ANTIBACTERIAL FACTORS IN COWS' MILK AND COLOSTRUM: IMMUNOGLOBULINS AND LACTOFERRIN

by

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ABSTRACT

The objectives of this study were: 1) to test the antibacterial activity of a pepsin digest of bovine lactoferrin, containing the peptide lactoferricin, in complex media; 2) to detect specific antibodies to human enterotoxigenic *Escherichia coli* in bovine milk or colostrum; and 3) to investigate the potential of a cell culture system to study and estimate the biological activity of milk immunoglobulins.

The pepsin digest of lactoferrin was bactericidal against *Salmonella enteritidis* in 1% peptone, but no substantial antibacterial activity could be demonstrated in trypticase soy broth or in some selected foods. Calcium at a concentration of 5 mM was sufficient to inhibit the antibacterial activity of the digest. Addition of lysozyme or EDTA enhanced the antibacterial activity of the digest, but not sufficiently to overcome the effect of inhibitors in the foods of interest. The activity of the digest was also inhibited by bile salts. These findings raise doubts about the potential for addition of lactoferricin to foods.

Antibodies to the colonization factor antigen CFA 1 of enterotoxigenic *E. coli* were detected in bovine colostrum by hemagglutination inhibition. Concentrations of antibodies to CFA 1, estimated by ELISA, ranged from 0.55 to 5.2 μg/ml in colostrum samples of non-vaccinated cows. Samples of milk immune concentrates from vaccinated and non-vaccinated cows were also tested. Vaccination increased the concentration of specific antibodies relative to the total IgG content of the samples tested.

Invasion of HeLa cells by *Salmonella enteritidis, S. typhimurium* and enteropathogenic *E. coli* was inhibited by addition of bovine colostrum to cell culture medium. Inhibition levels ranged from 73% to over 99%. The immunoglobulin-containing fraction, isolated from colostrum by affinity chromatography on a protein G-agarose column, inhibited invasion by *S. typhimurium*. An unidentified high molecular weight factor in the non-immunoglobulin fraction also inhibited invasion of HeLa cells. No inhibitory activity

was found in low molecular weight fractions. The results suggest that bovine colostrum contains both immunoglobulin and non-immunoglobulin inhibitors of invasion of HeLa cells by the bacteria tested.

HeLa cell cultures have the potential to be a convenient method for the study and evaluation of antibacterial properties of bovine milk.

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I. INTRODUCTION

The dairy industry is faced with the problem of cheese whey disposal or utilization. The recovery and commercialization of the whey solids has been seen as a possible solution to this problem. Immunoglobulins and lactoferrin, which have antibacterial activity, are present in cheese whey at low levels. It has been suggested that they could be extracted, concentrated, and used for supplementation of infant formula, or foods for the elderly and immunocompromised persons, in order to prevent diarrheal illnesses caused by enteropathogens.

Supplementation would imitate a phenomenon commonly observed in nature: the passive transfer of immunity from mothers to infants, which insures the survival of newborns until their own immune system is sufficiently developed.

In the literature review, the concept of passive transfer, which is the foundation of the proposal for supplementation, will be examined in the context of the main mechanisms of host defense against enteropathogens (humoral and mucosal immunity). The evidence supporting a role for milk in the protection of infants against infectious gastroenteritis will be presented. Experiments designed to demonstrate that colostrum or milk immune concentrates obtained from one species are effective in another species will then be reviewed. Similar studies have been done with human infants whose diets have been supplemented with immunoglobulins obtained from cows' milk or eggs, and with adults, where protection against challenge with enteropathogens was demonstrated. Finally, the reported *in vitro* antibacterial effects of lactoferrin and of lactoferricin, a recently discovered peptide obtained by pepsin digestion of lactoferrin, will be reviewed. This peptide has been shown to have a much greater antibacterial activity than lactoferrin in simple media.

There is a long history of experimentation aimed at the use of immunoglobulins or lactoferrin in foods, and many problems remain to be addressed. In this thesis two general

questions were asked:

- 1) can the bactericidal activity of a pepsin digest of bovine lactoferrin, containing the peptide lactoferricin B, be demonstrated or enhanced in complex media?

The results of experiments to compare the bactericidal activity of bovine lactoferricin in simple and complex media and in foods, and to examine the effect of added lysozyme or EDTA, will be presented.

- 2) can the antibacterial activity of milk immunoglobulins be detected and estimated in a relatively simple manner, using a method that would be more informative than immunoassays but less cumbersome than *in vivo* experiments?

The results of experiments to detect in bovine milk or colostrum, by hemagglutination and immunoassays, antibodies to the colonization factor antigen of a strain of human enterotoxigenic *Escherichia coli* will be presented. This is of interest because such antibodies have been found to be very effective in animal studies. Adherence to or invasion of the intestinal epithelium are virulence characteristics of most enteropathogens. But an anti-adherent or anti-invasive effect of milk immunoglobulins cannot be tested by immunoassays.

Experiments *in vivo* with human adult volunteers, with children and with a variety of animals are not routine. On the other hand, cell culture methods are in widespread use in studies of microbial pathogenesis, and adherence to or invasion of mammalian cells in culture by pathogens has been correlated with virulence *in vivo*. The results of *in vitro* experiments, using HeLa cells, to test the anti-invasive properties of bovine colostrum and colostrum fractions against *Salmonella enteritidis, S. typhimurium* and *Escherichia coli* will be presented.

II. LITERATURE REVIEW

A. The problem of whey utilization

Whey is the liquid fraction that remains following manufacture of cheese. It is produced in very large amounts and its utilization has been a continuing challenge for the industry.

The problem can be appreciated by the following figures: More than half of the solids in milk remain in the whey; the quantity of liquid whey produced is roughly ten times that of cheese. In 1991, the amount of whey produced in North America was about 885 thousand metric tons of solids, in Western Europe 1380 thousand metric tons, and in the Pacific Rim countries 155 thousand metric tons (Horton, 1993). The utilization was 75% in Europe and probably less than 50% in the rest of the world, and as a result a very large amount of material with potential value as food or feed is wasted. The corresponding volume of liquid can be calculated on the basis that solids are about 6-7% of the whey. It can be seen that the disposal of whey is a considerable problem. It has been calculated that simple discharge of 1,000 gallons of whey into a river would require the dissolved oxygen contained in 4,500,000 gallons of water for its oxidation (Gillies, 1974). Whey is comparatively concentrated compared to normal municipal waste and therefore puts great demand on municipal sewage systems, so that dairies have or will have to pay a surcharge to municipalities for disposal of their excess whey. The traditional methods of handling whey have been to feed it *as is* to livestock (pigs and cattle); to dry it for use in food or feeds; or to use it as fertilizer. The use of liquid whey as livestock feed is limited largely because of handling cost and labor, and excessive feeding may lead to digestive disturbances in some animals. Whey is beneficial as a fertilizer as long as the risk of pollution is limited, and the main problem again is that of handling; in addition the demand is seasonal and limited by geographical location (Gillies, 1974).

Since simple discharge of whey into waste disposal systems is expected to become increasingly regulated, and since cheese production is increasing, research efforts have focused on the recovery and use of whey solids. The dairy industry is also becoming more concentrated, and this creates another incentive to switch from simple disposal to large scale processing. In practical terms this means ultrafiltration and separation of the liquid whey into a permeate, mainly lactose, and a whey protein concentrate that may be spray-dried. In North America about 500 thousand metric tons of permeate solids were produced in 1991 (Horton, 1993). Some of the permeate can be spread on fields in very limited amounts because it is not a balanced fertilizer (insufficient nitrogen). There is interest in industrial use of the lactose-permeate in such products as glues in plywood manufacture, as a fermentation medium or in production of ethanol or lactic acid. Concentrated whey or whey protein concentrate are used in dairy and bakery products, in confectionery products, soups, soft drinks, and in meat products (Clark, 1987).

In the whey protein concentrate, the main proteins are bovine serum albumin, α-lactalbumin, ß-lactoglobulin, immunoglobulins, lactoferrin and lactoperoxidase, and this fraction of the whey is attracting more interest. It should be noted that a significant amount of research into utilization of whey appears to be proprietary and therefore information is limited. Specific information on the economics of whey utilization is difficult to find but it appears that most uses of whey are often in competition against other established products, and therefore whether whey or whey fractions are profitable or have a market depends on the market price of the competing products. As a result there is an incentive to look for new end uses of whey with high value added, with products marketed to industry and product development supported by a high level of research (van Hoogstraten, 1987).

Antimicrobial agents in milk whey have attracted attention as potential products for value-added processing of whey (Goldman, 1989), and of these, immunoglobulins and lactoferrin are the topic of this presentation.

B. The impact of gastrointestinal infections

Intestinal infections are common in infants where conditions of hygiene are poor. The result is often diarrhea. In the case of infants in underdeveloped countries, as many as ten episodes of diarrhea in the first year are not unusual (Black et al., 1989). Diarrhea is the main cause of infant deaths in some countries (Wadstrom, 1975). For example, it has been reported that in Nigeria, about 300 children die every day from the consequences of infection with enteropathogens (Ogunsanya *et al.*, 1994). Worldwide mortality has been estimated at 4 to 6 million/year (Guerrant *et al.*, 1990).

