The relationship between intestinal flora and the immunity of the intestinal tract

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Summary: Mechanisms of immune protection in the intestinal tract provide for a wide range of investigation of intriguing complexity. In farm animal species, research has advanced from a position of almost exclusive concern with colostrum uptake to one of detailed examination of cellular activities. Thus, the underlying mechanisms of immune protection of the young, whether mediated passively through maternal antibody, or actively through the gut mucosal response in the developing offspring, have consistent features regarding uptake of antigen from the lumen, the isotypes of immunoglobulin favoured in the gut and the effector mechanisms for protecting the mucosal surface. The origin of immune progenitor cells, when available for antigen-driven proliferation and migration, is discussed. Certain features of the gut-mammary axis in mucosal immunity link active and passive immunity, and suggest novel approaches to immunisation.

The ontogeny of the gut immune response, the characteristics of localisation of immunocytes within the lamina propria and their population dynamics in affording antibody for the intestinal lumen have implications for development of oral vaccines for young animals. Finally, some unique characteristics of mucosal antibodies which influence microbial virulence have major implications for herd health, the farm environment and the human food chain.

KEYWORDS: Bacterial colonisation - Colostral immunity - Escherichia coli - Immune response - Intestinal flora - Intestines - Newborn animals - Swine.

INTRODUCTION

The pressures in favour of infectious agents in modern animal production are intense. Owing to the rapid movement of animals through most modern farm units, the control of infection by good management and supported by routine use of drugs, frequently breaks down. In past years the numbers of drug-resistant bacteria have increased significantly. The spread of drug resistance plasmids (R factors) through a population of pathogenic and non-pathogenic bacteria is enhanced by regular use of antibiotics. Animal health management in agriculture is therefore in an interesting phase of change, with environmentalist lobbies becoming more aggressive in their opposition to the continued use of a variety of health and growth promoters. As interest in natural processes to preserve the integrity of the food chain increases, products which enhance natural biological systems will have prime value in intensive

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animal production. In this context, therefore, it is important to examine the relationship between the intestinal flora and the immunity of the intestinal tract. This relationship is most clearly demonstrated in the developing intestinal immunity of the young animal, especially to Gram-negative enteropathogens. The present paper will therefore concentrate on this aspect.

The observations on local intestinal immunity, whilst highly pertinent to animal health, also have major implications for animal nutrition. The antigens which enter the alimentary tract are of diverse origin, arising from maternal colostrum and milk, dietary proteins and polysaccharides, as well as all the viruses, bacteria and parasites which may colonise the gut. The gut is the largest lymphoid organ in the body. The surface area of the intestinal tract can extend to many square metres and within its tissues are located many millions of lymphoid cells. At this interface with the environment, the lymphoid cells are not only responsible for presenting antibody to the epithelium for local defence, but also for regulating the production of antibody, destroying invading pathogens, rendering the host tolerant to harmless antigens, and generally attending to beneficial functions which secure the animal’s health and performance. Good animal performance is most certainly dependent upon the maintenance of integrity and function of the intestinal epithelium. The fundamental question is therefore how to enhance resistance to infection, regulate detrimental responses to nutrients and reduce the numbers and virulence of pathogens in the environment. Under these circumstances, the lymphoid function of the gut is a prime focus of attention, but the role of the mammary gland must also be considered, since this is the only source of passive immunity for the progeny of farm animals. The role of maternal immunoglobulins in the gut of the suckled offspring brings us to the consideration of passive and active immunity in the developing animal, and thus to a final evaluation of the most desirable features of maternal immunisation.

THE EFFECT OF MATERNAL IMMUNOGLOBULIN ON HEALTH AND PERFORMANCE IN NEWBORN ANIMALS

Immunoglobulin molecules cannot pass to the fetus in pigs and ruminants, owing to the epitheliochorial placentation which interposes several epithelial layers between the maternal and fetal circulations. It is therefore inevitable that the newborn of these species are entirely dependent for survival, upon intestinal absorption of adequate amounts of colostral immunoglobulins. Intestinal permeability in these animals is of a relatively short duration, seldom effective for more than 24 hours (32). It is thus absolutely essential that, in the conventional farm environment, the newborn absorbs large quantities of maternal antibodies quickly if it is to survive and thrive. It is well known that in the calf, major disease problems attributable to Gram-negative organisms are generally related to deficiency in absorption of colostral antibody (19). It would be superfluous here to try to review all the excellent work on colostral immunoglobulin and passive immunity. However, it is pertinent to examine the role of colostral status of the young animal as it influences nutritional performance as well as disease susceptibility. It is also as well to examine how passive immunity integrates with the development of active immunity in providing an effective continuum for protecting the intestinal tract, thereby participating in the host’s control of virulence of the organisms in its environment.
In early studies of colostral status in cows, Irwin (27) showed that half the calves tested had not acquired sufficient amounts of colostral antibody. In a detailed study using a simple radial immunodiffusion method for evaluating colostrum IgG in calves, again more than half the animals were found to have a low colostral status (61). This resulted in higher mortality, increased medication and a lower growth rate (Figure 1).

Furthermore, colostrum absorption alone cannot be regarded as a sure measure of protection. The colostrum must contain antibodies specific for the enteropathogens likely to be encountered by the young animal. This point is well demonstrated in a study in which the levels of specific antibodies to bovine enteropathogenic E. coli

**FIG. 1**

Relationship between colostrum status, mortality, requirement for medication and weight gain up to weaning of a group of 56 calves
and *Salmonella* species were determined in sera from a group of calves aged 2-4 days, of both high and low colostral status (Figure 2). More than half of the calves showed specific antibody titres of 1:8 (or less) to most of the enteropathogens studied, irrespective of their colostral status. Furthermore, 58% of the animals possessed no antibody to *Salmonella typhimurium* and 67% possessed no antibody to *E. coli* O8. These animals would have been especially vulnerable to infection by these pathogens. This observation is important in the case of animals which pass through markets at an early age, and are subjected to considerable stress, which increases their susceptibility to disease.

