND	8	24	ND

Counts expressed as the means of the numbers of cells per twenty fields
(X40 objective)

ND = Not Determined

Table 5.3 Numbers of cells containing IgM, IgA and IgG in the intestinal lamina propria of 4 week old suckling or weaned pigs

		Suckling		Weaned	
No. Animals		9		3	
Organ	Ig	Count	S.D.	Count	S.D.
Duodenum	M	1542	(414)	1117	(377)
	A	1039	(360)	1433	(688)
	G	95	(103)	329	(184)
Jejunum	M	208	(76)	353	(164)
	A	145	(91)	255	(52)
	G	60	(32)	23	(10)
Ileum	M	110	(55)	73	(50)
	A	91	(41)	212	(133)
	G	21	(9)	7	(3)

Counts expressed as the means of the numbers of cells per twenty fields (X40 objective)

SD = standard deviation

Table 5.4 Proportion of IgA cells (expressed as percentage of total IgA & IgM cells) in the intestine of pigs of different ages

Age	<u>DAYS</u>						<u>WEEKS</u>			
	2	4	5	9	12	15	4	4	9	12
SUCKLING OR WEANED	S	S	S	S	S	S	S	W	W	W
Duodenum	-	6	5	43	24	43	40	57	82	90
Jejunum	-	25	16	32	53	35	41	42	82	90
Ileum	-	50	-	81	70	73	45	74	58	87
Colon	-	-	-	72	54	36	-	47	87	92

Legend

Figure 5.1. Numbers of cells containing immunoglobulins IgM; IgA and IgG in the duodenal lamina propria of pigs of different ages.

Sequential Development of Intestinal IPC.

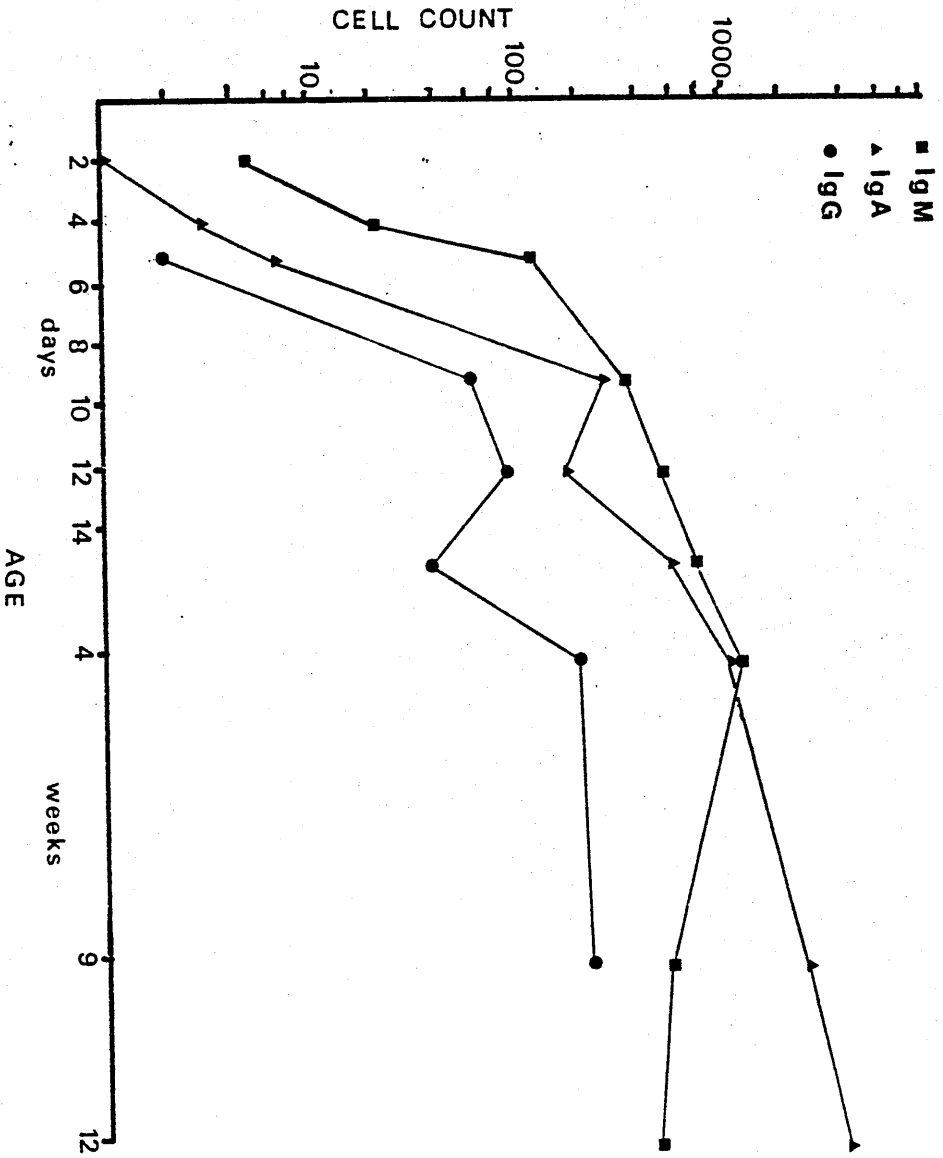


Figure 5.1

SECTION 6

INTESTINAL SECRETION OF ANTIBODY IN FOURTEEN DAY OLD PIGLETS
IN RESPONSE TO EARLY ORAL IMMUNIZATION WITH ESCHERICHIA COLI
SOMATIC ANTIGENS

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BACKGROUND AND OBJECTIVES

The preceding studies have shown that uptake of macromolecules by the intestinal mucosa of young piglets persists after 'closure' to maternal immunoglobulins and moreover that the rate of uptake declines with increasing age. Furthermore a small number of cells containing IgM has been demonstrated in the intestinal lamina propria of animals only 2 days old. The population of IgM cells in the lamina propria has earlier been shown to exceed that of IgA cells in 4 week old suckling pigs. Together these findings give rise to the possibility of stimulating the production of specific intestinal secretory antibodies to meet the needs of early weaning.

Enteric colibacillosis in the young pig is essentially local in nature with bacteraemia seldom being a feature. Enteropathogens proliferate in the gut lumen in close proximity to the absorptive epithelial cell lining. Frequently they possess plasmid determined adhesion factors which enable them to attach firmly to the epithelial cell membrane (Smith & Linggood, 1971). They synthesise a variety of enterotoxins and endotoxins which are absorbed via the enterocytes into the mucosal tissues causing serious functional impairment of the absorptive capacity of the epithelial cells as well as giving rise to inflammatory reactions in the underlying lamina propria. It is therefore

desirable that to be most effective antibody should be present on the luminal surface. Consequently for the demonstration of a complete protective immunity it is not sufficient to show that cells synthesising secretory antibody are present in the intestinal lamina propria, secretion of these antibodies into the intestinal lumen must also be demonstrated.

Earlier studies of intestinal antibody secretion have relied on experiments conducted in surgically prepared animals with Thiry Vella loops in the small bowel (Porter, Noakes & Allen, 1970). Such animals required more than one weeks post-operative convalescence to enable the immunoglobulin profile of intestinal secretion to return to normal, since inflammatory lesions and serous exudations arising from surgery frequently show the presence of the majority of serum proteins. These preparations are consequently unsuited to studies in the very young pig.

Furthermore the amounts of antibody secreted in the earliest stages of immunity are extremely small and have proved difficult to quantitate by conventional techniques. The development of quantitative microscopical assays employing activated sepharose beads (DASS) capable of detecting antigens in nanogramme amounts, (Capel, 1974; Deedler & Ploem, 1974) offers a much better prospect of examining the effects of oral immunization on intestinal antibody secretion in the very young pig. The technique is also conveniently consistent with the methodology for enumerating immunoglobulin containing cells used earlier.

In the present study an assay for the detection of antibody secretion using in vitro organ maintenance techniques and quantitative microscopy is used to examine intestinal responses of neonatal piglets to very early oral administration of bacterial antigens.