Travelers to underdeveloped countries are also at risk (Sack, 1990), as well as military personnel during large scale operations (Oldfield et al., 1991). An epidemiologic survey reported the incidence of diarrhea in travelers to tropical or subtropical areas favored by tourists to be over 30% on average, up to 50% in selected locations, while the incidence was about 5% for travelers to the United States and Canada (Steffen, 1986). Outbreaks in day care centers and nurseries in developed countries can result in very high rates of incidence (Bower *et al.*, 1989). In the United States, it has also become apparent that the greatest annual number and rate of diarrheal deaths occur among the elderly (Lew *et al.*, 1991). Diarrheal diseases are a significant concern with hospital patients and immunocompromised persons in developed countries (Guerrant *et al.*, 1990). In the United States, an incidence of between 6.5 million and 275 million cases of infectious gastroenteritis per year have been estimated (Archer and Kvenberg, 1985; Hedberg *et al.*, 1994). This last number is rather astonishing and illustrates the uncertainties in estimating the incidence of illnesses that mostly go unreported.

Treatment or prophylaxis with antibiotics is a possibility in the case of bacterial infections, but there are problems: given the course of most episodes of gastroenteritis, it is often not practical or possible to identify the pathogen or to test for sensitivity to antibiotics, so that often empirical treatment is practiced; gross abuse of antibiotics is common; in some

countries they can even be bought over the counter, and microbial resistance is widespread (Levy, 1982). Development of vaccines has been slow (Levine, 1991) and to date the most significant success has been in the protection of calves or piglets against enterotoxigenic *E. coli* by passive transfer of immunoglobulins through the colostrum or milk of vaccinated dams (Tacket, 1991).

The principles of prevention of diarrheal diseases in underdeveloped countries are simple and well-known: improved hygiene, improved sanitation, safe water. Their application has been hampered by a general lack of will to bring progress to these areas. In developed countries where a high level of hygiene already exists with a resulting lower incidence, improvements may be difficult to achieve because of the intractable nature of some of the problems: relative crowding in day care centres; increasing proportion of elderly people; a large number of immunocompromised persons and a high level of antibiotic resistant microorganisms in hospitals.

The understanding of the role of milk in the prevention of diarrheal diseases, which will be reviewed in a following section, has led to studies into the possibility of supplementing foods, targeted at some sections of the population, with antibacterial and antiviral factors isolated from the large supplies of whey produced by the dairy industry.

C. The enteropathogens

The pathogens most frequently implicated are enterotoxigenic *Escherichia coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorragic *E. coli* (EHEC), *Shigella*, *Vibrio cholerœ*, *Campylobacter*, *Salmonella* and rotavirus (Blanco *et al.*, 1991; Black *et al.*, 1989; Cravioto *et al.*, 1990). Some of them are also found in domestic animals. Infection occurs through the food, water, feces, by contact with animals or person-to-person. To give a few examples, infections with *E. coli* have been linked to ground beef, *V. cholerœ* to unsanitary water supplies and *Salmonella* to eggs and poultry (IFT,

1988). Prevalence of these pathogens varies depending on geographical location or environmental circumstances. For instance, the agent of travelers' diarrhea is overwhelmingly enterotoxigenic *E. coli* (Black, 1986), while *Salmonella* are a major cause of infections in hospitals, and rotavirus and *Shigella* are most frequent in day care centers (Guerrant *et al.*, 1990). More detailed reviews of the relationship between these pathogens and foods can be found in the literature (IFT, 1988).

Bacteria have been grouped in a variety of ways. Gram-negative or Gram-positive bacteria are defined by their ability to take up particular stains. All bacteria mentioned in the previous paragraph are Gram-negative. The distinction between Gram-negative and Gram-positive bacteria is a reflection of their outer membrane structure, which affects the effectiveness of many antibacterial agents. *E. coli* and *Salmonella* are also organized in numerous serogroups on the basis of the antigenicity of structures on their outer membrane. The groups of *E. coli* mentioned above (ETEC, EPEC, EHEC, EIEC) contain a variety of serogroups and are defined by the mechanisms by which they cause illness. Enteropathogens cause disease by producing toxins, by damaging the microvilli or by invading the cells of the intestinal epithelium. Adherence to the intestinal epithelium is a requirement for most if not all enteropathogens, and for some of them the mechanisms of adherence have been studied in great detail.

Adherence of enterotoxigenic *E. coli* (ETEC) is mediated by colonization factors or adhesins. Numerous fimbrial adhesins have been identified. These adhesins are proteins that attach to glycoconjugate receptors, and exhibit organ and species specificity (Krogfelt, 1991). Antibodies to ETEC fimbrial adhesins are highly protective and this has been the basis of a successful vaccine against enteropathogens in cattle. Adherence of ETEC to the intestinal epithelium is accompanied by production of toxins, proteins which are responsible for increased secretion of fluid and electrolytes and resulting diarrhea. Heat labile and heat stable toxins are produced. Only the heat labile toxin is of sufficient size to be immunogenic.

Enteropathogenic *E. coli* exhibit several different patterns of adherence. The adherence of some strains of enteropathogenic *E. coli* is correlated with the presence of an EPEC adherence factor (EAF) which results in a typical pattern of microcolonies adhering to cultured epithelial cells. In addition, such EPEC produce a characteristic lesion called adhering-effacing, which is believed to be associated with diarrhea as a result of the effacement of the intestinal brush border (Levine *et al.*, 1985; Knutton *et al.*, 1987). These strains of enteropathogenic *E. coli* have also been shown to be invasive (Donnenberg *et al.*, 1989), and the invasiveness is correlated with the presence of a 94 kDa outer membrane protein (Francis *et al.*, 1991).

Adherence of invasive enteropathogens such as *Yersinia*, *Salmonella* or *Shigella* is also mediated by proteins secreted or present on the outer membrane. The interaction of these proteins with host cell membrane receptors results in invasion by the bacteria. The best characterized of these proteins is the *Yersinia pseudotuberculosis* invasin (Isberg *et al.*, 1987). In *Shigella,* a number of outer membrane proteins associated with a virulence plasmid have been identified (Oaks *et al.*, 1986), while in *Salmonella* gene products associated with invasiveness have not yet been characterized (Finlay, 1994).

While this thesis is concerned with protection against bacterial pathogens, it should be noted that rotaviruses are responsible for a large proportion of intestinal illnesses in children (Hilpert *et al.*, 1987). At least as many studies have been carried out on the protective effects of milk against rotavirus infections as on its antibacterial effects. The results of some of these studies will be presented in later sections to illustrate some aspects of this topic, since the same principles are involved.

D. Host Defenses

Pathogens in the digestive tract need to survive, to colonize by adherence to receptors, and for some of them to enter the intestinal epithelium (invasion) or to generate toxins. These steps are potential targets for the antibacterial factors that may be present in milk.

To fight pathogens, the hosts have evolved many different mechanisms that have a bactericidal or bacteriostatic effect: they may deprive the bacteria of required nutrients, or generate toxic products, or prevent adhesion or invasion. Resistance to infections can be defined in different ways:

- specific vs non-specific host defense or immunity,
- active vs passive immunity,
- cellular vs humoral immunity,
- mucosal vs systemic immunity.

It is possible to combine these groups to further define various aspects of immunity or host defense; they will be discussed in greater or lesser detail to the extent that they relate to the topic of this presentation.

1. Non-specific host defenses

The organism is protected against infectious agents by a variety of "non-immune" mechanisms. Such mechanisms are of a general nature and do not depend on previous exposure to a pathogen or toxin, and are sometimes called "native immunity". The skin and mucous membranes provide a physical barrier to the penetration of microorganisms, and secretions associated with the mucous membranes contain chemicals such as lysozyme which are harmful to the bacteria, or create an unfavorable environment such as low pH which inhibits colonization. Lactoferrin and lactoperoxidase in milk or saliva are other examples of chemicals that may have a protective effect. Lactoferrin has attracted a great

deal of attention as a possible candidate for supplementation of foods and will be discussed in greater detail in another section. A protective effect has also been attributed to oligosaccharides in milk and this will be discussed briefly later. At the cellular level, phagocytosis of bacteria by neutrophils and macrophages is another important defense mechanism.

2. Specific host defenses

Infection leads to the development of an immune response that is specific to the infectious agent. The immune response involves both cellular and non-cellular (humoral) components (Tizard, 1992). A main feature of the immune response is the production of antibodies against antigenic determinants present on the pathogen. This aspect of the immune response is most relevant to the topic of this presentation

3. Specific humoral immunity: the immunoglobulins

The humoral components of specific immunity are the immunoglobulins (Igs), which are found in the circulatory system and in secretions such as milk. Antibodies are immunoglobulins with specificity for an antigen. Immunoglobulins are glycoproteins originally defined by their electrophoretic mobility at pH 8.6 (γ globulins). They all have a basic structure of four polypeptide chains: two heavy chains (approximately 450 amino acids, 50 kDa) and two light chains (approximately 220 amino acids, 23 kDa). The four chains are held together by disulfide bonds. Multiples of this basic structure exist and in part define classes of Igs. The general structure of Igs was elucidated by clever experiments involving separation of the chains by reduction and alkylation, as well as by papain digestion of the immunoglobulins and characterization of the corresponding fragments (Porter, 1959; Fleischman *et al.*, 1962). It was also possible to group immunoglobulins into several classes. Classes were defined by serological methods, by their biological activity and by some

structural differences; for example in humans the following classes have been identified: IgG, IgA, IgM, IgD and IgE. Subclasses of IgGs are present. Similar classes are found in other mammals. An immunoglobulin class is defined by a particular class of heavy chain. Two classes of light chains also exist but they associate with heavy chains of any class. The immunoglobulins classes in different species are sufficiently different antigenically that they can be distinguished by serological methods.