<table>
<thead>
<tr>
<th>Specific antibody titre</th>
<th>O8</th>
<th>O9</th>
<th>O26</th>
<th>O101</th>
<th>O114</th>
</tr>
</thead>
<tbody>
<tr>
<td>High colostrum status - serum IgG &gt; 12mg/ml</td>
<td>○</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low colostrum status - serum IgG ≤ 12mg/ml</td>
<td></td>
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**FIG. 2**
Specific antibody against bovine enteropathogens in serum from a group of 2-4 day old calves

Recognition of the importance of maternal colostrum containing the relevant specific antibodies for neonatal survival, has led to the practice of vaccinating the pregnant animal. This ensures that the newborn receives the best possible maternally-derived protection. More recently, in an attempt to broaden the range of this protection, attention has turned to the production of vaccines which utilise new knowledge of the determinants of pathogenicity and virulence, for example, plasmids which code for the production of adhesins, pili and enterotoxins.

This evidence that the passive immune status of the newborn has an impact on a number of parameters of economic importance to farm management — namely, the cost of medication, rate of growth, as well as the obvious losses from increased mortality — provides important support for the economic implications of gut immunity throughout all stages of animal production. In particular, appropriate oral
immunisation to control *E. coli* in the first few weeks of life can have significant benefits on the health and performance of both pigs (56) and calves (58). In this context it is important to understand the ontogeny of gut immunity and its link with maternal immunity.

**CHARACTERISTICS OF EARLY INTESTINAL IMMUNITY**

The major stimulus to the development of immune mechanisms in the alimentary tract arises from the microflora which colonises the organ so quickly after birth. In the germ-free state, the pig shows virtually no development of lymphoid tissues even at five weeks of age; whereas within ten days of monocontamination with a single strain of *E. coli*, the intestinal tissues provide essentially the same picture as that of a conventional animal of the same age (29).

Mucosal immunity is normally dominated by IgA, but in both pigs and calves the lymphocytes which infiltrate the intestinal mucosa during the first week of life are predominantly concerned with the synthesis of IgM (1, 3). In the mechanisms of transport involving j-chain and its affinity for secretory component, IgM is as well equipped as IgA to behave as a secretory immunoglobulin. It is now clear that IgM plays a primary role in intestinal defence and that IgA dominates as the gut immune function matures over a matter of weeks (Figure 3).

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**FIG. 3**

*Numbers of cells synthesising IgM and IgA in the duodenal lamina propria of young pigs*

(1) Expressed as mean number of cells per 20 unit volume of tissue
Detailed studies by immuno-electron microscopy indicate that the mode of transport across the intestinal epithelium for both IgM and IgA is in the form of membrane-bound vesicles (2, 4, 12, 45). The vesicles pass through the cytoplasm to accumulate in the supranuclear region before being released into the gut lumen (Figure 4). Unlike the uptake mechanisms for colostral immunoglobulin from the gut, the secretory mechanisms release the immunoglobulins with their transport receptor intact. Secretory component thus appears in the gut, complexed with the secreted immunoglobulin to participate in other biological functions, such as mucin binding and blocking the proteolytic activity of gut enzymes, which might otherwise destroy the antibodies (33, 70). A schematic representation of the transport mechanism mediated by the secretory component is shown in Figure 5.

The development of the immunoblast population in the lamina propria of the gut (Table I) is peculiarly orientated towards the duodenum (5). The source of antigenically primed cells is the Peyer's patches (13) which are located predominantly in the ileum. Owen and Jones (47) have identified specialised antigen-sampling epithelial cells (M cells) in Peyer's patches which are responsible for antigen uptake from the lumen and its presentation to the recipient cells in the lymphoid tissue. Thereafter the activated lymphocytes pass through the mesenteric lymphatics, enter the bloodstream from the thoracic duct, and eventually reemerge in the tissues at various mucosal sites.

<table>
<thead>
<tr>
<th>TABLE I</th>
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<tbody>
<tr>
<td>Numbers of cells containing IgM, IgA and IgG in the intestinal lamina propria of pigs of different ages</td>
</tr>
<tr>
<td>Days</td>
</tr>
<tr>
<td>No. animals</td>
</tr>
<tr>
<td>Suckling or weaned</td>
</tr>
<tr>
<td>Organ</td>
</tr>
<tr>
<td>Duodenum</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Jejunum</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Ileum</td>
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</table>

Counts expressed as the means of the numbers of cells per twenty fields (x 40 objective)
ND = Not determined

The fact that disproportionate immunoblast activity is orientated towards the upper small intestine is provident from the point of view of overall gut immunity, but it probably signals a regulating function induced by the intestinal flora. Changes in the lymphocyte population might be due to greater vascularity of the duodenum, and this region is more sensitive to the influence of microbial toxins, in particular
Note the numerous stained vesicles in the sub-microvillous cytoplasm.