MATERIALS AND METHODS

Activation of Sepharose beads with Cyanogen Bromide

Sepharose 4B beads (Pharmacia, Uppsala, Sweden) were prepared for protein coupling by activating with cyanogen bromide (CnBr). 100 mls of well washed sedimented beads were added to 20g CnBr in 100 ml H₂O. The pH was raised to 10.5 with 1 M NaOH and the reaction allowed to proceed for 10 minutes, with gentle stirring. Following activation, the beads were washed 4 times in 0.2 M sodium citrate buffer pH 6.5 (500 mls per wash). Throughout the whole procedure the temperature of the solutions was maintained below 10°C.

Coupling antigens to beads

To 10 gms of sedimented activated beads, was added 10 mls of a solution of O somatic antigen specific for one of the Escherichia coli serotypes (vide infra), in phosphate buffered saline pH 7.1 (PBS). The coupling reaction was allowed to proceed for 48 hours at 4°C with constant stirring. Following coupling the beads were washed 3 times in PBS. The coupled beads were deactivated to block any remaining active groups, by incubating, at room temperature, in 0.2 M ethanolamine in 0.05 M carbonate buffer pH 10.0. Incubation times varied from 1-6 hours. Following deactivation the beads were washed for 2 hours in 3 changes of PBS and stored at -20°C until required for use.

Staining techniques

Prior to use the beads were thawed and given a further wash in 0.1 M sodium phosphate buffer pH 7.1 containing 0.5 M sodium chloride (HSB).

The staining reaction was performed by mixing 1 drop (0.03 ml) of coupled beads with 1 drop of fluid under assay and 1 drop of carrier beads added. The mixture was shaken for half an hour at room temperature (20⁰) on a Dynatech AM 69 shaker. After shaking, 2 drops of HSB were added and the tray shaken for 2 minutes before being centrifuged at 1,000 RPM for 30 seconds. The supernatant was removed and replaced by a further 3 drops of HSB. The mixture was again shaken for two minutes and then recentrifuged. The washing procedure was repeated a total of 6 times.

Following washing the bead mixture was treated with 1 drop of rabbit anti-pig globulin serum and shaken for half an hour. It was centrifuged and rewashed as previously.

Finally the bead mixture was stained with the sheep anti-rabbit globulin FITC conjugate for half an hour with continuous shaking and rewashed as before.

Following the final washing the supernatant was removed and replaced with 1 drop of the glycerin phosphate buffer pH 8.5 and the beads resuspended. One drop of the suspension was placed on a microscope slide, covered with a coverglass and sealed with nail varnish.

Measurement of Fluorescence

The intensity of the fluorescent emission of the beads was measured on a Reichert Zetopan microscope equipped with a Reichert microphotometer having a Phillips photomultiplier tube X.P. 1010 and a digital voltmeter (Exel XL 15). The beads were viewed by incident light using an HBO 200 light source (Osram) with a 2FITC 3 + 2.5 BG 38 exciter filter and a 1 GG + 2 OG 515 barrier filter in conjunction with an interference beam splitter reflecting up to approximately 490nm. The readings were made using a X40 0.9 N.A. objective in conjunction with a X8 PK eyepiece.

The microphotometer interference wedge was set to read at 527nm and calibrated against a fluorescence demonstration slide having permanent phosphor emitting at a peak of 528 nm (Becton Dickinson).

For calibration the measuring diaphragm was set at 1mm diameter. The photomultiplier control unit was set to range 3, and the band width adjusted to give a reading of 30 on the digital voltmeter when a previously selected phosphor crystal was viewed. (This crystal was selected for each subsequent calibration step.) Permanent recordings were made on a Servoscribe RE 5W.20 potentiometric chart recorder adjusted to give a value exactly twice that of the digital voltmeter.

The fluorescent intensity of the beads was measured using the 4mm diameter diaphragm. They were selected from different areas of the slide to ensure that they had not previously been irradiated since it was found that the fading of fluorescence was considerable. The beads selected were those with a diameter approximating 10mm and the central 4mm diameter portion was measured. A minimum of 10 beads per sample were measured.

Assays were made on the luminal fluid from each of the three levels of intestine against beads specifically labelled with 'O' somatic antigen from each of the seven serotypes of E. coli used for the oral immunization, in turn.

In addition control background values were obtained by treating the antigen coupled beads with rabbit anti-pig globulin reagent followed by staining with the sheep anti-rabbit globulin FITC conjugate.

Figure 6.1 shows a typical recording from a series of beads. The fade rate of the fluorescent emission is clearly demonstrated. Because of this values were taken as being the peak of each individual curve.

Animals

Two groups of animals were used for the experiments, one to evaluate the gut assay technique, the other to study the efficacy of oral vaccination of piglets at an early age.

The animals involved in the evaluation of the assay comprised a group of four 5 week old pigs. They were suckled on the sow until 3 weeks old when they were weaned. Prior to weaning they had access to Creepcare diet (B.O.C.M.S.) from 4 days of age and following weaning they were fed Growercare (B.O.C.M.S.). Both diets were supplemented with "Intagen" vaccine (B.O.C.M.S.) at levels known to give adequate stimulation to the intestinal secretory immune system (Porter, Kenworthy & Allen, 1974). The vaccine "Intagen" comprises selected serotypes of Escherichia coli which are known porcine enteropathogens. Culture concentrates of each serotype are inactivated by heating to release the 'O' somatic antigens 0,8; 0,45; 0,138; 0,139; 0,141; 0,147; and 0,149 (Porter, Kenworthy, Holme & Horsefield, 1973).

The key objective of this study was to evaluate the efficacy of early oral vaccination and this was examined in 7 piglets. Four animals were orally dosed with 0.1ml of Intagen vaccine in 1.0ml of physiological saline at 4 and 7 days of age. Each dose contained 200 haemagglutination inhibition units/serotype (Porter, Kenworthy & Allen, 1974). The remaining three animals were maintained as a placebo group.

In vitro assay of antibody production

Animals were sacrificed at 14 days of age by intravenous administration of Euthatal (May & Baker) and the whole of the intestinal tract, distal to the pylorus, was immediately removed and placed into Eagles minimum essential tissue culture medium (M.E.M.) (Wellcome Laboratories) at 4°C.

Segments of duodenum, jejunum and ileum 5-7 cms in length, were prepared and flushed through with fresh MEM and one end ligated. Sufficient MEM was introduced into the lumen to induce a slight distension and the other end ligated. The segment of gut was immersed in a bath of MEM at 37°C with continuous aeration. Incubation was continued for 4 hours after which time the luminal MEM was extracted and its volume measured. It was then stored at -20° until required for analysis. The length of intestinal tissue between the two ligatures was measured and its weight determined.

The luminal MEM was thawed and assayed by the DASS technique and the results calculated.

Calculation

The mean of the ten readings per sample was determined and the mean for the control subtracted. The resulting figure was then adjusted to take into account the dilution of secreted antibody by the volume of MEM introduced into the lumen and the weight of tissue from which the antibody was derived using the following formula $\frac{N \times V}{W}$ where N is the value of the mean of the test minus the mean of the control. V is the volume of MEM in the lumen and W is the weight of tissue.

RESULTS

A. Development of D.A.S.S. Assay

1. Deactivation of CnBr activated Sepharose beads

A series of investigations were undertaken to examine the effect of blocking in buffer for different times.

CnBr activated beads were deactivated in buffer and then treated with rabbit antisera specific for *E. coli* antigen O:8 or O:149, after washing, the beads were stained with goat anti-rabbit/FITC conjugate and evaluated. The results showed that there was no great difference in background staining levels of beads treated for longer than 3-4 hours (Table 6.1). A time of 4 hours was selected for the deactivation of beads in all future assays.

2. Influence of staining time

The effect of different staining times was studied using beads labelled with sheep anti-*E. coli* 0:78 serum. They were stained with a rabbit anti-sheep globulin FITC conjugate (Nordic) diluted 1:20 for periods varying from 15-20 minutes. The results (Table 6.2) showed that peak activity was reached after a staining time of 60 minutes.