IgGs are the most abundant class of immunoglobulins in human serum and are involved in neutralization of toxins and viruses and binding and opsonization of bacteria. IgMs are made up of five basic units, and are the first class of antibody to appear following original stimulation by an antigen (primary response) while IgGs appear later (secondary response). IgMs are very efficient at fixing complement, a group of proteins involved in lysis of bacteria. IgAs are present at low concentration in serum but are the predominant immunoglobulins in milk and other secretions where they exist as dimers to which is attached a secretory component (sIgAs). The secretory component is attached to the IgA dimer during transport through the epithelium; it also appears to protect IgAs from degradation by proteolytic enzymes, which is beneficial to its protective role in the digestive tract. The main role of IgAs is to prevent adherence of bacteria to the intestinal epithelium. IgDs are present in very small amounts in the serum and are associated with cells of the lymphoid system. IgEs are also present in extremely low amounts in the serum but are of great importance because of their property of binding to basophils and mast cells and subsequently to participate in allergic reactions. Similar classes of immunoglobulins are found in other mammals. In cattle, a subclass of IgG called IgG1 is the predominant immunoglobulin in milk, while IgG2 is predominant in serum (Tizard, 1992; Kimball, 1986).

The enzymatic digestion of immunoglobulins by papain permitted the identification of three fragments: two identical fragments called Fab or antigen binding fragments, which were later determined to be made up of the light chains and the N-terminal half of the heavy

chains, and the Fc fragment, which is made up of the C-terminal half of the heavy chains (Figure 1). An intact antibody molecule is bivalent, which means it can bind to two antigens or two antigenic sites, whereas the Fab fragments are monovalent. Agglutination or precipitation reactions which are the most visible sign of an antigen-antibody reaction in the laboratory, require an intact antibody molecule. The monovalent Fab fragment, however, retains the ability to bind to the antigen.

The specificity of immunoglobulins is determined by the primary structure of some portions of their light chains and heavy chains. Light chains are made up of two domains of equal lengths, and heavy chains are made up of four or five domains. Homology between the sequences of the domains reflect the evolutionary origins of immunoglobulins. The N-terminal domains of both heavy chains and light chains are called the variable regions because of their great variability in amino acid sequence. This diversity is generated by multiple germ-line genes for the variable regions, by gene rearrangement and by somatic mutations (Tizard, 1992). The variable regions define the specificity of a given antibody molecule for an antigen. The other domains define the constant region of the light and heavy chains. They also define the classes to which they belong. The heterogeneity of immunoglobulins was quite an obstacle to their analysis: for instance it would have been impossible to obtain a complete amino acid sequence of a given immunoglobulin because of the great heterogeneity of the variable regions. The study of myeloma proteins, which are homogeneous immunoglobulins produced in large amounts by clones of malignant plasma cells, allowed for the sequencing of many different immunoglobulins and the understanding of the basis of the specificity of antibodies.

4. Active vs passive immunity

The distinction between active and passive immunity is important and is the foundation from which the idea of supplementation of diets with antibacterial factors was

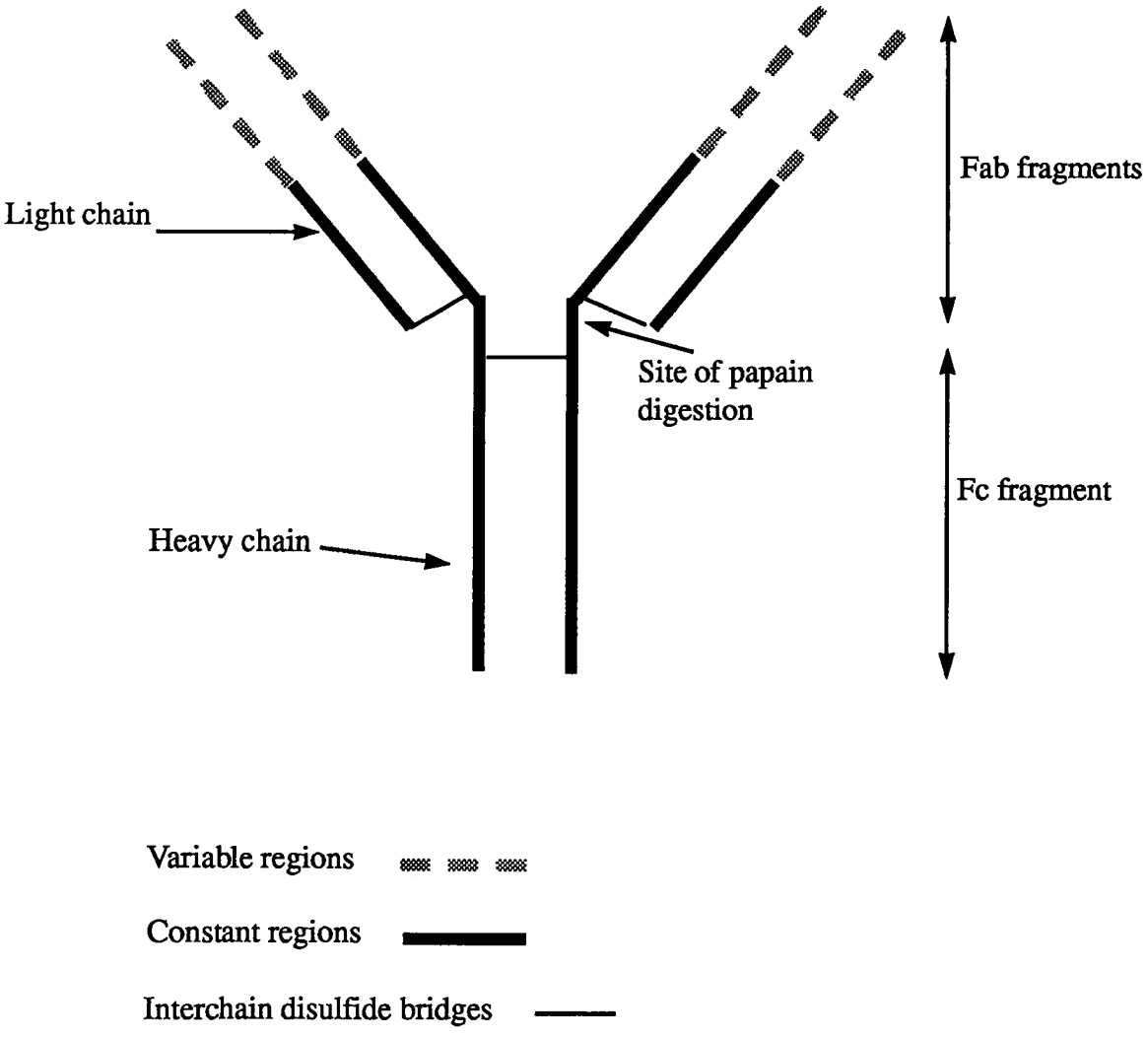


Figure 1. The basic structure of immunoglobulins.

developed. Active immunity refers to the immune response that develops following exposure to an antigen, and which results in expansion of cell lines with a specific interest in the particular antigen, or in increased production of immunoglobulins. Both infection (natural) and vaccination (artificial) may produce an active immune response, the result of which is to provide the individual with protection against the particular pathogen for a period of time, of varying duration depending on the antigen. Active immunity, however, requires time to develop, and time may be a critical factor in the defense of the body against an infection, in the case of a first exposure to an antigen. Passive immunity refers to the transfer of immunoglobulins from a donor, who has been immunized against an antigen, to a recipient who may be at risk of exposure, or who has been exposed to the antigen but for some reason is not immune to it. The classic example of passive immunity is that of the use of antiserum against the tetanus toxin. A person infected with the tetanus bacillus, and who is not immune, may succumb to the toxin before being able to develop active immunity. The passive transfer of immune globulins provides the recipient with temporary protection against the toxin. The tetanus antitoxin has been produced by immunization of horses, but injection of horse immunoglobulins, which are antigenic in humans, may have negative consequences for the recipient in the long term. A better approach has been to use human immunoglobulins isolated from pooled plasma, which have the advantage of being less immunogenic to humans than foreign proteins (Kimball, 1986).

This type of passive transfer is of course an artificial situation but nature has provided a striking and widespread example of successful passive transfer. Ironically, the therapeutic use of antiserum was developed long before there was any understanding of the passive transfer of immunity in mammals. A newborn has had no exposure to pathogens and therefore while he may be able to mount an active immune response, this response may be too slow. A primary immune response following first exposure to an antigen requires several days before production of an effective amount of specific antibodies, by which time an infection may have

taken a fatal course. While newborns are rapidly colonized by bacteria after birth, and are exposed to pathogens in the environment, most survive as a result of the passive transfer of immunity from the mother to the infant. Several variations on this mechanism exist in mammals. Humoral immunity can be transferred from the mother in three different ways during late pregnancy and after birth: 1) transfer of immunoglobulins *in utero* to the circulation of the fetus; 2) immediately following birth with the colostrum, by gut absorption into the circulation of the newborn; and 3) later with the milk, where there may or may not be selective gut absorption of the immunoglobulins. Combinations of these mechanisms are found in nature, so that it has been possible to classify mammals into three different groups (Butler *et al.*, 1985): Group I mammals are those where the transfer of Igs is strictly *in utero* (humans and rabbits); group III mammals where the transfer is strictly through the colostrum and gut absorption for a very limited period following birth (pigs, cows, sheep, goats); and group II mammals where both mechanisms are found (dogs, rodents) and where selective absorption through the gut may continue for a significant length of time (18 days in mice and rats). Newborns of group III rarely survive in a natural environment without colostrum. In all groups, passive transfer of immunoglobulins continues during lactation, the main component of milk Igs being secretory IgAs, except in bovines where IgG1 is the main component. Group III mammals, being born without any detectable amount of immunoglobulins, have provided an excellent system for the study of the development of humoral immunity. In piglets, following normal colostrum intake, immunoglobulin concentration in serum, which is highest 12 hours after birth, steadily declines for several weeks before *de novo* synthesis results in increasing concentrations of Igs. For instance, IgM concentration is at the lowest after 2 weeks, IgG after 4 to 5 weeks and IgA after 2 to 3 weeks (Klobasa *et al.*, 1981). The concentration of IgAs in sows milk at that time is higher than that of the piglets serum. It has also been reported that the daily oral intake of immunoglobulins by a seven day old piglet is greater than the immunoglobulin content of its entire circulatory system (Wilson, 1974),

which illustrates the importance of passive transfer.