**FIG. 4**

Immunoelectron micrographs of pig intestinal tissue treated with peroxidase conjugated rabbit anti-pig globulin and hydrogen peroxide 3,3 diaminobenzidine followed by OsO₄ fixation.
Secretory component mediated transport of secretory immunoglobulin
enterotoxin (66), than more distal sites. In this respect Parrott and Ottaway (50) have suggested that infiltration of mucosal tissues may be associated with an early inflammatory response, enabling increased numbers of immunoblasts to infiltrate the challenged tissues. Obviously the participative role of T-cell subsets in regulating this arm of local gut immunity, together with macrophages, has to be taken into account. It is through such mechanisms that appropriate regulation of gut immunity will arise, allowing a wide-ranging influence to be maintained over damaging elements of the microflora through natural mechanisms. To understand this process it is worth examining the character of infectious processes with Gram-negative flora and the nature of the host response.

**NEONATAL INFECTION AND ANTIBODIES OF THE GUT-MAMMARY AXIS**

Intestinal colonisation is the natural route of the stimulus that influences colostral antibody secretion (64). Thus colostrum and milk are a convenient source of antibody, and can be investigated for local and systemic response, the dam being beyond lethal susceptibility to infection. Paradoxically, the dam is a major source of infection for the newborn, which is fatally susceptible to enteropathogenic infection. Therefore, the overall model of dam and its offspring is ideal for studying protective function of antibody. This model has been used effectively to examine the host-pathogen relationship and novel antibody effector mechanisms which influence microbial virulence.

Earlier we pointed out that IgM is the class of antibody which is so important in the early development of mucosal surface immunity. Returning to this theme of the desirability of initiating the natural aspects of immunity, it is significant that the principal class of antibody produced in the colostrum of the sow when undergoing infection in late gestation, is also IgM (59). This immunoglobulin proves to have immense potency for protecting the newborn but it also has other advantages. IgM functions both locally and systemically and, unlike IgG, exerts no suppressive action on the active development of immune response in the young animal which passively acquires it. Thus it exhibits many ideal characteristics, but the main test of its efficacy is obviously in the protective function. Using the infection model of Saunders et al. (65), a 76% mortality was achieved in four litters of piglets maintained on control sows, whereas in litters suckled on sows which had received live *E. coli* in the last period of gestation, mortality relating to infective challenge was reduced to 7%.

This level of protection may be so impressive as to suggest that infection should be allowed to take its course. Indeed many farmers have practised "feedback", the feeding of infected tissues or dung to sows during gestation, in order to achieve this protection. However, maintaining our theme of performance-associated immunity, one of the major drawbacks of immunisation by infection is that, despite the protective function afforded by the antibody, the birth weight and subsequent growth potential of the newborn pig may be detrimentally affected. When sows were dosed with pathogenic *E. coli* before parturition, the mean birth weights of the litters were reduced by 26% compared with those of uninfected controls, while piglet weight at 14 days of age was almost half of that of the controls (54) (Table II). Such a policy is likely
TABLE II

Effect of "feedback" on birth weight and growth of newborn piglets

<table>
<thead>
<tr>
<th></th>
<th>Gilts (O149)</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. litters</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Mean birth weight</td>
<td>0.97</td>
<td>1.31</td>
</tr>
<tr>
<td>(kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survival to 14</td>
<td>81.48</td>
<td>96.70</td>
</tr>
<tr>
<td>days of age (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean 14-day weight</td>
<td>2.69</td>
<td>4.50</td>
</tr>
</tbody>
</table>

To increase the reservoir of virulence plasmids within the herd and although the level of resistance may be increased in individual animals, the danger of serious breakdowns is very real.

The central role of the gut in this immune function and its ready access for antigenic stimulus makes it an ideal target organ for the presentation of vaccine orally. However, oral presentation of heat-inactivated bacterial antigens failed to generate effective antibody levels in the colostrum of sows. In a new approach, Chidlow and Porter (15) combined oral with intramuscular antigen administration and were able to stimulate the production of IgM antibodies by judicious timing of the parenteral administration during continuous oral boosting. Effective antibacterial function was demonstrated in colostrum from sows vaccinated by vaccine given in feed and by injection, tested by in vitro and in vivo techniques. Virulent pathogenic *E. coli* were killed rapidly and cleared quickly from the blood of sucking piglets. Lethal oral infection using the technique of Saunders *et al.* (65) demonstrated excellent natural protection. Furthermore, in major farm trials involving more than 2,300 farrowing sows, this type of immunisation provided major benefits in reduction of mortality, reduction of piglet medication, increase in the number of pigs weaned and significant improvements in weight of animals at weaning (Table III). The impact of such an approach on animal performance leads immunology into close association with nutrition, through the maintenance of integrity and function of the intestinal mucosa against the assault of environmental pathogens. An extension to this theme is the prospect of exerting a natural control over herd environment through intestinal immunity at the interface between the host and its microflora.

From the foregoing it is clear that mucosal antibodies can provide effective neonatal defence against severe enteropathogenic challenge. However, oral vaccination of the sow will also reduce a dangerous source of virulent pathogens, thereby reducing the infection load imposed on the newborn pig. This form of prophylactic immunisation of the maternal intestinal tract attacks the problem of neonatal enteritis on two fronts, successfully breaking the cycle of infection through sow and piglet. The alimentary tract of the dam is a reservoir for many pathogens which infect the offspring, and this approach attacks the problem at its root in addition to meeting the objective of providing effective passive antibody for the suckled offspring.
TABLE III

Benefit of combined oral "in feed" and parenteral vaccination of pregnant sows with inactivated E. coli antigens: comparative farm trial results