3. Reproducibility of Readings

The reproducibility of the assay was determined on replicates of sample of luminal contents from a 4 week old immunized pig using beads labelled with E. coli 08 antigen. The variation from the average value of the means of four replicate assays was well within the value established for a significant difference at $p = 0.05$ (Table 6.3). Hence the reproducibility of the test was judged satisfactory.

B. Evaluation of organ bath assay

Assessment of the feasibility of using an organ bath assay to measure the production of secretory antibody by intestine was made on samples of secretion from the duodenum, jejunum and ileum of four 5 week old pigs which had received a course of oral immunization with "Intagen" via the feed. Assays of antibody against all seven serotypes of E. coli included in the vaccine were made. Table 6.4 shows the readings from the 4 pigs for each level of intestine. The ability to measure antibody production by this method was clearly demonstrated although wide variation of response to oral immunization to the various antigens was revealed. Pig 1 showed a particularly high response in practically all regions of the gut to every serotype whilst conversely pig 4 only showed a low level of response to antigens 0 8 and 0 141 and failed to respond to antigens 0 45 and 0 149. Similarly pigs 3 and 5 showed no response to 0 45 and pig 3 failed to produce antibody to 0 149 also.

It is interesting to note that taken overall the highest level of antibody production occurred in the duodenum (Table 6.5).

C. Efficacy of Early Oral Immunization

The feasibility of stimulating an early active secretory immune response in the intestine by oral immunization was examined in seven piglets, 4 vaccinates and 3 placebo controls. The vaccinated animals were given two oral doses of Intagen one at 4 days and the second at 7 days of age. This regimen was selected as one which fitted in with other farm management activities such as ear marking and iron injection and thus minimised effort expended should the approach be used in any practical farming situation.

The piglets were killed at 14 days of age and tissues taken from 3 levels of the small intestine, duodenum, jejunum and ileum. Secretion by these tissues, of specific antibody, against each of the 7 E. coli serotypes present in the vaccine, was assayed by the DASS method.

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The antibody assays for each serotype at the various levels of intestine of the two groups of pigs are given in full in table 6.6.1. The variation in antibody response of the individual pigs seen in the earlier experiment on the evaluation of the technique was again apparent.

A consistent pattern of reaction to all serotypes was obtained in the duodenum and jejunum of two of the vaccinated pigs (Nos. 3 & 4), whilst a similar pattern was found in the jejunum but not the duodenum of a third vaccinated animal (No. 1). One vaccinated pig (No. 2) gave a poor response throughout to all serotypes. In contrast a similar pattern was not observed in any of the placebo pigs, one of which (No. 5) showed little evidence of activity at all.

As a consequence of this variation in response, the differences between the means of antibody level to individual serotypes in duodenum and jejunum of the two groups of pigs are not significant. However, the means of the vaccinated group are consistently higher than those of the placebo group indicating a direction of effect of the vaccination schedule which was significant at the 95% level.

The means of the antibody assays obtained for each serotype at the various levels of intestine of the two groups, vaccinate and placebo, are given in table 6.6. A comparison of these means shows that in both the duodenum and jejunum the levels of antibody detected in the vaccinated group was consistently higher than in the controls. There was a significant direction of effect ($P < 0.05$) for all 7 E. coli serotypes. Earlier studies of macromolecular uptake in intestinal tissues of pigs of this age demonstrated an absence of competition between antigens (Section 4), it is therefore reasonable to expect this common direction of effect. The levels of antibody secreted by the ileal tissues of the two groups of pigs did not differ significantly.

Some activity against the various serotypes was evident in the controls, particularly the jejunum and ileum. This was presumably due to background stimulation from the microflora which had become established during the first weeks of life. The piglets were maintained with the dam in a conventional herd and although bacterial analysis of the luminal flora was not undertaken a number of enteropathogenic E. coli serotypes were known to be present in the environment. It is to be expected therefore that the animals would be subjected to challenge from microbial pathogens. The essential question is whether or not oral administration of small doses of antigen, at an early age, would significantly effect antibody secretion to lift it above the normal base-line and thereby enhance the level of resistance to any subsequent proliferation of pathogens which might occur.

DISCUSSION

The prospect of stimulating active production of intestinal antibody early in the life of the pig is of special interest in view of the current trend towards weaning at a very early age when the animals immunological defenses are minimal. The results of this series of experiments, in which comparatively small doses of antigen gave rise to a significant antibody response are particularly encouraging.

In these studies, assay of antibody activity has been confined to 0 antigen in order to maintain a consistency with the preceding investigations of uptake of macromolecules, including endotoxin. The levels of antibody demonstrated in the piglets orally vaccinated at 4 and 7 days of age were lower than those found in the 4 week old animals which had received oral vaccine daily from about 10 days of age. They were also low by classical serological standards. Nevertheless the results indicate that the intestinal immune mechanisms can be successfully stimulated at this early age.

Under normal circumstances secretory intestinal antibodies do not function exclusively within the luminal contents but are in very much higher concentration at the surface of the epithelium where they are bound into mucin (Porter & Allen, 1972). Here the control of infection and uptake of toxic molecules may be effected by a complex variety of mechanisms. These include bacterial agglutination (McClelland, Samson, Parkin & Shearman, 1972) bacteriostasis (Porter, Kenworthy, Noakes & Allen, 1974) elimination of virulence determinants (Porter, Linggood & Chidlow, 1978) and enterotoxin neutralization (Linggood, 1976).

Although no evidence is presented to indicate the class of the secreted antibody it is not unlikely that most of the activity is attributable to IgM. It has been shown earlier (Section 5) that in animals of this age cells synthesising this class of immunoglobulin form the largest proportion of immunocytes in the intestinal lamina propria. Further, lipopolysaccharide antigen from gram negative organisms promotes the preferential synthesis of IgM (Friedman, 1973; Melchers, Braun & Galanos, 1975) and this class of immunoglobulin plays a dominant role in the early phase of intestinal response to E. coli infection in the gnotobiotic pig (Porter, Kenworthy, Noakes & Allen, 1974). The presence of IgM antibody in intestinal secretions at an early age will greatly enhance the efficacy of the defense system since it is a particularly potent antibacterial agent (Michael & Rosen, 1963) and affords protection against the lethal effects of endotoxins (Kim & Watson, 1965).

The finding of higher levels of antibody in the anterior small intestine than in the ileum is consistent with the demonstration that the numbers of immunoglobulin containing cells were highest in the duodenum (Section 5) and accords with the view advanced earlier (Section 4), that as macromolecules are more readily absorbed in the upper gut this would be the area in which the synthesis of secretory antibody was likely to be greatest.

The emphasis on the role played by the upper gut, which has been a constant feature of the results throughout the research in this presentation, deserves consideration particularly with respect to its relevance to possible mechanisms of oral immunization against colibacillosis. The environment of a conventional intensive pig unit is continually laden with pathogens. These organisms can be isolated in small numbers from apparently healthy piglets (Kenworthy & Allen, 1966) but rapidly proliferate to high levels following stress, when they infect the upper gut (Smith & Jones, 1963; Smith & Linggood, 1971) giving rise to clinical symptoms of lethargy, diarrhoea, dehydration and emaciation which may lead to death (Dunne, 1975).

Although oral vaccination of piglets at 4 and 7 days of age successfully stimulated antibody production in the duodenum and jejunum it was ineffective in the ileum since antibody levels in this tissue were similar for both the vaccinated and placebo groups. It is likely that the response in ileal tissue was influenced by the presence of low levels of indigenous pathogens providing an immune stimulus. The failure of the orally administered antigen to raise antibody production in the ileum significantly above the baseline of the controls may have been due to the dose being too small to reach the lower gut in sufficient quantity to effect a response, most being absorbed in the upper intestine. Alternatively the presence of naturally occurring antibody in the ileum could have blocked further uptake of antigen since secretory antibody interferes specifically with macromolecular uptake (Walker, Isselbacher & Bloch, 1974).