While this appears to be a rather gratifying picture of nature at work in a beneficial way, it is complicated by the fact that newborns are unresponsive for a period of time to some antigens even though they are generally immunocompetent, and it is thought that the presence of Igs transferred from the mother may contribute to the slow development of active immunity (Klobasa *et al.*, 1981).

5. Systemic vs mucosal immunity

The distinction between systemic and mucosal immunity is of interest in this context because while much of the knowledge of the immune system was obtained by the study of systemic immunity, the exposure of the body to the environment and to microorganisms is greatest at the mucosal level, for instance the lungs and the digestive tract. Obviously, it is easier to obtain serum from the circulatory system in a reproducible manner than to get samples from the intestinal epithelium or from the lungs. Also, it has been found easier to immunize an animal or a person in a way that would result in a significant systemic immune response, whereas it is still rather difficult to immunize an individual so as to achieve a good level of mucosal immunity (de Aizpurua and Russell-Jones, 1988), and this has been one of the reasons why vaccines to enteropathogens have been slow to appear.

The concept of compartmentalization of the immune system derives in part from the observation that in humans and other mammals the classes of immunoglobulins that are predominant in the circulation (IgGs) are different from the classes predominant on mucosal surfaces (sIgAs). The passive transfer of immunity in the newborn is a good example of the distinction between mucosal and systemic immunity. In the human, IgGs are transferred through the placenta from the circulation of the mother to the circulation of the fetus, and after birth sIgAs are transferred with the milk to the digestive tract of the infant. In domestic animals, the situation is not so obvious because there is no placental transfer of immunity,

but studies of colostrum and milk composition in sows have shown that IgGs are predominant in colostrum, while sIgAs become predominant in the milk after a few days. The IgGs from the colostrum can be absorbed from the intestine into the circulation, thereby fulfilling the same function as placental IgGs in humans, while after a short time the piglet intestine becomes impermeable to immunoglobulins and therefore the sIgAs then present in the milk remain in the intestine. An interesting point is that the IgGs in the colostrum have been found to originate predominantly in the serum of the sow, while the IgAs in the milk are synthesized in the mammary gland (Salmon, 1989). Antibody-producing cells mature in the lymphoid tissues of the intestine, from where they migrate to other tissues on body surfaces, for instance the mammary gland, thereby establishing a connection between antigen stimulation in the intestine and antibody secretion in the milk (Tizard, 1992).

Even though this presentation is concerned with the use of immunoglobulins and other factors isolated from milk, it should be noted that hens eggs are another potential supply of immunoglobulins that could be used in passive transfer of immunity. Research to study the protective effect of immunoglobulins obtained from eggs of vaccinated chickens has demonstrated their efficacy, and methods have been developed to purify immunoglobulins from eggs (Fichtali *et al.*, 1992). While this will not be reviewed in detail, evidence from a number of experiments will later be presented briefly in support of the concept of passive transfer of immunity across species.

E. Milk and resistance to enteropathogens in humans

In humans, placental transfer of immunoglobulins reduces the dependency of the newborn on the intake of milk or colostrum, and therefore the use of infant formula has been a practical or even popular alternative to breast feeding. There has been a long-standing controversy over the advantages of one or the other, and the evidence supporting a protective

effect of milk is mostly circumstantial.

Colonization by pathogens does not necessarily result in diarrhea. In a study of 315 children in Nigeria, it was found that 75% of children with diarrhea were infected with enteropathogens, while of the control group, with no symptoms of diarrhea, 28% were infected with enteropathogens (Ogunsanya *et al.*, 1994). Cravioto *et al.* (1990) studied a population of 75 infants in a Mexican village for one year, and found an average of 4 episodes of diarrhea per child in the first year, during which the children were colonized with the following pathogens: enteropathogenic, enterotoxigenic, enteroinvasive and enterohemorrhagic *E. coli*; *Salmonella, Shigella, Campylobacter jejuni* and rotavirus. Incidence of colonization did not necessarily correlate with incidence of diarrhea. In other words, it is possible for a child to be colonized and not show symptoms. It was suggested that consumption of milk by breast-fed infants conferred some protection against appearance of symptoms of disease, while colonization did occur and led to development of immunity.

Studies have shown that there is an inverse correlation between levels of specific antibodies in mothers milk and the likelihood of the child showing symptoms of infection. For example, Cruz *et al.*(1988) surveyed a number of infants in a poor urban area of Guatemala and found that among the infants infected with enterotoxigenic *E. coli*, those who became sick ingested milk with significantly lower titers of antibody to the heat labile toxin of ETEC than the children who remained asymptomatic. Glass *et al.* (1983), following up on an observation that children past the breast feeding age are hospitalized more frequently for cholera than children who are breast fed, found a lower incidence of symptoms in children receiving milk with a high level of specific antibodies, while there was no relationship between antibody levels and colonization. Production of specific antibodies in the milk has been observed following natural infections. Stoll *et al.*, (1986) found that milk antibody titers to cholera toxin increased in 80 to 90% of Bangladeshi patients following natural infection; anti-LPS titers also increased. High titers of anti-*Campylobacter*-flagellin IgA antibodies

were found in the milk of all women tested in an area of Central Africa (Renom *et al.*, 1992). Similarly, an association between *Campylobacter* antibodies in human milk and protection from diarrhea caused by *Campylobacter* was found in a prospective study of Mexican children (Ruiz-Palacios et al., 1990).

Antibodies to viruses can also be found in human milk: For instance, in a survey of 49 mothers, McLean and Holmes (1980) found evidence of secretory IgA specific for rotavirus in every sample of milk.

Antibodies are thought to inhibit adherence of bacteria to the intestinal epithelium, or to have some antitoxin action. However, levels of immunoglobulins in milk are variable (Cruz et al., 1982), levels of specific antibodies in any individual mother's milk are also highly variable over time (Cruz & Arevalo, 1985) and some children are raised on infant formulæ, which are devoid of biologically active immunoglobulins.

Other agents in human milk that may have a protective effect were reviewed by Goldman (1989). Of these, lactoferrin will be discussed later. A variety of oligosaccharides, which are believed to act as receptor analogs for various adherence factors of enteropathogens and enterotoxins, have been found in human milk (Cravioto *et al.*, 1991; Ashkenazi and Mirelman, 1987; Newburg *et al.*, 1992). Lægrid *et al.* (1986) showed that gangliosides from human milk completely inhibited fluid accumulation caused by cholera toxin in rabbit ileal loops. As far as bovine milk oligosaccharides are concerned, few *in vivo* experiments have been done. In one study, glycoconjugates obtained from adult bovine plasma were shown to protect colostrum deprived calves against lethal doses of enterotoxigenic *E. coli* (Mouricout et al., 1990). While milk oligosaccharides are not part of the research presented here, they may be potential factors in the interpretation of results and therefore deserve this brief mention.

1. Passive transfer in animals: experimental evidence

Many experiments have shown that immunoglobulins obtained from domestic animals can protect against enteric pathogens in the same or another species. Uncontrolled experiments are practiced by some farmers who freeze excess colostrum for later use in an emergency, and at times colostrum from one species is fed to a newborn of another species on the same farm. Failure to receive colostrum results in a well-defined end point for domestic animals and therefore success is easily measured.

Besides such circumstantial evidence, experiments have demonstrated the role of milk immunoglobulins in passive transfer of immunity. For instance, newborn seronegative calves fed milk replacer supplemented with colostrum from vaccinated cows were fully protected against challenge with rotavirus, whereas control calves were not (Saif *et al.*, 1983). Similar results were obtained by Castrucci *et al.* (1984).

Gnotobiotic piglets infected orally with *E. coli* and treated with colostrum or milk from vaccinated sows survived significantly longer than control piglets. Survival depended on continuous intake of milk or colostrum (Wilson, 1974)

Experimental evidence is also available to support the concept of passive transfer across species. Boesman-Finkelstein *et al.* (1989) immunized pregnant cows with cholera toxin and *Vibrio cholerae* outer membranes and collected the colostrum for up to four days post partum. The recovery of purified colostrum immunoglobulins ranged from 143 to 794 g per cow. Titers were determined against the respective antigens. The immunoglobulin preparations were fed to 6-day-old rabbits prior to and following challenge with virulent *V. cholerae*. The rabbits who received the immunoglobulins showed significantly delayed onset of diarrhea compared to the control. Rivier and Sobotka (1978) showed that a serum preparation from rabbits vaccinated with enterotoxigenic *E. coli* gave 100% protection to rats challenged orally with the bacteria. Interestingly, the serum had only weak bactericidal effect *in vitro* and none could be detected *in vivo*. Yoshiyama and Brown (1987) immunized

pregnant rabbits with live virulent *V. cholerœ*. Milk collected from the rabbits inhibited *V. cholerœ* induced water secretion in rat ileal loops (the cholera toxin induces fluid secretion in ligated loops). Immunoabsorption studies showed that the immunoglobulins were the protective agent, and that they were specific for the enterotoxin. Lecce *et al.* (1991) found that antibody from cows immunized with a simian strain of rotavirus protected piglets challenged with a porcine strain of rotavirus. Gnotobiotic rats were protected against dental caries by ingestion of whey obtained from the milk of cows hyperimmunized with several strains of *Streptococcus mutans* (Michalek *et al.*, 1987).