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccinated</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Litters</td>
<td>1,182</td>
<td>1,117</td>
</tr>
<tr>
<td>Pigs born alive</td>
<td>12,153</td>
<td>11,469</td>
</tr>
<tr>
<td>Pigs weaned</td>
<td>11,144</td>
<td>9,882</td>
</tr>
<tr>
<td>% Survival average</td>
<td>93.0*</td>
<td>87.0</td>
</tr>
<tr>
<td>% Loss</td>
<td>7.0</td>
<td>13.8</td>
</tr>
<tr>
<td>Average litter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Born</td>
<td>10.0</td>
<td>10.27</td>
</tr>
<tr>
<td>Weaned</td>
<td>9.58*</td>
<td>8.88</td>
</tr>
<tr>
<td>Extra</td>
<td>0.60</td>
<td>-</td>
</tr>
<tr>
<td>Antibiotic treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Litter average</td>
<td>3.26*</td>
<td>12.38</td>
</tr>
<tr>
<td>Pig average</td>
<td>0.35*</td>
<td>1.40</td>
</tr>
<tr>
<td>Treatment ratio %</td>
<td>25</td>
<td>100</td>
</tr>
</tbody>
</table>

* Significant (P < 0.05)

DEVELOPMENT OF INTESTINAL ANTIBODY RESPONSES TO BACTERIAL ANTIGENS FROM THE LUMEN

We have previously stated that lymphoid development does not take place in animals reared in the germ-free state, but arises quickly in response to bacterial challenge, and that the early plasma-cell response is dominated by IgM synthesising immunocytes. Lymphoid differentiation along plasma-cell lines occurs in the absence of antigenic stimulus in fetal life. In pigs, follicular distribution of IgM cells in Peyer's patches occurs as early as 55 days of gestation (14). Oral immunisation of fetal lambs with E. coli antigens, administered into the amniotic fluid some two weeks before parturition (26), is accompanied by an increase in the number of IgM cells in the mesenteric lymph. The rate of development of IgA cells in the lamina after birth was markedly increased as well. The competence of this very early development of gut lymphoid activity was tested with a Salmonella typhimurium vaccine delivered prenatally followed by a subsequent challenge at birth (26), and significant protection was obtained.

Emerging from the sterile environment of the uterus, the intestine of the newborn animal experiences major antigenic challenge due to rapid microbial colonisation. In the absence of prenatal stimuli, responses are too slow to maintain the newborn free of infection in a normal farm environment, without the benefit of maternal antibody. We should now focus on the question whether maternal antibody reduces the competence of the intestine to respond, even though it is perfectly equipped for immune function prepartum (24).

In this context, poliovirus immunisation in children provides an interesting precedent (62). After dosing at birth, copro-antibodies of the IgA class appear within
three weeks (28). In fistulated baby pigs, local intestinal secretions were examined for antibody activity in response to local application of heat-inactivated E. coli antigens; peaks of response were observed in the first three weeks of life, in animals either weaned at four days or maintained wholly on the sow (57). This creates the opportunity to build a competent local immunity in preparation for weaning. In a systematic study of oral immunisation in colostrum-deprived and colostrum-fed piglets (71), a diminished response to E. coli antigens was related to colostrum feeding, when evaluated at 19 days. However, the response was normal at 31 days, indicating that there was merely a delay rather than a suppression of the local immune development. Therefore, oral immunisation of the animal before weaning in order to protect against infection after weaning is immunologically feasible.

There are various ways in which secretory antibodies may mediate in local interference with microbial pathogenicity. Antibacterial agglutinins and antibodies producing bacteriostasis, mainly associated with IgA, appear within the lumen from the intestinal mucosa after only a few days of antigenic challenge. However, intestinal antibody secretion in response to immunisation with inactivated bacterial antigen persists at elevated levels for no more than three or four weeks, and a second dose of the same antigen is necessary to induce an almost identical short-lived response of similar intensity and duration (53). In order to sustain consistent antibody secretion it is necessary to apply repeated doses. In earlier studies, Freter and Gangarosa (18) recorded essentially similar observations in relation to oral immunisation with killed Vibrio cholerae, repeated doses of which were necessary to generate detectable levels of copro-antibody.

Access of antigen to Peyer's patch lymphoid follicles is an essential prerequisite for establishing “memory” in the mucosal immune system. The failure of inactivated bacterial antigens to initiate a mucosal amnestic response may be due to an ineffective uptake of these antigens by memory (M) cells. Owen et al. (48) found that viable Vibrio cholerae were phagocytosed by M cells and carried, in vesicles, through the epithelium overlying Peyer's patches, to be discharged into the underlying lymphoid follicles. However, V. cholerae inactivated in a variety of ways (including heating) were not. High doses of heat-killed Shigella flexneri instilled into the intestinal tract of rabbits failed to initiate a mucosal immune memory response although instillation of live Shigella produced a significant memory response, persisting for more than 60 days (30). In contrast, inoculation of killed bacterial antigens directly into the Peyer's patch lymphoid follicles primes the mucosal immune system, resulting in a memory response which may persist for a year (7, 31).