Topical application of antigen to different levels of intestinal mucosa stimulate an immune response both at and distal to, the site of administration, but not proximally (Pierce & Gowans, 1975). The results of the present studies accord with this finding. In the placebo pigs although there was a measurable level of antibody in the ileal tissue the amount in the duodenum was negligible. It is clear from this result that the stimulation of an antibody response by indigenous pathogens in the lower small intestine affords little immediate protection against infection in the upper gut. It would appear than one

of the important features of oral vaccination for protection against colibacillosis is that this route of administration not only ensures topical application of the vaccine to the mucosal epithelium but also ensures that the greatest concentration of antigen is delivered to the region of the tract most at risk.

Intestinal immunocyte precursors originate in the G.A.L.T., principally Peyer's patches. This lymphoid tissue is most extensive in the lower small intestine. This suggests that the continual presence of a low level of pathogens in this region ensures a constant supply of precursor cells which migrate to other areas of the alimentary tract where they are available to respond to antigenic challenge. There is no amnestic response in the intestinal secretory immune system (Freter & Gangarosa, 1963; Ogra & Karzon, 1969; Lee & Foo, 1978) and in order to produce an effective level of immunity antigen must be administered continually. The simplest and cheapest way to achieve this with domestic livestock, is to include it in the feed. In young suckling animals however consumption of fabricated feeds is either low or non-existent and for these, the vaccine must be given directly in order for early onset of secretory immunity to be obtained.

Table 6.1

Comparison of the effect of time of incubation on the efficiency of blocking CnBr activated sepharose 4B by 0.2 M ethanolamine in 0.05 M carbonate buffer pH 10.0.

Incubation time (HRS)	Results (FI Units)	
	Rabbit anti <u>E. coli</u>	
	<u>0 8</u>	<u>0 149</u>
1	28	24
3	13	14
4	17	14
6	17	19

Table 6.2

Influence of staining times of fluorescent intensity of activated sepharose beads labelled with sheep anti-E. coli 0 78 stained with rabbit anti-sheep FITC conjugate.

Staining time (minutes)	15	30	45	60	90	120
Reading (F.I. Units)	28	29	31	50	42	51

Table 6.3

Replicate assays of Escherichia coli 0 8 antibody in luminal fluid by DASS technique.

Assay No.	1	2	3	4
Readings	24	23	17	28
	29	23	22	25
	26	26	23	18
	24	24	18	24
	27	21	23	19
	28	18	25	19
	25	31	28	30
	23	19	22	18
	20	25	26	18
	24	21	22	16
Mean	25.0	23.1	22.6	21.5

Overall mean 23.05

Significant difference of means (P = 0.05) 3.38

The assay was performed on 4 aliquots of a sample of luminal fluid and the means of 10 readings from each sample are compared.

Table 6.4

Evaluation of organ bath assay for the determination of secretory antibody production.

Antibody levels (F.I. Units)

Antigen	Pig No.	Duodenum	Jejunum	Ileum
0 8	1	11.7	7.1	20.2
	2	8.6	3.6	7.4
	3	1.2	0	0.9
	4	2.7	10.0	0.9
<hr/>				
0 45	1	31.6	19.5	9.6
	2	0	0	0
	3	0	0	0
	4	0	0	0
<hr/>				
0 138	1	29.2	26.4	13.7
	2	22.0	12.7	4.5
	3	14.0	14.1	13.0
	4	16.0	22.3	9.6
<hr/>				
0 139	1	28.9	31.8	23.7
	2	22.3	16.8	8.0
	3	19.8	24.3	5.0
	4	40.5	13.8	15.6

contd/.....

Table 6.4 contd.....

Antibody levels (F.I. Units)				
Antigen	Pig No.	Duodenum	Jejunum	Ileum
0 141	1	22.8	41.7	13.5
	2	9.6	11.2	0.7
	3	5.5	0	0
	4	5.0	0.9	6.5
<hr/>				
0 147	1	33.3	9.6	40.7
	2	13.1	9.1	6.2
	3	25.2	13.4	3.9
	4	25.7	26.1	9.8
<hr/>				
0 149	1	26.4	7.8	0
	2	0	0	0
	3	0	0	0
	4	N.D.	23.2	27.1

N.D. = Not Done

Table 6.5

Evaluation of Organ Bath Assay.

Means of results of secretory antibody levels in four pigs.

Antigen	Antibody levels (F.I. Units)		
	Duodenum	Jejunum	Ileum
0 8	6.0	5.2	7.4
0 138	20.3	18.9	10.2
0 139	27.9	21.7	13.1
0 141	10.7	13.5	5.2
0 147	24.3	14.6	15.2

Note:- Results for antigens 0 45 and 0 149 have been omitted because of the large number of null results.

Table 6.6

Results of Immunoglobulin Assays on lumen fluid by DASS
 Method expressed as means of assays in vaccine and placebo
 groups of 14 day old pigs.

E. coli serotype 08. 045. 0138. 0139. 0141. 0147. 0149.

Organ.	Treatment	No of								
		Animals								
Duodenum	Vaccine	4	2.06	3.98	4.30	4.65	4.08	4.13	3.63	
	Placebo	3	0.00	0.23	1.80	0.80	1.80	2.17	0.13	
Jejunum	Vaccine	4	9.33	9.75	12.38	9.05	6.85	8.13	6.78	
	Placebo	3	1.83	5.13	7.80	2.70	5.60	5.37	2.40	
Ileum	Vaccine	4	3.55	2.83	4.68	1.80	3.63	1.90	2.88	
	Placebo	3	3.00	4.67	5.93	0.93	3.00	3.33	2.10	

Table 6.6.1. Results of secretory antibody assays on lumen fluid by DASS method of Vaccine and Placebo groups of 14 day old pigs.

Group	Pig No.	ORGAN																				
		D	J	I	D	J	I	D	J	I	D	J	I	D	J	I						
Vaccine	1	1.9	12.4	3.6	0.0	6.2	3.3	0.3	14.4	4.5	0.8	6.9	2.2	0.0	2.8	2.4	1.2	10.0	0.8	0.0	9.6	1.5
	2	2.4	4.3	3.1	0.0	0.0	0.4	1.5	8.8	4.5	0.0	0.0	0.2	1.2	0.0	1.4	0.3	0.4	2.9	0.0	1.9	1.4
	3	0.0	1.4	0.6	9.0	14.6	2.2	8.5	12.2	1.5	7.6	11.7	0.7	6.1	7.0	0.6	6.1	10.4	0.3	0.0	9.5	4.9
	4	4.0	19.2	6.9	6.9	18.2	5.4	6.9	14.1	8.2	10.2	17.6	4.1	9.0	17.6	10.1	8.9	11.7	3.6	14.5	6.1	3.7
Mean	2.06	9.33	3.55	3.98	9.75	2.86	4.30	12.38	4.68	4.65	9.05	1.80	4.08	6.85	3.63	4.13	8.13	1.9	3.63	6.78	2.88	
Placebo	5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	4.0	1.6	0.0	0.0	0.4
	6	0.0	3.8	5.2	0.7	4.8	12.2	3.6	12.1	16.2	0.0	1.5	0.7	3.8	8.9	6.3	5.7	6.8	7.4	0.4	7.2	5.9
	7	0.0	1.7	3.8	0.0	10.6	1.7	1.8	11.3	1.6	2.4	6.6	2.1	1.6	7.9	2.7	0.7	5.3	1.0	0.0	0.0	0.0
	Mean	0.00	1.83	3.00	0.23	5.13	4.67	1.80	7.80	5.93	0.80	2.70	0.93	1.80	5.60	3.00	2.17	5.37	3.33	0.13	2.40	2.10
S.D.	2.50	12.48	5.16	7.11	14.12	8.86	6.49	9.26	11.84	7.84	12.12	2.97	6.82	13.21	7.72	7.28	8.09	5.01	11.02	7.56	4.85	