Calves and piglets fed cow colostrum were protected against rotavirus (Bridger and Brown, 1981). Dose-dependent protection of piglets by bovine antibodies against human rotavirus was demonstrated in one study (Schaller *et al.*, 1992).

Colostrum-deprived piglets have been raised fairly successfully by supplementing their milk replacer with immunoglobulins isolated from bovine serum: the 21-day survival rate of piglets receiving milk replacer supplemented with bovine serum Igs was 75%, compared to 92% for the group receiving porcine Igs and 22% for a control group receiving only milk replacer (Drew and Owen, 1988).

Similar results have been obtained with egg yolk immunoglobulins (IgY). Mice were protected against challenge with human rotavirus by IgY immunoglobulins isolated from the yolk of eggs from immunized hens (Ebina *et al.*, 1990). Colostrum deprived piglets were protected against enterotoxigenic *E. coli* infection by an antibody powder obtained from eggs of chicken immunized with K88, K99 and 987P fimbrial adhesins. Piglets receiving the highest titer preparations had a 100% survival rate following challenge, while control piglets had mortality from 80 to 100% (Yokoyama *et al.*, 1992). In a similar study, Peralta *et al.* (1994) immunized chickens with a preparation of purified fimbriæ from *S. enteritidis*. Test mice fed the egg-yolk antibody preparations had a survival rate of 78% compared to 32% for control mice, when challenged with the homologous strain of *S. enteritidis*.

2. Passive transfer in humans: experimental evidence

Besides the correlation between presence of specific Igs in human milk and resistance to enteric infections found by surveys, a few experiments have been carried out with human subjects, primarily studying protection against *E. coli* and rotavirus by passive transfer of immunoglobulins obtained from milk or eggs. In an early experiment, cows were vaccinated with a mixture of pathogenic *E. coli* (Mietens and Keinhorst, 1979). A milk immune concentrate was obtained, containing about 45% immunoglobulins, mostly IgG1. The concentrate was fed to infants suffering from diarrhea caused by pathogenic *E. coli.* Stool cultures in 84% of the patients in the treatment group became negative, whereas 8 out of 9 patients in a control group remained positive.

Milk immunoglobulin concentrates obtained from vaccinated cows were used to treat 75 infants, up to two years of age, against rotavirus infection (Hilpert *et al.*, 1987); the infants had been admitted to hospital with acute gastroenteritis. Following identification of rotavirus as the causative agent, the infants received 2 g of concentrate per kg of body weight per day for five days. Concentrates with three different levels of neutralizing activity were used. A therapeutic effect was defined as the reduction in excretion time of rotavirus. A high neutralizing activity titer was required for the concentrate to achieve this therapeutic effect. Ebina *et al.* (1985) found that infants fed 20 ml daily of cow colostrum obtained from vaccinated cows were protected against infection during an outbreak of rotavirus in an orphanage, but that the colostrum had no therapeutic effect on infants suffering from diarrhea prior to receiving the colostrum. Feeding of cow colostrum containing antibodies to rotavirus also protected infants during a prospective study (Davidson *et al.*, 1989). It appears, from the various studies, that the effect of ingesting colostrum was protective, rather than therapeutic.

Tacket *et al.* (1988) immunized pregnant cows during the last eight weeks of gestation with enterotoxigenic *E. coli* belonging to 14 different serogroups, with cholera

toxin and with heat labile toxin. After removal of casein, fat, lactose and salt, the milk was concentrated and lyophilized. The resulting milk immune concentrate, containing 45% immunoglobulins, was fed three times a day, with antacid, to 10 adult volunteers in an amount equivalent to a total daily dose of 4.8 g of immunoglobulins for 7 days. A control group received a control milk concentrate. On the third day, the volunteers were challenged with an enterotoxigenic strain of *E. coli* (H10407). None of the volunteers in the test group had diarrhea, while 9 out of 10 controls did. All volunteers excreted the pathogen for the same number of days, even though at a lower level for the group receiving the immune concentrate, indicating that the protective activity was not entirely bactericidal. While the study by Tacket *et al.* (1988) was at that time the most detailed study of supplementation in adults, there was no determination of minimum effective dose, or of the antibody specificity that was most protective (anti-toxin, anti-LPS, anti-fimbriæ, etc).

It is interesting to speculate on the number of doses of immune concentrate that could be obtained from one immunized cow on the basis of the above results combined with data from another paper. According to the data of Boesman-Finkelstein *et al.* (1989) it is possible to project an average recovery of about 400 g of IgGs during the first four days of lactation. If a cow produces about 8,000 liters of milk a year with an IgG concentration of 0.5 mg/ml, one is looking at a total production of over 4,400 g of IgG, approximately 10% of which is produced during the first few days of lactation. This amount would translate into about 1,000 doses from each cow per year.

In a similar experiment, Tacket *et al.* (1992) immunized pregnant cows with a preparation of formalin treated *Shigella flexneri*. The resulting milk immune concentrates were used to protect adult volunteers against challenge with *S. flexneri*. A preparation with a high titer against *S. flexneri* LPS provided full protection, whereas a preparation with a lower titer only provided limited protection. Again the preparations were not tested for the presence of antibodies with different specificities.

Supplementation with bovine colostrum or immunoglobulins of immunodeficient patients suffering from gastroenteritis has been reported. Malnourished children suffering from prolonged infantile diarrhea associated with low level of intestinal IgA were reported to improve following feeding of bovine colostrum (Bustos Fernandez *et al.*, 1978). Two children with immunodeficiency and chronic diarrhea were reported to improve following extended supplementation with immune globulins (presumably of human origin) (Melamed *et al.*, 1991). Three immunodeficient children suffering from chronic diarrhea associated with the presence of rotavirus were treated with human serum globulin possessing antirotaviral activity. It was found that the immunoglobulins were capable of binding to rotavirus in the digestive tract so that excreted rotavirus was in the form of immune complexes (Losonsky *et al.*, 1985). Finally, AIDS patients suffering from *Cryptosporidium* - associated diarrhea have been treated with hyperimmune bovine colostrum (Nord *et al.*, 1990; Ungar *et al.*, 1990).

These experiments are evidence that passive transfer of immunoglobulins obtained from vaccinated cows or eggs provides protection against enteric pathogens. One recurring shortcoming has been a lack of information on the effective amounts and specificity of antibodies in the preparations.

F. Assessment of biological activity of immunoglobulin preparations

Should a preparation of antibacterial agents be obtained from milk or whey, it is necessary to estimate its biological activity. For example, it would be necessary to determine or define a dose of antibody that achieved a desired therapeutic or preventive effect. Given the heterogeneity of immunoglobulins, a dose may have to be defined by a specific effect in a controlled laboratory situation and related to the desired result *in vivo*.

Measurement of total immunoglobulin content is not sufficient because it does not provide information on the specificity of any antibody present. Measurements of antibodies

against a specific antigen (colonization factor antigen, lipopolysaccharide, enterotoxin) is more informative but may not reflect activity given the possible effects of processing on activity, or differing affinities of the antibodies for the antigen. Few of the experiments where humans or animals have been protected against an infection by colostrum or a milk immune concentrate have attempted to relate dose to effect, or to identify the antigens against which the immunoglobulins were effective. Ebina *et al.* (1985) protected infants against infection with rotavirus by feeding colostrum from vaccinated cows. Antibody neutralizing titers were determined but all infants were fed the same amount of colostrum. Total immunoglobulins levels were also measured but no relationship was apparent with the antibody neutralizing titer. In another experiment to protect calves against rotavirus using colostrum from immunized cows, the antibody titer only was determined and all calves received the same amount of colostrum (Castrucci *et al.*, 1984). In experiments with ETEC *E. coli*, Tacket *et al.* (1988) measured antibody titers against LPS, enterotoxin and colonization factors, by ELISA or hemagglutination, in an immune concentrate obtained from the milk of vaccinated cows. No dose response relationship was presented, and the respective effectiveness of the various antibodies could not be determined. Schaller *et al.* (1992), in a study on the prevention of rotavirus induced diarrhea in gnotobiotic piglets using bovine antibody, defined an *in vitro* virus neutralizing titer from which in turn a functional antibody dose was determined. They established a dose dependent relationship between viral shedding, diarrhea and antibody. The functional antibody dose was defined as that required to reduce the incidence of diarrhea or viral shedding by 50%.

The problems associated with the use of human volunteers to test antibacterials are obvious, and a need for practical substitutes has led to the use of animal models or organs obtained from animals, or cell culture models.

1. Animal models.

The virulence characteristics of enteropathogens (invasion, adherence or enterotoxin production) can be studied in laboratory animals. Mice are good models to study infection and invasion by *Salmonella* (Takeuchi, 1967; Hohmann *et al.*, 1978). An infant mouse model has been used to study ETEC virulence, but mouse strains showed great differences in susceptibility to ETEC of various origins (bovine, porcine or humans) and generally showed only weak susceptibility to human ETEC (Duchet-Suchaux *et al.*, 1990). Guinea pigs have been used to study adherence of ETEC (Ashkenazi and Mirelman, 1987), and rabbits have been used to study adherence of rabbit and human EPEC strains (Demierre *et al.*, 1975; Moon *et al.*, 1983). Ligated loops models can be used to study the effect of enterotoxins (Svennerholm, 1975).

Problems associated with the use of laboratory animals are numerous: cost, inconvenience, low productivity, variability and ethical considerations. Alternative models that are less controversial have been developed.