Despite these observations, it would appear that intestinal immunocytes involved in secretory antibody production have a very short duration of activity, and may not play a significant role in long-term memory. Mattioli and Tomasi (40) found that IgA plasma cells in the gut of mice had a half-life of only 4.7 days. Therefore persistence of antibody production will depend on the recruitment of further immunocompetent cells. Indeed, studies of responses in Thiry-Vella loops in sheep (25) provide evidence for the influence of antigen on the localisation of specific antibody-producing cells. There is little or no serological response to orally administered bacterial antigens in young pigs. In gnotobiotic pigs, immunocytes containing IgA were present only in tissues of the gastro-intestinal tract (57). Of course one might infer from this that oral immunisation gives rise to lymphoid cells restricted mainly to the lamina propria. However, this may be related to the dose of antigen.
MICROBIAL VIRULENCE AND ANTIBODIES OF THE GUT-MAMMARY AXIS

Direct evidence of anti-enterotoxin activity has been identified in ligated gut studies (35). In young calves orally immunised with *E. coli* antigens, the onset of anti-enterotoxic activity was surprisingly rapid (10), and more rapid than expected from the development of specific antibodies, suggesting that non-specific functions may yet have to be delineated which provide protection. However, a ligated gut model with cell-free preparations of *E. coli* enterotoxin (9, 34) has shown that enterotoxin neutralisation can be solidly attributable to intestinal antibody secretions. This activity could be passively transferred with intestinal secretions from one gut lumen to another (34), demonstrating the local function of the antibody produced.

Such experiments throw light on host resistance following immunisation. Anti-enterotoxin activity is important in providing a wide protective function against enteropathogens. It is essential to maintain a broad perspective in vaccine development in order to cope with the changing pattern of microbial virulence in intensive agriculture. The most desirable phenomena that could be derived from the immune response would be a nonspecific regulation of microbial virulence leading to a safer environment.

The phenomenon of microbial adhesion to the intestinal wall is of major biological importance in the infectious process (20). Antibodies at the site of bacterial adhesion are known to play an important defensive role in preventing microbial colonisation. This can be demonstrated by employing host cell membranes and antibodies against specific adhesion determinants (17, 51, 72).

The pig provides an excellent animal model for studying the influence of *E. coli* virulence determinants. Over the past decade, specific adhesion determinants K88ab, K88ac, K88ad, K99 and 987p have been identified as prevalent pilus antigens (43).

Experience with immunisation schedules for the sow and the infection models in the newborn piglet has led to the discovery of new mucosal antibody mechanisms which interfere with plasmid-mediated virulence determinants (59). The introduction of colostral antibodies into cultures of porcine enteropathogenic *E. coli* induced the loss of K88ab and K88ac antigens (46, 49, 60). Confirmation that the plasmids had been effectively eliminated was demonstrated indirectly, by the failure of the organisms to produce K88 antigens when cultured in the absence of antibody. In experiments with organisms having a K88-linked plasmid function for rapid raffinose fermentation, the antibodies induced the formation of raffinose-negative strains as well (36). This particular activity was not associated with antibodies to K88 antigens. In fact the antibodies were induced by a heat-stable antigen. The antigen was common to porcine enteropathogens because antibodies induced by immunisation with one heat-inactivated serotype induced the loss of K88 plasmids from several other serotypes.

Interesting aspects of this important phenomenon are the nature of the antigen involved, the mechanism whereby plasmid loss was induced, the isotype efficiency in promoting the effect, and whether the phenomenon is confined to *in vitro* circumstances or whether its effects are evident *in vivo*. Additionally, it is important to determine whether these plasmid “curing” functions extend to other pilus antigens.
These observations are interesting because, under normal circumstances, bacterial modification takes place to the disadvantage of the host, and it is surprising that natural antibody mechanisms have this potential to "unmask" the pathogen.

Mainil et al. (39) found that K99-negative variants were detected more frequently from suckled newborn piglets, inoculated with a K99-positive strain of a porcine enteropathogenic E. coli, than from colostrum-deprived pigs similarly infected. In contrast to the K88 studies reported earlier (36), this loss appeared to be associated with K99 antibody in colostrum, since the litter with the highest number of piglets excreting K99-negative variants was that of the dam with the highest titre of K99 antibody in her colostrum. Furthermore, a greater shedding of K99-negative variants occurred in colostrum-deprived piglets fed K99 monoclonal antibody compared to those fed K88 monoclonal antibody.

Loss of surface antigens was discovered in studies of the effect of human milk antibodies on the biological properties of an E. coli strain from an infant (21). The effect was to render the bacteria more sensitive to serum bactericidal activity and there was more spontaneous agglutination, characteristic of the loss of surface antigen. It was suggested that the breast milk factor exerts a selective pressure in the gut, favouring the proliferation of mutant strains of decreased virulence. Experimental cholera infection in germ-free mice has indicated a similar phenomenon, antibody production being frequently associated with serotypic conversion and the emergence of rough mutants (63). A major difference between these phenomena and that of K88 plasmid elimination is that the latter is irreversible, whereas the former may be reversed. For example, reintroduction of the rough form of Vibrio cholerae to germ-free mice devoid of antibody brings about the return of smooth forms and the recovery of virulence.

The question of selection pressure is important, for plasmid "curing" agents can eliminate plasmids from a bacterial population by two different mechanisms. Some agents such as the acridine dyes and ethidium bromide have a direct effect on replication of the plasmid itself, while others, like the surface-active agent sodium dodecyl sulphate, select for any plasmid-negative variance that may have arisen spontaneously. Experiments with antibodies from immune milk, able to exert the "curing" effects, indicate that the plasmid-eliminating antibodies belong to the latter category of "curing" agent. For example, when enteropathogenic E. coli positive and negative for K88 were grown in nutrient broth containing 10% control immunoglobulin, the two bacterial strains gave very similar growth curves. However, in the presence of curing antibodies, growth of the K88-positive form was initially much slower than that of the negative form. Total viable counts of all cultures were similar after 24 hours of growth, but the K88-positive form no longer comprised 100% of the bacteria present in the culture inoculated with curing antibodies. In fact, K88-negative variants had begun to emerge after about 10 hours of culture in the presence of the antibodies, and subsequently they began to dominate. Subculture of this mixture in the constant presence of the antibodies, continued to favour the elimination of the K88-positive strains. Schematic representation of growth curves for K88-positive and negative strains of E. coli in the presence of "curing" antibodies is shown in Figure 6, and from this the underlying principles of selection can be appreciated.