Key: D - Duodenum J - Jejunum I - Ileum S.D. - Significant Difference between means (P = 0.05)

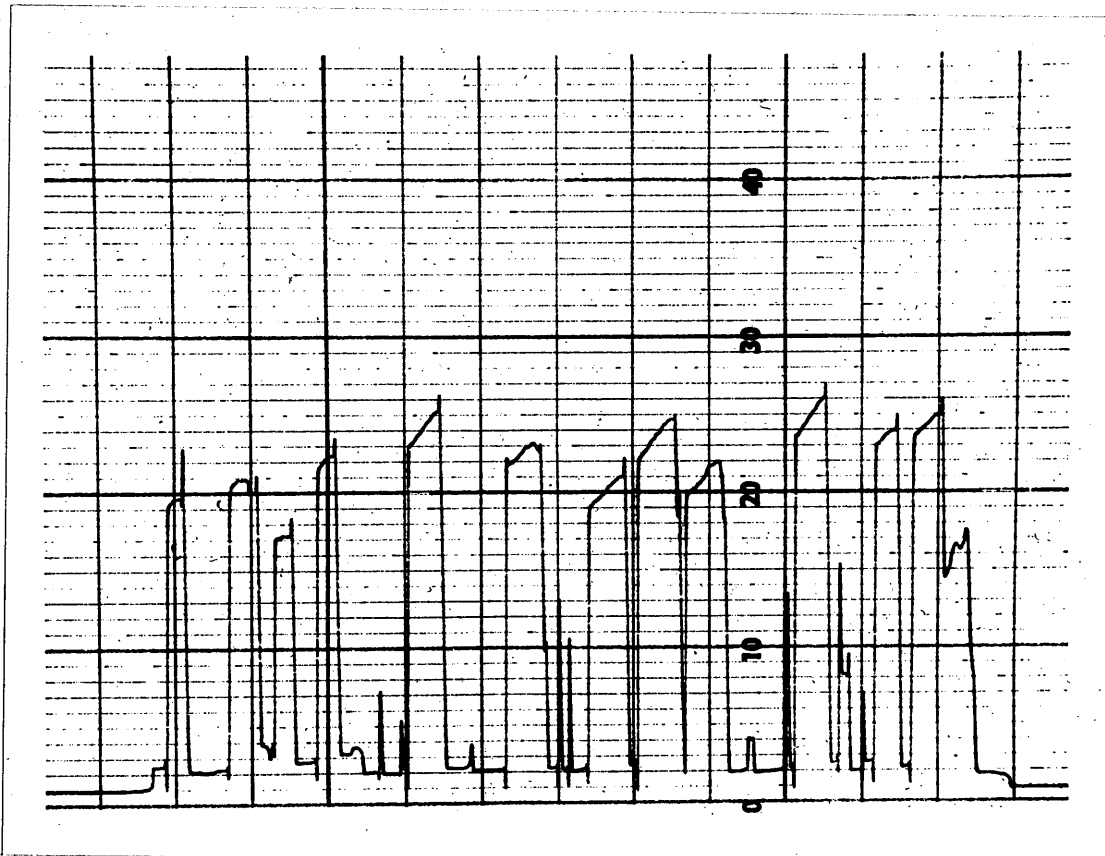


Figure 6.1. An example of the recording obtained when screening defined antigen substrate spheres (DASS) labelled with fluorescein isothiocyanate.

Each peak represents the reading from one bead.

The decay in emission due to irradiation is clearly shown by the slope. The reading is taken from the top of the peak.

SECTION 7

AN EVALUATION OF EARLY ORAL IMMUNIZATION OF PIGLETS
WEANED AT 14 DAYS OF AGE.

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BACKGROUND AND OBJECTIVES

Oral immunization, by inclusion of antigens from enteropathogenic E. coli in the feed, has been shown to confer protection against post weaning enteritis, in pigs weaned at 3 weeks of age, during the critical 2 weeks after weaning. This was reflected by a more rapid clearance of the infecting pathogen, a reduction in both the intensity and duration of clinical symptoms, fewer deaths and improved growth (Porter, Kenworthy & Allen, 1974). Extensive field trials on conventional farms further demonstrated the practical advantages of oral vaccination in economic terms (Porter, Kenworthy, Holme & Horsefield, 1973).

The findings of the studies reported in this presentation have shown that the younger piglet is equally capable of producing an intestinal immune response to oral immunization. Small doses of antigen given per os during the first week of life elicited a production of specific antibody in the intestinal mucosa by 14 days of age. However, the activities of the secreted antibodies were low and the results give no indication of their potential value in conferring enhanced resistance to the young animal against enteric infection. The present experiment was undertaken to examine this question. The resistance conferred by oral immunization, against enteric infection by a pathogenic E. coli serotype is evaluated in terms of health and performance, in piglets weaned when 14 days old.

Earlier attempts to reproduce colibacillosis in pigs by oral administration of enteropathogens has met with varied success. A number of different protocols have been tried. These have varied from the feeding of minced viscera from infected animals (Timoney, 1950) to the daily administration of large volumes of broth cultures (Sojka, Lloyd & Sweeney, 1960). The age of the pig appears to be a factor which influences success. Smith & Jones (1963) failed to reproduce coli enteritis in 9 week old pigs but were successful in piglets 12-24 hours old. More recently Porter, Kenworthy & Allen (1974) successfully infected 3 week old weaned pigs by giving, per os, 2 consecutive daily doses of pathogenic E. coli serotype 0 149 (Abbotstown strain). In the light of this information it was considered that by using a similar protocol and the same strain an infection model could probably be established in 14 day old pigs.

MATERIALS AND METHODS

Animals

The infection study was performed on a litter of 10 Large White/Large White/Landrace piglets. They were housed, with the dam, in a concrete floored pen on straw bedding which was renewed daily. At birth they were randomly allocated to either the vaccinated or placebo control groups. They were suckled on the sow until they were weaned at 14 days of age and had access, ad libitum, to creep diet (BOCMS Baby Suckler care) from 7 days old until the termination of the experiment.

Weights were taken at birth, at 12 day old and at 1, 1½ and 2 weeks post weaning.

Oral vaccination Schedule

The oral vaccine was prepared from Sow Intagen Injectable (BOCMS). This is a sterile preparation of antigens from the same group of selected E. coli serotypes that are present in Intagen "in feed" (Vide Supra-section 6) at a concentration of 500 HIU/serotype/ml for use it was diluted 1:10 with distilled water.

Each animal in the vaccinated group was given 2.0 mls of the diluted vaccine orally on each of 5 consecutive days, commencing when they were 4 days old, i.e. a total dose of 500 HIU/serotype.

Infection protocol

The piglets were infected at 12 days of age with a single oral dose of 2.0mls of an overnight broth culture of pathogenic E. coli, 1×10^9 organisms/ml. The pathogen used was a synthesised Nalidixic acid resistant strain of serotype 0149:K91:K88 ac(L) Abbotstown.

Bacteriology

Faecal samples were collected by anal stimulation with a sterile swab. A specimen was obtained from each piglet 2 days prior to infection and daily for 7 days post infection and at intervals thereafter until 4 weeks of age. The numbers of nalidixic acid resistant E. coli per gram of faeces were estimated on sheep blood agar plates containing 40 ug/ml Nalidixic acid by the technique of Miles and Misra (1938). The dry weights of the faeces were determined to give an estimate of faecal moisture content.

Bacterial Antibody Assay

The milk whey from the sow taken 2 days after parturition was assayed for antibody to E. coli 0 149 by the haemagglutination test described by Porter, Noakes & Allen (1970).

RESULTS

The sow had not been subjected to any immunisation schedule but it was observed that her milk secretion contained a low level of anti-E coli antibody specific for the 0149 strain used in the infection challenge and also during the pre-weaning immunisation. Although this antibody would be routinely present in the gut with regular suckling, previous studies of the induction of secretory antibodies in fistulated suckled animals (Porter, Kenworthy, Noakes & Allen, 1974) leads one to expect no serious impairment in immunisation.