2. The study of bacterial adherence or invasion in cell cultures

Cell culture systems have been used to study bacterial adherence, bacterial invasion and the effect of enterotoxins. The systems differ in the origin of the cells used: either cell suspensions obtained directly from the organ of interest, or permanent cell lines. Each have advantages and disadvantages related to ease of use and similarity to actual *in vivo* situations.

Enterocytes have been obtained from human biopsies to study adhesion of enterotoxigenic *E. coli* (Knutton *et al.*, 1984) or of enteropathogenic *E. coli* (Knutton *et al.*, 1987). The advantage of cells suspensions is that they are obtained from the organ of interest. The problem with the use of cell suspensions is the need and inconvenience to go back to the original source, animal or human, when required.

Permanent cell lines have the advantage of relative ease of use compared to human or animal subjects, uniformity, control of experimental conditions and availability. A variety of permanent cell lines such as chinese hamster ovary cells (CHO), Madin-Darby canine kidney cells (MDCK), human cervical epithelium cells (HeLa) or human larynx epithelium cells (HEp-2) have been used to study invasion or adherence by enteropathogens. Some cell lines such as Caco-2 cells (human colon) and HT-29 cells (human colon) have the ability to differentiate in culture and display characteristics of intestinal cells, which is convenient for the study of the pathogens that require an enterocyte brush border for adherence.

Campylobacter jejuni was shown to adhere to and invade HEp-2 cells (Konkel and Joens, 1989); the invasiveness of *S. choleræ-suis, S. flexneri* and *Yersinia enterocolitica* were studied in CHO, HEp-2 and MDCK cells (Finlay and Falkow, 1988); the invasiveness of *S . typhimurium* has been studied in Caco-2 cells (Gahring *et al.,* 1990); the invasiveness of *Listeria monocytogenes* was studied in Caco-2 cells (Gaillard *et al.*, 1987) and RPMI-4788 and HT-29 cells (Meyer *et al.*, 1992) as well as the invasiveness of *Shigella flexneri* (Mounier *et al.*, 1992) and the adherence of enterotoxigenic *E. coli* (Darfeuille-Michaud *et al.*, 1990). Heat labile enterotoxins from *E. coli* can be detected with the use of Y1 mouse adrenal cortex tumor cells ((Donta *et al.*, 1974, Sack and Sack, 1975).

The validity of the cell culture models relies on the ability to demonstrate that *in vitro* invasion of, or adherence to permanent cell lines is correlated with the same phenomenon either in primary cell suspensions of the corresponding *in vivo* target or with virulence *in vivo*. This has been accomplished in many cases. For example, Giannella *et al.* (1973) correlated invasion of HeLa cells by *S. typhimurium* with invasion of rabbit ileal mucosa. Strains that were able to invade HeLa cells were also able to invade rabbit ileal mucosa, while strains that did not invade HeLa cells could not invade rabbit ileal mucosa. Day *et al.* (1981) compared the invasiveness of strains of *Shigella* and *E. coli* in HEp-2 cells with the results of the Sereny test. Of 63 strains tested, 37 were positive in both tests, 25

were negative in both and only one strain was positive in one test and negative in the other. Similarly, the ability to adhere to cultured cell lines correlated with virulence for EPEC and ETEC. Levine *et al.* (1985) demonstrated that EPEC strain E2348/69, isolated during an outbreak of infant diarrhea, adhered to HEp-2 cells and to the intestinal mucosa of colostrum deprived piglets, whereas strain MAR 20, derived from E2348/69 and cured of a 60-MDa plasmid, could not adhere to HEp-2 cells or to piglets' intestinal mucosa. Ingestion of the parent strain by volunteers resulted in diarrhea and production of antibodies against a plasmid associated 94-KDa outer membrane protein, whereas ingestion of the derived strain resulted in a lower proportion of cases of diarrhea and no detectable production of antibodies against the outer membrane protein. The outer membrane protein was called EPEC adherence factor (EAF) and was found in other EPEC strains but not in ETEC. Similar results were obtained by Knutton *et al.* (1987). Darfeuille-Michaud *et al.* (1990) demonstrated that ETEC H10407 (CFA 1), Pb 176 (CFA II), 1373 (CFA II), and 2230 adhered to differentiated Caco-2 cells with a brush border, but not to other cell lines. Adherence to the Caco-2 cells was correlated to the presence of the respective plasmids involved in the production of the adhesion factors, and to adherence to the brush border of human enterocytes. Inhibition studies with purified antigens and with antisera to the purified antigens also demonstrated the specificity of adherence to Caco-2 cells and it was concluded that Caco-2 cells behaved in the same way as human enterocytes.

3. Methods to estimate adherence or invasiveness of bacteria in cell cultures

While adherence of bacteria to cultured cells has most often been estimated visually by microscopy following staining of the monolayers, invasion of cell cultures by bacteria has been evaluated by plating techniques and by microscopy. Two general schemes to study invasion, one relying on bacterial enumeration by plating techniques, the other on observation by microscopy, will be described.

Bacteria within mammalian cells are protected from the effect of some antibiotics. If monolayers of mammalian cells are exposed to the bacteria of interest for a sufficient length of time to allow invasion of the cells, it is possible to eliminate the extracellular bacteria by incubation with the antibiotic gentamicin; intracellular bacteria are then released by treatment with a detergent and counted by dilution and plating on bacteriological medium (Falkow *et al.*, 1992). This method is relatively easy and efficient.

Bacterial adherence can be determined by running a parallel experiment in some wells in which the monolayers are washed, prior to lysis of the cells, instead of being treated with gentamicin. This treatment will give an estimate of the total number of adhering and invading bacteria, from which the number of adherent bacteria can be obtained by subtracting the number of invasive bacteria obtained with the gentamicin treatment. Alternatively, it is possible to block invasion by treatment of the mammalian cells with cytochalasins, which prevent uptake of the bacteria (Finlay and Falkow, 1988), and adherent bacteria can be estimated by enumeration or microscopy. From a methodological point of view, it is apparent that invasion can be evaluated with more confidence than adherence.

The second method, microscopy, relies on the fact that antibodies cannot normally enter mammalian cells (Heeseman and Laufs, 1985), so that external (adherent) bacteria can be stained with a fluorescein labeled antibody, for instance, and then, following permeabilization of the mammalian cells with a mild detergent, the internalized bacteria can be stained with a rhodamine antibody. The monolayer of mammalian cells can then be examined with a fluorescence microscope. Internal and external bacteria can also be quantitated with this method, even though it is not as efficient as the gentamicin assay.

4. Use of cell culture systems to study inhibition of adherence or invasion

Cell culture systems are also suitable to study or test factors that may inhibit

adherence or invasion. In particular, cell culture systems have been used to study the anti-adherence properties of human milk or colostrum against a variety of pathogens, and mammalian cell suspensions and permanent cell lines have been used. Attachment of *Streptococcus pneumoniæ* and *Hæmophilus influenzæ* to human buccal epithelial cells was inhibited by human milk and receptor oligosaccharides (Andersson *et al.*, 1986). Adhesion of EPEC *E. coli* to HEp-2 cells was inhibited by IgAs or oligosaccharides purified from human colostrum (Cravioto *et al.*, 1991). In other studies, adhesion of human EPEC to HeLa cells was inhibited by human colostrum or high molecular weight fraction of colostrum, but not by any low molecular weight fraction (Camara *et al.*, 1994; Silva and Giampaglia, 1992).

Adherence of enterotoxigenic *E. coli* to porcine duodenal and ileal cells *in vitro* was inhibited by anti-fimbriæ antibodies isolated from egg yolk (Yokoyama *et al.*, 1992). Adherence of *Salmonella enteritidis* to mouse intestinal epithelial cells *in vitro* was also reduced by anti-fimbrial antibody (Peralta *et al.*, 1994).

No examples of the use of cell culture systems to test for anti-invasive effect of human milk, or for anti-adherent or anti-invasive properties of bovine milk have been found. Therefore one of the goals of the research reported here was to investigate the suitability of a cell culture method to determine whether bovine milk, or colostrum, or immunoglobulins had any anti-invasive effect against some enteropathogens, so that at some point in the future, the *in vitro* and *in vivo* antibacterial activity of bovine milk immunoglobulins could be correlated.

G. Lactoferrin: antibacterial properties

Lactoferrin is a glycoprotein related to the iron binding transferrins. It is found in most external secretions as well as in the granules of neutrophils, where it is present at very high concentrations (Lehrer and Ganz, 1990). Lactoferrin is present in the milk of some, but not all, mammals. It was reported that a one month old breast-fed infant receives around

1200 mg of lactoferrin a day (Butte et al, 1984). The concentration of lactoferrin in human colostrum is about 7 mg/ml and in milk approximately 1 to 2 mg/ml. (Hennart *et al.*, 1991). The concentration in cows milk is much lower: approximately 0.1 to 0.3 mg/ml (Nonnecke and Smith, 1984). Even though lactoferrin has been studied extensively, its biological role remains undefined. The various functions that have been suggested for lactoferrin were reviewed by Sanchez *et al.* (1992).

The structure of lactoferrin is now well understood. Similar to all transferrins, the lactoferrin molecule is made up of two lobes, each with a binding site for iron (Figure 2). Binding of iron requires bicarbonate ions and is inhibited by citrate ions (Brock, 1985). The outstanding feature of transferrins and lactoferrin is their high binding affinity for iron. The existence of transferrin receptors on mammalian cell surfaces, and of lactoferrin receptors on intestinal microvillous membranes in some species, point to a role of transferrins and lactoferrins in the transport and absorption of iron. However, there does not seem to be any *in vivo* experimental evidence at this point that supports a role for lactoferrin in iron absorption, or that demonstrates improved iron absorption by supplementation of diets with lactoferrin (Sanchez *et al.*, 1992).