Guinnea and Jansen (22) identified a further epitope on K88, namely K88ad, and suggested that this new variant was emerging in porcine enteropathogenic E. coli due
to selection pressure on the bacterial K88-positive population resulting from the widespread use of K88ab/ac vaccines. In previous antibody studies, the heat-inactivated oral vaccine did not contain a K88ad plasmid. There might be a virulence factor capable of evading the newly-discovered plasmid elimination effect afforded by the antibodies so far investigated. A direct comparison can be made with the capacity of milk antibodies in culture to cure K88ab, K88ac and K88ad from field strains of E. coli belonging to the O8 sero-group (60). There was no evidence to suggest that the K88ad plasmid differs in stability. Furthermore, K88ad was eliminated with equal facility from an O9 serogroup, and since this was not incorporated in the vaccine, this finding provides strong evidence of a lack of association of the antibodies with the O antigen.

Clearly, the loss of the K88 plasmid from K88-positive serotypes in culture, due to the selective action of "curing" antibody, can be achieved comparatively simply in culture when antibody pressure is the only force operating. However, the situation in vivo will be very much more complex. In the newborn piglet, possession of the K88 plasmid is important because the adhesion factor enables the strains to colonise the upper gut. Under normal circumstances, therefore, if a litter of piglets is infected with a mixture of E. coli positive and negative for K88, the adhesive K88-positive type will rapidly form 100% of the population, since they proliferate most rapidly in the intestine. The intestine is not a closed fermentation vessel wherein dosed antibody conveniently remains in suspension with the microbial population. Obviously, K88-positive strains have the benefit of adhesion onto the intestinal surface epithelium, and antibody can be lost from the lumen by various mechanisms. Therefore, very strong antibody pressure will be required to keep the balance of the E. coli population

**FIG. 6**

Schematic representation of growth curves of K88+ and K88− strains of E. coli in the presence of "curing antibodies"
in favour of the K88-negative form, and presentation of maternal antibody to the lumen in the newborn will have to be fairly continuous, through regular suckling.

The maternal sow is a major source of enteric pathogens infecting its offspring, and the excretion rate is greatest during the farrowing period (8, 41, 42). Any animal experiment to examine the environmental effects of immunisation should therefore take into account both the dam and its offspring. The objective should be to reduce the level of environmental contamination with fecal pathogens by disrupting the proliferation cycle of infection/excretion/reinfection through sows and piglets.

Within this context Linggood and Porter (37) set up an infection model to examine the effect on the virulence of an infecting dose of pathogenic E. coli of active antibodies in the intestines of the sow and passive antibodies in her offspring. Two primiparous sows, due to farrow at about the same time, were selected. The sow expected to farrow first was immunised by a combined oral and parenteral schedule with heat-inactivated vaccine designed to take advantage of the antibodies of the gut-mammary axis. The second sow was not immunised, and both were kept in adjacent pens in an isolated unit. Three days before parturition, the immunised sow was given feed containing 400 ml of a broth culture of a mutant of the porcine enteropathogenic E. coli O149 K91 K88ac resistant to nalidixic acid. The organisms excreted by this animal into the environment infected her litter of piglets, and also the unimmunised sow which farrowed five days after the immunised sow. The E. coli pathogen was isolated from the faeces of the sows and their piglets by daily culture of the samples on blood agar containing nalidixic acid. Colonies of the strain were purified and tested for K88ac by slide agglutination. Colostrum and milk samples were obtained from the sows and blood samples from the piglets for testing of their K88 “curing” activity. The culture of the strain given to the immunised sow three days before parturition was 100% K88-positive. Shortly after parturition, fewer than 30% of the colonies isolated from the faeces of this sow were K88-positive. This strain was naturally acquired by its progeny of eleven piglets. One of the piglets acquired little or no colostral antibody and died within two days from enteric infection; organisms recovered from different levels of the intestinal tract proved to be 100% K88-positive. In the remaining ten piglets there was a general trend from initially high numbers of K88-positive strains in the faeces, followed by a daily decrease in their proportion, until the K88-negative strains predominated. Meanwhile, the unimmunised sow and her litter acquired the O149 pathogen from the environment. In all the piglets, a K88-positive variant rapidly formed 100% of the O149 organisms present and more than half of the progeny of the control sow died from the infection during the first 48 hours (Table IV).

**Table IV**

*Elimination of K88ac from an O149:K91, K88ac strain of E. coli in vivo and in vitro*

<table>
<thead>
<tr>
<th>Sow treatment</th>
<th>Piglet mortality</th>
<th>Loss of K88 in vivo</th>
<th>Loss of K88 in vitro after 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>In sow</td>
<td>In piglets after 72 h</td>
</tr>
<tr>
<td>Immunised</td>
<td>9%</td>
<td>70%</td>
<td>65% *</td>
</tr>
<tr>
<td>Control</td>
<td>69%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

* Average of 10 surviving piglets
Experiments with cultures supported these findings. Incorporating samples of colostrum or milk in culture resulted in the elimination of K88 from O149 colonies in the case of the immunised sow whereas negligible K88 loss occurred when the strain was grown in the presence of similar samples from the control sow.