Bacterial Antibody Assay

Infection Study

Pathogenic E. coli were not demonstrated in any faecal samples from the suckling pigs taken prior to infection on the 12th day of age.

Nalidixic acid resistant E. coli 0 149 were present in specimens taken during the 2 days after infection but the numbers were low. It was therefore not possible to obtain reliable counts until the strain proliferated as anticipated during the post weaning period.

One piglet (No. 277) in the vaccinated group passed a faecal specimen which had a moisture content in excess of 80% (< 20% solids) and was classified as being abnormally loose, i.e. scour, according to the criteria of Kenworthy & Allen (1966). In contrast 3 animals (Nos. 271, 272, and 279) in the placebo group passed similarly loose stools for 1 or more days post weaning (Table 7.3.).

Following weaning the numbers of resistant pathogens increased rapidly. Within 24 hours the mean count in both groups was approximately 10^6 organisms/gram faeces, indicating that infection had been successfully established. Thereafter the mean count in the vaccinated pigs remained at around this level for a further 5 days before declining. In contrast the count in the placebo group continued to rise reaching a peak of 10^8 organisms/gram 3-5 days after weaning and then declined (Tables 7.1 and 7.2, figure 7.1).

One animal (No. 277) in the vaccinated group had a count of 10^7 organisms/gram over a period of 4 days during the first week post weaning, whilst in one placebo pig (No. 274) the count remained below this level throughout the trial period.

Faecal Solids

Estimation of faecal solids showed that faecal moisture content broadly reflected the pathogen count (Table 7.3). The percentage faecal solids were generally lower in animals at the time when the E. coli count exceeded 10^7 although there were exceptions, especially in the later stages of the trial. Overall the faecal moisture content of the placebo pigs was higher than that of the vaccinated animals, although at no time was a persistent diarrhoea syndrome established.

Health

The difference in pathogenic E. coli count and faecal moisture levels between the 2 groups was reflected in the better health of the vaccinated pigs. Whereas at the height of the infection the placebo animals showed typical symptoms of ill health, listlessness, loss of bloom and starry coats, the vaccinates remained alert and healthy throughout, including the animal (No. 277) which showed a high faecal count. Interestingly the two placebo pigs worst affected were the heavier of the group (Nos 271 & 279). One showed some scour and the other (No. 279) died 9 days post weaning. A visual assessment of health condition is presented in table 7.6.

Weight Gain

The weight gains of the 2 groups of animals are shown in tables 7.4 and 7.5. The vaccinated pigs gained weight on average approximately 15% faster than the placebo animals. After the first week post-weaning their mean weight was 0.5 kg greater than that of the placebo groups, despite their being the lighter group of pigs at birth. This was significant $P = 0.1$ by the Wilcoxon ranked pairs test. By the end of the trial this had increased to a mean of 0.7 kg. Even the pig which had excreted a high level of enteropathogen and suffered a little diarrhoea (No. 277) gained 3.95 Kg during the 2 weeks after weaning. This was more than any placebo pig gained.

DISCUSSION

The earlier work reported in this presentation demonstrated that the neonatal pig is equipped with the necessary cellular mechanisms to initiate a secretory immune response. Antigen was absorbed across the intestinal mucosa in quantities sufficient to stimulate an immune response and cells containing secretory immunoglobulins infiltrated the intestinal lamina propria from a very early age. Oral administration of antigens elicited secretion of intestinal antibodies, with a range of specificities, albeit in very small amounts. The findings of this experiment confirm and extend these observations showing that enhanced resistance to post weaning set back due to E. coli in early weaned pigs can be conferred by oral immunization in the first few days of life.

Early attempts to establish a model for post weaning E. coli infection have met with varied success. Oral dosing, with broth cultures of pathogenic coli, to pigs weaned at 3 weeks of age, housed individually in cages failed to establish the organism (Kenworthy & Allen, 1966a, Porter, Kenworthy & Allen, 1974) but administration of a relatively large dose of the infecting pathogen on two consecutive days to 3 week old weaned pigs, housed collectively in a pen, shortly after they had been weaned

was successful (Porter, Kenworthy & Allen, 1974). In the present experiment a lower dose of E. coli was administered to the suckling pigs some 48 hours before weaning and it is interesting to note that during the ensuing 2 days, although the serotype could be detected in the faeces of the piglets the numbers were too small to permit reliable counting. However within 24 hours of their being weaned large numbers of pathogens were being excreted by all animals demonstrating a successful implantation.

The faecal pathogenic coli counts of the vaccinated group of pigs, although considerably lower than those of the controls remained comparatively high throughout the first week after weaning. Because of the difficulties in establishing an infection model, experienced by other workers, both groups, vaccinates and control were housed together in a single pen throughout the experiment. The vaccinated pigs were therefore subjected to constant re-infection from the large reservoir of pathogens excreted by the unvaccinated animals which would account for the continued high counts in this group. Indeed they did not fall appreciably until the counts of the control pigs declined. The vaccinated group although excreting a mean of 10^6 faecal E. coli per gram remained healthy and grew well whilst the unvaccinated placebo group, excreted some 100 fold more organisms and were in very poor condition, one animal dying. It is possible that had the vaccinates been moved to a clean environment at weaning the pathogenic coli would have been eliminated and their performance would have been even better.

The vaccination schedule used in this experiment differed from that used earlier in the study of intestinal antibody production in 14 day old pigs (Section 6). On that occasion 2 doses of antigen, 200 HIU/dose, were administered orally when the animals were 4 and 7 days old, whilst in this experiment 5 consecutive daily doses of 100 HIU per day were given from 4 days of age making a total dose of 500 HIU 20% higher than previously. There were two main reasons for this change, firstly the level of antibody measured in the earlier study was judged to be on the low side and secondly the experimental design of keeping the pigs together throughout, used to establish the infection model would, if successful, result in a severe challenge. Under normal practical farm conditions suckling piglets begin to eat artificial creep feeds in reasonable quantities between 8 and 10 days of age. It is therefore reasonable to expect that if the vaccine is included in the creep feed, supplementary doses of antigen given per os on two occasions during the first week would be sufficient to ensure an effective level of antibody by the time the animals are weaned at 12-14 days of age.

The greatest source of economic loss in pig production is due to morbidity. Animals which, although surviving infection suffer serious gut damage or impaired function which markedly reduces their growth potential. One of the major features of earlier studies of oral vaccination for porcine colibacillosis has been the continued improvement in growth of vaccinated pigs compared to untreated controls. The present investigations were undertaken to establish an early onset of secretory antibodies against pathogenic E. coli mainly to enhance the success of weaning at a very early age. However, if the findings of the morphological study, which showed that inflammatory changes occur in the intestinal mucosa of apparently healthy suckling piglets as early as 10 days after birth, are taken into account, there is a very good case for the oral vaccination protocol which has been developed, being administered to pigs, irrespective of their intended age of weaning. This would be of particular benefit in herds where "milk scour" occurring in suckling pigs at about 12-14 days of age is a problem.