Transferrins maintain the extracellular concentration of unbound iron at a level which is too low to sustain the growth of bacteria, and injection of iron compounds reduces the lethal dose of infectious pathogens (Williams and Griffiths, 1992). Such finding would suggest an antibacterial role for lactoferrin, but for the fact that pathogens grow well in body fluids. Bacteria have developed mechanisms to compensate for the unavailability of free iron: the iron scavenging siderophores, which remove iron from the transferrins, and the bacterial transferrin or lactoferrin receptors similar to the mammalian receptors (Griffiths, 1993; Williams and Griffiths, 1992; Modun *et al.*, 1994). These bacterial receptors often show species specificity for the host transferrin or lactoferrin.

In spite of this paradoxical situation, lactoferrin has repeatedly been shown to have

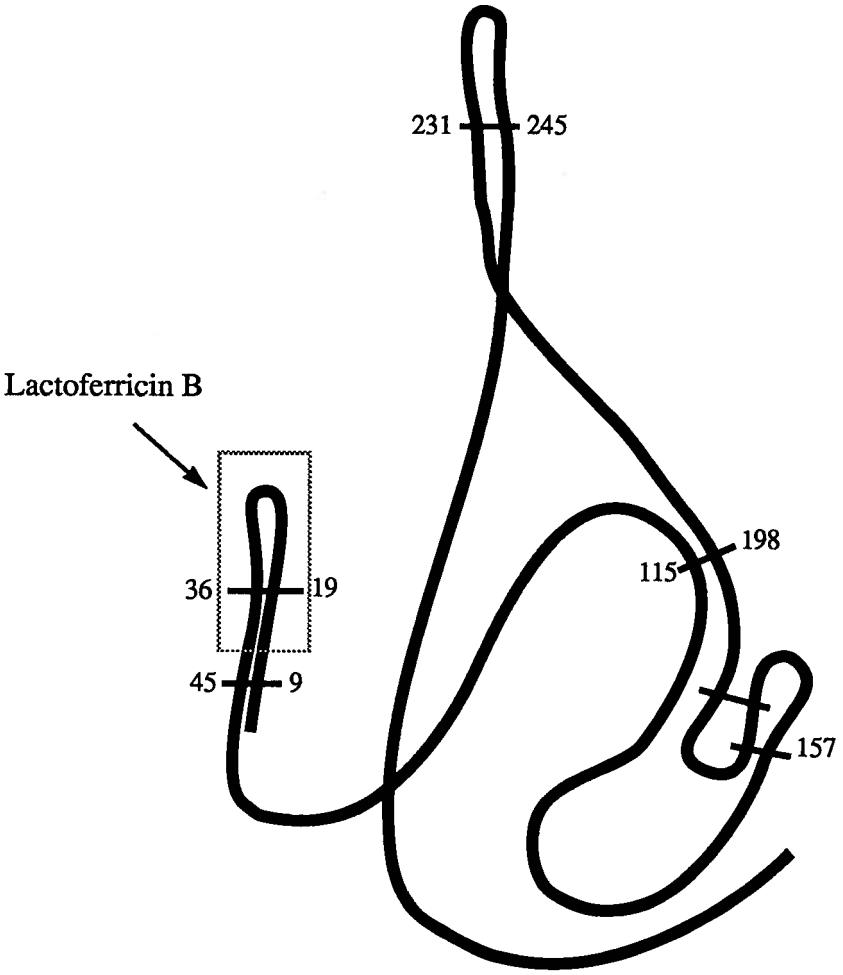


Figure 2: Model of the N-terminal lobe or domain of transferrins (adapted from Brock, 1985).
The most conserved disulfide bridges are shown, with numbers indicating their position in bovine lactoferrin. The iron binding amino acids are Asp60, Tyr92, Tyr192, His253 (Pierce *et al.*, 1991)
The location of the lactoferricin B peptide is shown .
The sequence of the peptide is: Phe17-Lys-Cys-Arg-Arg-Trp-Gln-Trp-Arg-Met-Lys-Lys-Leu-Gly-Ala-Pro-Ser-Ile-Thr-Cys-Val-Arg-Arg-Ala-Phe41 (Yamauchi *et al.*, 1993).

some bacteriostatic activity *in vitro*. Reiter *et al.* (1975) found that dialysed colostrum had a bacteriostatic effect on two strains of *E. coli*. A bacteriostatic effect of human milk or bovine colostrum was attributed to lactoferrin by Griffiths and Humphreys (1977). In both cases citrate and iron were reported to be inhibitors of the lactoferrin activity, and addition of bicarbonate was beneficial. Law and Reiter (1977) demonstrated bacteriostatic activity of bovine lactoferrin isolated from cheese whey. Saturation with iron inhibited the effect of lactoferrin. Spik *et al.* (1978) found inconsistent activity of iron binding proteins in 1% peptone, except for human lactoferrin, which was strongly bacteriostatic. There was good activity in heat treated human milk, in conjunction with IgA. The addition of bicarbonate was without effect. Stephens *et al.* (1980) found that human lactoferrin and sIgA individually had no effect on the growth of *E. coli*. In combination, they had significant bacteriostatic activity on enteropathogenic serotypes, but not on commensal serotypes. On the other hand, Arnold *et al.* (1980) showed that a virulent culture of *Streptococcus pneumoniæ* was less sensitive to lactoferrin that the avirulent strain. From other reports it appears that the effect of lactoferrin is highly dependent on the medium composition and on the condition of the bacteria. For instance, Rainard (1986a) demonstrated a strong reduction of growth of *E. coli* B117 and 11 other *E. coli* isolates from mastitic cows using only 0.1 mg/ml of bovine apo-lactoferrin. The bacteria had been grown in an iron-poor, semi-defined medium, and a long incubation time in the presence of lactoferrin was required, presumably to deplete the stored iron in the bacteria. Addition of IgG1 resulted in little improvement to the inhibiting effect of lactoferrin. In another experiment, Rainard (1986b) found that addition of 10% brain-heart infusion broth to the medium abolished the bacteriostatic effect of lactoferrin. Given that iron-saturated lactoferrin has no bacteriostatic effect, it was proposed that it is effective by making iron unavailable to microorganisms (Finkelstein *et al.*, 1983). However, experimental evidence that lactoferrin is bactericidal independently of its ability to remove iron from the growth medium was presented (Arnold *et al.*, 1982).

More recent work has suggested that lactoferrin may act by causing damage to the outer membrane of Gram-negative bacteria. The outer membrane of Gram-negative bacteria is made up of a bilayer with lipopolysaccharide (LPS) molecules occupying most of the outer layer (Nikaido and Vaara, 1987). Ellison *et al.* (1990) found that lactoferrin at a concentration of 2 mg/ml caused the release of LPS from *S. typhimurium* SL696. Ethylene diamine tetraacetic acid (EDTA) (2×10^{-5} M) had the same effect. The LPS release could be blocked by the addition of calcium or magnesium. They also observed that, similar to EDTA, lactoferrin increased the susceptibility of a wild type *E. coli* strain to rifampicin and that this effect was reversed by the addition of calcium. It was suggested that the action of lactoferrin on the LPS was similar to that of EDTA or to that of other permeabilizing agents of the Gram-negative outer membrane. Further studies by Ellison and Giehl (1991) showed that lactoferrin did not have any ability to chelate calcium, and the antibacterial effect required direct contact between lactoferrin and the bacteria.

Whether lactoferrin is bacteriostatic in two different ways (binding of iron or release of LPS) appears to still be an open question. It has been suggested that the binding of iron, which results in a conformational change of the protein (Anderson *et al.*, 1990; Grossman *e t al.*, 1992) prevents binding of lactoferrin to the LPS and thereby inhibits its bacteriostatic activity.

The effect of lactoferrin on the bacterial membrane led Ellison and Giehl (1991) to investigate a potential synergistic effect of lactoferrin and lysozyme on Gram negative bacteria. Lysozyme, which is used as a food preservative in some countries (Proctor and Cunningham, 1988) is another antibacterial agent that causes lysis of some bacteria by enzymatic hydrolysis of the peptidoglycans found in the cell wall of both Gram-positive and Gram-negative bacteria. The peptidoglycans of Gram-negative bacteria, however, are protected by a layer of lipopolysaccharide (LPS), and therefore are not accessible to lysozyme under normal conditions. The LPS are strongly anionic and are stabilized by

calcium and magnesium. They bind to lysozyme and inactivate its enzymatic activity (Ohno and Morrison, 1989). The activity of lysozyme against Gram-negative bacteria can be promoted by EDTA in tris buffer at pH 8.0 (Wooley and Blue, 1975). Disruption of the bacterial outer membrane by polycations or chelating agents such as EDTA can be demonstrated by the increased sensitivity of the bacteria to antibiotics, lysozyme or bile salts (Nikaido and Vaara, 1987).

Ellison and Giehl (1991) found that lactoferrin and lysozyme in combination had a slight bactericidal effect in 1% peptone or similar simple medium, while individually lactoferrin was only bacteriostatic and lysozyme had little or no effect against strains of *V. cholerœ, E. coli* and *S. typhimurium.* Binding studies showed that lactoferrin and poly-*l*-lysine had similar ability to bind LPS and in general the properties of lactoferrin in those experiments appeared to be similar to those of polycationic agents. Synergy between apolactoferrin and lysozyme was also demonstrated by Suzuki *et al.*, (1989) in 1% peptone.