The plasmid "curing" activities were associated with all isotypes of immunoglobulin in serum and secretions. In sow colostrum, where immunoglobulin levels are high for the first 24 h, IgG is the principle immunoglobulin present (11, 52). However, this falls rapidly during the first two days of lactation and IgA predominates, being maintained at a fairly consistent level thereafter (55). Thus IgA will be able to maintain the antibody pressure in piglets, in favour of the K88-negative forms of *E. coli*, throughout lactation. Since these antibody activities are undertaken exclusively in the lumen of the intestine, immunisation schedules must take this into account. Parenteral vaccination schedules designed to hyperimmunise sows with the formation of IgG antibody would be less successful owing to the brief function of this isotype in the gut. IgG lasts a long time in the blood circulation of the piglet, but it does not act at mucosal sites. Moreover, IgG can suppress the subsequent response of the newborn to antigenic challenge (44) and so result in greater susceptibility to *E. coli* infection as the piglet develops.

During the past decade, emphasis has been placed on the development of pilus vaccines, K88 for the pig, K99 for the calf, and so on. We have already pointed out that anti-K88 antibodies do not induce the plasmid elimination effect and therefore cannot be expected to exert any control environmentally. Furthermore, the vaccines have been designed to produce IgG antibodies, which in the long term may be detrimental for the development of active immunity. Significantly in this field, Chidlow, Blades and Porter (16) demonstrated clearly, in various immunisation and infection models in piglets, that the protective function against *E. coli* was more greatly associated with IgA and IgM classes than IgG, and that K88 antibodies played a less significant role in host defence. Of critical importance is the ability to properly integrate the characteristics of maternal immunity with those of the developing immune system in the young suckled animal in order to protect it effectively and secure good health and performance throughout early life.

While these plasmid-mediated antibody effector mechanisms are obviously important, they are not solely responsible for effective protection. Solid immunity from disease is more likely to result from a combination of defence mechanisms, antibacterial and antitoxic, acting together, rather than one single activity. For example, Svennerholm and Ahren (68) have demonstrated synergy between antibodies to certain somatic antigens and anti-enterotoxins which together give protection against homologous and heterologous strains of *E. coli*. Since the natural disease process depends on many interlocking mechanisms, not all of which are fully understood, effective immunity is most likely to be achieved by a combination of as many relevant factors as possible.

**EFFECT OF MUCOSAL ANTIBODIES ON TRANSMISSIBLE DRUG RESISTANCE**

The major issues considered so far have been the host protective functions of antibodies and the broader implication for the host-pathogen relationship.
Observations on adhesion determinants lead to a new field of investigation, relating to the environment. If the appropriately immunised host can suppress the virulence of the pathogens which may colonise its gut, then the more susceptible animals are less at risk in the herd. Furthermore, there are clear implications for antibodies which reduce virulence by their effect on microbial plasmids; they improve not just the environment of the herd but also have a bearing on the food chain. In particular, microbial drug resistance becomes a focus of opportunity for influence by intestinal immunity.

Two genetic components are necessary for infectious drug resistance: the determinant gene conferring resistance and that coding for a sex pilus. Synthesis of a sex pilus is essential before drug resistance transfer can be effected by conjugation. As these two components are linked, the strain carrying the sex factor becomes a potential donor of drug resistance.

Experiments to examine the effect of colostral antibodies on the maintenance of the sex factor and drug resistance in a replicating *E. coli* population have shown that the presence of antibody leads to the emergence of variants lacking the sex factor (38). This is similar to the K88 plasmid loss described earlier. Whereas strains lacking the sex factor but carrying the drug resistance determinant do occur, those carrying the sex factor but not the drug resistance determinant have never been recovered. It would therefore appear that the presence of the sex factor (or the resulting synthesis of the sex pilus) is the more disadvantageous for the organism with regard to the antibody. Thus, while drug resistance may be present in a population of cells, it cannot be transferred without the sex factor.

Under natural conditions, pathogenic *E. coli* have to compete with the rest of the intestinal flora. Antibody activity, which may be only marginally effective against an organism when grown in pure culture, should be more potent against the same organisms when grown in a competitive environment. This hypothesis was confirmed by growing a strain of *E. coli* possessing transmissible drug resistance in a mixed culture. It had to compete with the same *E. coli* strain which lacked both the drug resistance determinant and the sex factor, and a different strain similarly devoid of either factor. All three organisms grew equally well in the absence of colostral antibody but in the presence of immune wheys, the strain carrying the sex factor was completely eliminated from the mixed culture (38).

The use of antibiotics is virtually mandatory in modern intensive animal production to achieve a profitable level of output. The continual throughput of animals, coupled with high stocking densities, creates ideal conditions for the proliferation of pathogenic organisms. In addition, the abnormal stress imposed on livestock by these practices considerably increases their vulnerability to infection. Indeed, a point has now been reached where, because of the build-up of drug resistance in the enteric flora, drugs which previously were of benefit are no longer effective. Not only does this create problems for farm animal production, but it is also a source of increasing public concern. The infectious transfer of drug resistance determinants from animal to human pathogens, coupled with the possibility of drug residues in animal products, presents serious potential hazards for human health. The introduction of controls on the subclinical usage of antibiotics for animal production (69), which were designed to reduce the level of drug resistance in the environment, has failed to have the desired effect. The incidence of drug resistance in enteropathogens in farm livestock has continued to increase over the intervening period (67).
Against this background, the implications of the findings of the in vitro studies, were they to occur in vivo, could be far reaching. The incidence of drug resistance occurring in E. coli isolated from pigs reared on farms routinely using drugs for the control of porcine enteropathogenic E. coli was compared with that of isolates from animals on units which practiced oral vaccination. A third group of animals, from farms which neither used drugs routinely nor practised oral vaccination, was also studied. Isolates were recovered from pigs of the three ages at which the animals were most likely to be susceptible to E. coli-associated enteritis: 1-4 days, 14-21 days of age and 5-10 days after weaning. Resistance to eleven of the more commonly used drugs was determined (6).