Table 7.1

Faecal counts of Nalidixic acid resistant E. coli 0 149 from orally vaccinated and placebo pigs infected at 12 days of age and weaned at 14 days of age

Treatment	Vaccinated					Placebo				
Pig No.	273	275	277	280	281	271	272	274	276	279
Days										
Post-Weaning										
1	5.5	6.3	6.5	6.7	6.0	5.5	6.5	6.0	4.9	5.0
2	N.S.	5.4	4.3	4.1	7.3	5.5	3.8	N.S.	6.7	6.2
3	4.4	4.8	7.6	5.6	5.6	8.7	5.6	6.9	7.0	7.6
4	4.8	5.1	7.3	4.4	5.5	8.4	4.7	6.6	7.3	7.7
5	4.3	5.9	7.2	N.S.	5.8	8.5	7.8	6.9	N.S.	N.S.
6	4.3	6.1	7.0	4.0	5.6	8.3	4.9	6.2	8.0	7.5
8	5.1	4.4	4.9	4.0	4.1	5.6	4.2	N.S.	4.7	DIED
14	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.0	0.0	

Counts expressed log₁₀ viable bacteria per gram faeces

Table 7.2

Means of faecal counts of Nalidixic acid resistant E. coli O 149 from orally vaccinated and placebo pigs infected at 12 days of age and weaned at 14 days of age

<u>Treatment</u>	<u>Vaccinated</u>	<u>Placebo</u>
Days Post weaning		
1	6.36	5.99
2	6.56	6.23
3	6.91	8.04
4	6.65	7.88
5	6.60	8.11
6	6.36	7.82
8	4.71	5.18
14	0.00	0.00

Counts expressed \log_{10} viable bacteria per gram faeces

Table 7.3

Faecal solids of orally vaccinated and placebo pigs experimentally infected with E. coli 0 149 at 12 days of age and weaned at 14 days of age

<u>Treatment</u>	<u>Vaccinated</u>					<u>Placebo</u>				
<u>Pig No.</u>	273	275	277	280	281	271	272	274	276	279
	<u>Faecal Solids (%)</u>									
<u>Days Post Weaning</u>										
1	40.7	31.1	54.1	25.5	31.5	22.0	19.6	31.1	23.6	44.1
3	36.7	40.2	22.6	37.1	31.0	8.8	N.S.	34.1	32.7	33.2
4	35.1	34.1	4.2	33.8	25.8	5.2	30.0	29.6	25.0	16.3
5	N.S.	30.0	N.S.	N.S.	21.7	5.7	32.3	30.8	N.S.	N.S.
6	35.3	31.8	N.S.	34.8	35.0	21.0	30.6	36.0	24.8	27.0
8	34.5	32.9	34.3	32.0	36.2	28.0	31.9	42.3	30.7	DIED

N.S. = No sample obtained.

Table 7.4

Weights of Orally vaccinated and Placebo pigs experimentally infected with E. coli 0 149 at 12 days of age and weaned at 14 days of age

<u>Treatment</u>	<u>Vaccinated</u>					<u>Placebo</u>				
<u>Pig No.</u>	273	275	277	280	281	271	272	274	276	279
<u>Age days</u>	<u>Live Weight (Kilo)</u>									
Birth	1.50	1.35	1.25	0.95	0.95	1.65	1.10	1.20	1.20	1.35
12	2.55	2.70	2.50	2.25	2.20	2.55	2.30	2.55	2.35	2.75
21	5.10	4.25	3.75	3.55	3.65	3.35	3.15	3.75	3.45	3.85
24	5.70	4.90	4.05	3.70	3.95	3.80	3.40	4.15	3.80	DIED
28	7.10	6.15	5.20	4.70	4.40	5.10	4.10	5.00	5.00	-

Table 7.5

Means of live weights of orally vaccinated and placebo pigs experimentally infected with E. coli 0 149 at 12 days of age and weaned at 14 days of age

Treatment	Vaccinated	Placebo	Difference
No. Pigs	5	5	vacinated minus Placebo
Age (days)	Mean Live weight (SD)	Mean Live Weight (SD)	
Birth	1.20 (0.21)	1.30 (0.19)	-0.1
12	2.44 (0.18)	2.50 (0.16)	-0.06
21	4.06 (0.57)	3.51 (0.26)	0.55*
24	4.46 (0.74)	3.79 (0.27)	0.67
28	5.51 (0.99)	4.80 (0.40)	0.71

* significant P = 0.1 (Wilcoxon ranked pairs test)

400107

Table 7.6

Visual assessment of the health of orally vaccinated & placebo pigs experimentally infected with E. coli 0 149 at 12 days of age and weaned at 14 days of age.

<u>Treatment</u>	<u>Vaccinated</u>					<u>Placebo</u>				
<u>Pig No.</u>	273	275	277	280	281	271	272	274	276	279
	<u>Health Score</u>									
<u>Days Post Weaning</u>										
1	+++	+++	+++	+++	+++	++	++	+++	++	+++
3	+++	+++	+++	+++	+++	+	+	+++	++	++
4	+++	+++	++	+++	+++	+	++	++	+	+
5	+++	+++	++	+++	+++	+	+	+++	+	+
6	+++	+++	++	+++	+++	+	++	+++	+	+
8	+++	+++	+++	+++	+++	++	++	+++	+++	DIED

Key:-
 +++ Bright, alert, smooth coat, good bloom.
 ++ Less alert, coarse coat, poor colour.
 + Listless, starry coat, white colour, drawn appearance.

Mean faecal counts of Nalidixic acid resistant
E.coli O 149

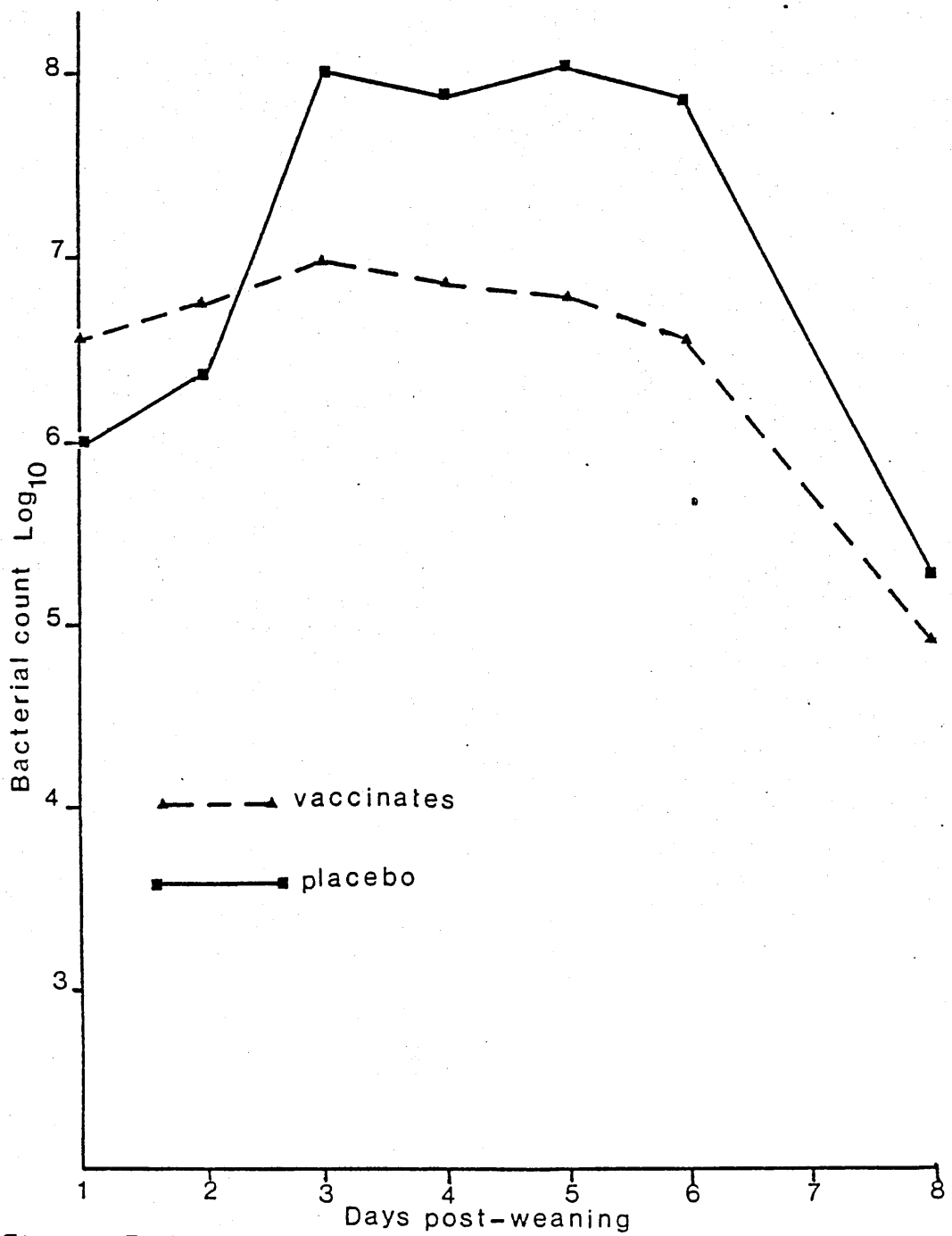


Figure 7.1

SECTION 8

GENERAL SUMMARY AND CONCLUSIONS

The continual demands being made on modern agriculture for improved productivity has resulted in more intensive management of livestock. In an endeavour to increase production all aspects of animal husbandry are under increasing pressure. Higher stocking densities have increased the incidence of disease enormously. The current practice of controlling microbial infection by chemotherapy is proving totally inadequate. Gross misuse of drugs has led to an alarming decline in their efficacy due to the increase in drug resistance. In the search for alternative methods of promoting the health of intensively reared livestock, immunobiological control of disease is receiving increasing attention.

One aspect of the quest for increased farm productivity is a continual move towards earlier and earlier weaning. Although current practice is for the majority of pig breeding units to wean piglets at 3-4 weeks of age, economics indicate that weaning at 10-14 days of age would be desirable. Unfortunately intestinal infections particularly by enteropathogenic E. coli, are the dominant problem in rearing young livestock, especially at weaning. The younger an animal is when weaned, the more prone it is to colibacillosis. The present research on intestinal immunity was undertaken against this background. The objective was to furnish information upon which the feasibility of promoting an early onset of secretory antibodies against pathogenic E. coli in piglets, could be established. The interaction of the cellular components of the intestinal mucosa with the luminal environment was studied in the developing animal to afford a better understanding of the mechanisms involved.

A morphological evaluation of the changes occurring in the intestinal mucosa of the pig during the first 4-5 weeks of life revealed that inflammatory changes occur in the intestinal lamina propria of apparently healthy animals as early as 10 days after birth. These changes, which become progressively more severe as the animal ages, are greatly exacerbated by infection with enteropathogenic E. coli. Initially microoedema of the villous lamina propria, accompanied by infiltration of the tissues with inflammatory cells occurs. This is followed by desquamation of villous epithelium giving rise to gross changes in structure. The long slender finger like villi seen in younger pigs are replaced by bizzarre stunted leaf-like structures comprised of fused villi with a deformed epithelium. The changes result in a diminution of the absorptive epithelial surface area. Quantitative comparisons between germ-free and affected animals demonstrated the potential magnitude of this loss. The surface area of the anterior small intestine of a 5 week old affected pig was up to 80% less than that of a germ-free animal of the same age. In all the studies, the anterior region of the small bowel was consistently found to be most severely affected.

This morphological study provided an essential background to the research programme. Derangement of pig gut mucosal tissues following E. coli infection is accompanied by malabsorption (Kenworthy & Allen, 1966a, 1966b) which is reflected in the poorer growth of the affected animal. The findings of the present study suggest that this may be caused by a combination of reduced surface area and a loss of functional efficiency of the absorptive epithelium.

The question arose whether these changes were severe enough to prevent antigen gaining access to the mucosal tissues in sufficient quantity to stimulate an intestinal secretory immune response and whether any particular area of small bowel was more affected than another.

The relative ability of different levels of the small intestine to absorb macromolecules was examined in vitro using everted sac preparations of tissues from 3-4 week old pigs which had been weaned at 14 days of age. Horse-radish peroxidase (H.R.P.) was selected as a model antigen. Macromolecular uptake was shown to occur in these tissues with the rate of absorption being greatest in the duodenum, an area not previously examined in similar studies of other species. Histochemical examination showed H.R.P. entering the lamina propria in apparently substantial quantities in areas of epithelial damage. As the earlier morphological studies had shown that epithelial damage was greatest both in the anterior gut and in older animals it was possible that this could be a contributory factor to the findings in the latter experiments and that the rate of macromolecular uptake in younger animals would be different.

The studies were therefore extended to investigate whether the characteristics of antigen absorption changed markedly in early development; thus pigs of varying age, both suckling and weaned were examined. In addition to the H.R.P. antigen E. coli endotoxin was included to provide data relevant to the needs of oral immunisation. Macromolecular uptake was greatest in the younger animals with the least inflammatory change and whilst antigen was able to enter the mucosa at sites of epithelial damage, amounts were small compared with those actively transported by healthy absorptive epithelium. This finding provided some further indication why piglets suffering from enteropathogenic infection fail to thrive. The study also showed that the rates of absorption of the two antigens, H.R.P. and endotoxin were independent of each other, suggesting a lack of competition for binding sites on the absorptive epithelium.

Having determined that bacterial antigen could be absorbed by the gut of the piglet in the post natal period following closure to maternal immunoglobulins it was necessary to establish at what age the intestinal secretory immune mechanisms would be capable of responding. A comparative immunohistochemical study was made of cells containing immunoglobulins. IgM, IgA and IgG, in the intestinal mucosa of pigs varying in age from 2 days to 12 weeks.

Cells with cytoplasmic IgM appeared in the lamina propria earlier than those containing either IgA or IgG. The duodenum appeared to be the preferred site of infiltration, small numbers of IgM containing cells being found in this tissue from 2 day old animals. The numbers of cells occurring in duodenal mucosa were consistently higher than at other levels of intestine, irrespective of immunoglobulin class.

IgM cells formed the majority of immunoglobulin containing cells in the lamina propria of the small bowel in suckling pigs up to 4 weeks of age. But in the weaned animal the proportion of IgM cells gradually declined with maturity until by the time the pigs were 12 weeks old nearly 90% of cells contained IgA.

Thus it appeared that the mechanisms required for the production of secretory intestinal antibodies in response to oral vaccination were present in the pig from an early age. There still remained, however, the need to demonstrate that these could be activated to produce specific antibodies in the suckling piglet during the first week of life. An in vitro organ bath assay was therefore developed using defined antigen specific spheres (DASS) and quantitative immunofluorescent microscopy. One advantage of this technique was that the reagents and methodology used were consistent with the earlier cellular enumeration studies.

Oral vaccination with a polyvalent bacterial vaccine was undertaken at 4 and 7 days of age and antibodies were satisfactorily demonstrated in the lumen. Again the importance of the anterior small intestine to the immune defense of the alimentary tract was demonstrated by the finding that this was the tissue producing the greatest level of specific antibody.

The amounts of antibody detected in 14 day old piglets orally immunized at 4 and 7 days of age were less than that in 4 week old pigs orally vaccinated daily from 10 days of age. They were also low by classical serological standards. The question remained whether they were sufficiently high to enhance resistance to colibacillosis, in the young pig weaned as early as 14 days.

This point was investigated by establishing an infection model in a litter of 10 piglets using a synthesised nalidixic acid resistant strain of enteropathogenic E. coli serotype O 149 (Abbotstown). Half the pigs in the litter were given a daily oral dose of polyvalent E. coli vaccine for 5 consecutive days commencing at 5 days of age. The remaining animals were retained as placebo controls. All animals were infected with the enteropathogenic E. coli when they were 12 days old and weaned at 14 days of age.

Bacteriological analyses of the faecal count of the infecting strain of organism were performed and the animals' health and weight gains recorded. The results showed that by all parameters the vaccinated animals substantially out performed the unvaccinated placebo group. Their faecal pathogen count was reduced by 100 fold, they were in better health and this was reflected in an improved weight gain which was 15% better than that of the unvaccinated group over the 2 weeks post weaning.

In conclusion the research recorded in this presentation has demonstrated that oral vaccination of piglets at an early age produces an immune response which has practical benefits for early weaning. Furthermore from the observations in the morphological study, inflammatory changes occur in apparently healthy suckling piglets as early as 10 days after birth. Thus there would appear to be a benefit from administering a short course of oral vaccine to piglets, irrespective of their intended age of weaning, especially where milk scour occurring in suckling pigs at about 12-14 days of age is a problem.

SECTION 9

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