Three salient points emerged from the study (Table V). Firstly, on the farms not using oral vaccination, the incidence of drug resistance increased as the animal aged.

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Age of pigs before weaning</th>
<th>Age of pigs after weaning</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>newborn</td>
<td>before weaning</td>
</tr>
<tr>
<td>I</td>
<td>0.358</td>
<td>0.384</td>
</tr>
<tr>
<td>II</td>
<td>0.381</td>
<td>0.391</td>
</tr>
<tr>
<td>III</td>
<td>0.397</td>
<td>0.454</td>
</tr>
</tbody>
</table>

Significance: values with the same superscript are significantly different

a = *  P < 0.05
b = *
c = **  P < 0.01
d = ***  P < 0.001

(1) ARI: Hinton and Linton (23)

Since virtually all animal feeds are medicated with antimicrobial drugs which exert a selective pressure on the enteric population favouring resistant strains, this might have been anticipated in the oldest group of animals, which had been weaned; but it was interesting to find a similar effect in two-week-old pigs. In contrast, on the farms using oral vaccine there was a significant decline in the incidence of antibiotic resistance in the older pigs compared with newborn piglets from the same herds, and also with pigs from the farms not using oral vaccine. Thus it would appear that the effect of mucosal antibodies in blocking the transfer of drug resistance factors by eliminating sex pili from the population, demonstrated in vitro, can also operate in animals to limit the spread of drug resistance. In pigs not immunised, the development of resistance was particularly associated with those drugs against which the resistance
factor was most likely to be plasmid-mediated, especially tetracycline and ampicillin. Conversely, there was less development of tetracycline resistance in the orally vaccinated group than in the other two groups.

Secondly, although it would have been expected that on farms where drugs were used routinely, the build-up of resistance would have continued, this did not occur. The results indicate quite clearly that while the level of resistance in the environment of this group of farms was higher than in the other two groups, the incidence of drug resistance in newborn pigs from all three groups was remarkably similar. It would have been expected that on the farms with high drug usage, the level of resistance in newborn piglets would have continued to rise as they became infected by increasingly resistant strains excreted by the older animals. That this did not happen suggests that processes are operating which tend to restrict the expression of resistance factors in isolates from newborn piglets. This activity is almost certainly due to maternal colostrum, because immune colostral antibodies also inhibit the transfer of drug resistance determinants.

Thirdly, the *E. coli* included both pathogenic and non-pathogenic isolates, but most of the organisms recovered were untypable and presumably non-pathogens. This commensal flora was largely responsible for the incidence of drug resistance. This finding, which is compatible with that of other workers (23), gives some indication of the location of the reservoir of drug resistance in pig herds and probably explains why the anticipated decline in the level of drug resistance following the restrictions of use, failed to materialise. It also serves to highlight the need to develop alternatives to drugs, particularly for use as growth promoters, if their effectiveness in the control of disease is not to continue to decline.

**CONCLUSION**

Reference was made earlier to the pressures created by modern methods of agricultural production which favour the development of disease. This applies to the relationship between the intestinal flora and the immune mechanisms which give protection against enteropathogens, since it is only by a better understanding of the manner in which natural protection is conferred that better strategies for disease control can be evolved.

By considering the ontogeny of the mucosal immune system and in particular the role of maternally-derived passive protection for the newborn, we have highlighted the synergy which exists between the two systems, because this contains lessons of importance to the development of vaccination schedules designed to reduce the incidence of enteric disease in the young.

We have, moreover, extended our review to cover not only specific immune responses to enteropathogens, but also included details of recent studies of the novel mechanisms affecting the control of virulence determinants in the microbial population. These, because they are more non-specific in character and thus have a broader spectrum of activity, may prove to be of even greater significance for the control of infections in the future. This latter aspect is of particular relevance when developing strategies to control infection pressures in the farm environment and it also has important implications for the human food chain.

**"**
Resumen: Los mecanismos de la protección inmune en el tracto intestinal ofrecen a la investigación científica un amplio campo de sorprendente complejidad. En la actualidad, la investigación relativa a las especies de animales de granja, que antes solía ocuparse casi exclusivamente de la toma del calostro, estudia detalladamente lo que ocurre a nivel celular. Así, los mecanismos en los que se basa la protección inmune de los animales jóvenes, ya sea que se trate de mediación pasiva, a través de los anticuerpos maternos, o de mediación activa, a través de la respuesta de la mucosa intestinal del neonato en desarrollo, presentan características comunes en lo que se refiere a la absorción de antígeno a partir del lumen, los isotipos de inmunoglobulinas que se forman de preferencia en los intestinos y los mecanismos encargados de proteger la superficie de la mucosa. También se comenta el origen de las células inmunes parentales, cuando su proliferación y su migración pueden ser provocadas por un antígeno. Determinadas características del eje intestino-mama en la inmunidad de la mucosa vinculan la inmunidad activa y la pasiva y sugieren nuevos enfoques de la inmunización.

El desarrollo de la respuesta inmune del intestino, las características de la localización de los inmunocitos en la lámina propia y de su dinámica de población respecto al aporte de anticuerpos al lumen propio, tienen incidencia en la elaboración de vacunas orales para animales jóvenes. Finalmente, algunas características únicas de los anticuerpos de la mucosa, que influyen en la patogenidad microbiana, tienen importantes repercusiones en la sanidad del rebaño, el medio ambiente de la granja y la cadena alimenticia humana.


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