As a result of these laboratory studies and of its presence in various secretions and within the neutrophils, lactoferrin has been widely presented in the literature as offering protection against infectious microorganisms (Hennart *et al.*, 1991). Goldman (1989) suggested that lactoferrin could be added to foods as a preservative agent or to protect individuals against gastrointestinal infections. Ambitious projects have been undertaken to produce lactoferrin on a large scale. A biotechnology company is planning to build a herd of several hundred transgenic cows and to extract from their milk several tons of human lactoferrin every year, which will be used for oral supplementation of immunocompromised patients (Seltzer, 1994; Hodgson, 1992).

It is interesting that, to this writer's knowledge, little if any direct experimental evidence for an *in vivo* antibacterial effect of lactoferrin has been presented. In an experiment designed to study the *in vivo* effects of lactoferrin, Moreau *et al.* (1983), attempted to set up conditions that would be most favorable to the effect of lactoferrin, such

as addition of sodium bicarbonate and trypsin inhibitors to a mouse diet deficient in iron. The gnotobiotic mice were inoculated with a strain of *E. coli* that had been found to be sensitive to lactoferrin *in vitro*. No differences were found in the extent of colonization of the test mice compared to control mice, as determined by fæcal bacteria counts. Similarly no differences were found in fecal counts of infants receiving either breast milk, infant formula or formula supplemented with lactoferrin. Balmer and Wharton (1989) found that breast fed and formula fed infants had significantly different fæcal flora. To test the hypothesis that the difference was caused by a lack of lactoferrin in the bovine milk derived formula, infants were fed a basic formula or a formula supplemented with bovine lactoferrin. The infants were observed for 14 days. It was found that addition of lactoferrin to the formula had no effect on the infants' fæcal flora (Balmer *et al.*, 1989). On the other hand, Teraguchi *et al.* (1993) showed that supplementing the diet of specific pathogen free mice with bovine lactoferrin prevented the large increase in the number of fæcal *Enterobacteriaceæ* that otherwise followed a switch from a solid to a milk based diet. This effect was not dependent on the iron content of lactoferrin, and was attributed to the bactericidal domain of lactoferrin named lactoferricin, which will be described in the following section. This hypothesis is not likely to be correct, and data addressing this question will be presented in a later chapter.

Finally, with regard to the supplementation of infant formula with lactoferrin, Drew *et al.* (1990) calculated that 833 g of lactoferrin are required to bind 1 g of iron. On this basis, approximately 10 g of lactoferrin would be required to bind the 12 mg of iron in 1 liter of a soy based brand of infant formula. The total protein content of the infant formula is 18 g/l, and as a result lactoferrin would have to make up the majority of the protein fraction of the formula.

H. Lactoferricin

Recently, it has been reported that peptides obtained by pepsin digestion of bovine

lactoferrin have a strong bactericidal activity against a variety of Gram positive and Gram negative bacteria in 1% peptone. Tomita et al. (1991) found that a pepsin digest of bovine lactoferrin inhibited the growth of *E. coli* O111 at concentrations of 0.25 mg/ml or more (in terms of lactoferrin), whereas a minimum concentration of 2.0 mg/ml was required for undigested lactoferrin to achieve the same inhibitory effect. In addition, it was demonstrated that the lactoferrin hydrolysate retained its activity under concentrations of iron which abolished the activity of undigested lactoferrin. The antibacterial activity of the digest was retained even following heating at 121°C for 15 min at pH 3 to 7.

Hydrolysates of both human and bovine lactoferrin were fractionated by reverse-phase HPLC and the active peptides were sequenced (Bellamy *et al.*, 1992b). The peptide obtained from human lactoferrin had a calculated molecular weight of 5,558 Da, whereas the peptide obtained from bovine lactoferrin had a molecular weight of 3,126 Da. The peptides were called lactoferricin H and lactoferricin B respectively. Lactoferricin B was found to be 25 amino-acids long, while lactoferricin H was 47 amino-acids long. The primary structure of each peptide was found to correspond exactly to a sequence of residues near the N-terminal end of the corresponding lactoferrin, and it was determined that disulfide bonds between cysteine residues formed a loop of 18 amino-acid residues (Figure 2). A high proportion of the residues in each peptide were lysines or arginines. The peptides are also located away from the iron-binding residues of undigested lactoferrin, leading to the conclusion that the antibacterial activity of the peptides is not related to the iron-binding ability of lactoferrin. Iron seemed to have little effect on the activity of lactoferricin against *E. coli* O111 (Bellamy *et al.*, 1992b). Yamauchi *et al.* (1993) demonstrated that 80 μM ferric chloride inhibited the antibacterial effect of 2 μM bovine lactoferricin toward *E. coli* CL99 1-2, but not of 18 μM bovine lactoferricin, while 20 μM lactoferrin was completely inhibited by the same concentration of ferric chloride. Pepsin digestion of related proteins such as human transferrin, murine lactoferrin or ovotransferrin failed to produce peptides with

antibacterial activity (Bellamy *et al.*, 1992b). The corresponding sequences of these proteins have a lower content of lysines and arginines than lactoferricin H or lactoferricin B.

The antibacterial spectrum of lactoferricin B was studied in some detail (Bellamy *et al.*, 1992a). Most gram-positive or gram-negative bacteria tested, including many foodborne pathogens, were susceptible to inhibition by lactoferricin B, with only *Pseudomonas fluorescens* IFO-14160 and *Bifidobacterium bifidum* ATCC 15696 being completely resistant to the action of lactoferricin B. The activity of lactoferricin B was not affected by addition of various carbohydrates and protein, but was sensitive to the addition of calcium or magnesium, as well as to the presence of citrate, succinate, lactate or acetate. The inhibition by calcium or magnesium appeared to vary depending on the target strain (two *E. coli* strains were tested).

Wakabayashi *et al.* (1992) demonstrated that 4 strains of *Listeria monocytogenes* were highly susceptible to inhibition by lactoferricin B in 1% peptone (minimum inhibitory concentration, 0.6 μg/ml) and that at a concentration of 31 μg/ml, bacteria numbers were reduced from over 10^6 CFU/ml to less than 100 CFU/ml in 30 min at 37°C.

Finally, Yamauchi *et al.* (1993) showed that lactoferricin bound to and released labelled LPS from strains of *E. coli* and *Salmonella* and compared this effect to that of other cationic peptides which also produce alterations in the outer membrane of Gram-negative bacteria.

Bellamy *et al.* (1992a) and Tomita *et al* (1991) suggested the use of lactoferricin B as a "natural preservative agent in foods and cosmetics" or in some "clinical foods and products for prevention or treatment of infectious diseases". The results published so far show that even though lactoferricin is a potent antibacterial agent under some well-defined laboratory conditions it may be susceptible to environmental effects that could make its application problematic. Should this be the case, the possibility of enhancing the antibacterial effect of lactoferricin with lysozyme and EDTA is worth investigating.

Conclusion

To conclude this brief survey of some of the mechanisms of host defense against enteropathogens, the supplementation of diet with antibacterial factors obtained from cows milk can be defined as :

- protection at the mucosal level;
- passive transfer of immunity;
- humoral rather than cellular;
- specific (immunoglobulins) or
- non-specific (lactoferrin or lactoferricin).

For supplementation by immunoglobulins to be implemented, a number of questions remain to be answered. For instance:

- how to extract immunoglobulins economically on large scale from milk or eggs;
- to what extent is biological activity affected after processing;
- how to measure the amount and effectiveness of any specific antibody in a sample of purified Igs;
- what amount of specific antibody is necessary to provide protection against a given pathogen in a given population;
- is it necessary or economical to immunize the donor animal;
- of the variety of antibodies produced against a given pathogen, which is the most effective (i.e. anti-LPS, anti-adhesins, anti-toxins, others?);
- is there a problem with allergenicity of milk Igs;
- is there a synergistic effect between antibodies and other constituents of the milk;
- how can sterile, safe and stable preparations of Igs be obtained without losing their activity;

- which is the target population that would benefit most from the supplementation, with the minimum of risks?

With regard to a food industry application of lactoferrin or lactoferricin, the main question remains: do they have any antibacterial effect *in vivo* or in more complex environments than those tested to date?

The experiments reported on the following pages addressed a few of these questions.

III. ANTIBACTERIAL ACTIVITY OF A PEPSIN DIGEST OF BOVINE LACTOFERRIN IN COMPLEX MEDIA.

Lactoferricin H and lactoferricin B, cationic peptides derived by enzymatic digestion of human and bovine lactoferrin respectively, have been shown to have strong bactericidal activity against a variety of pathogens in simple media, and it has been proposed that they could be used as food additives, either as preservatives or for the prevention of gastroenteritis (Tomita *et al.*, 1991). At the time the experiments reported below were undertaken the antibacterial effect of lactoferricin had not been tested in more complex media. The purpose of the present study was: to determine whether bovine lactoferricin, in the form of a pepsin digest of bovine lactoferrin, had a bactericidal or bacteriostatic effect in foods or similar complex media; to determine the limits on this effect; and whether the use of lysozyme and EDTA in conjunction with lactoferricin could improve its antibacterial activity.

A. Materials and methods

Bacteria.

S. enteritidis ATCC 13076 were obtained from the American Type Culture Collection, Rockville, MD, and were maintained on TSA slants at 4 °C.

Lysozyme

Chicken egg white lysozyme (Sigma L-6876) (E.C. 3.2.1.17) was used at a final concentration of 0.08 mg/ml, unless otherwise indicated. The lytic activity of lysozyme was confirmed by spectrophotometric assay of lysis of *Micrococcus lysodeikticus* according to the supplier's directions.

EDTA

EDTA was used at a final concentration of 0.25 mM, unless otherwise stated. This is in the range of concentration of EDTA allowed for use in some foods in Canada (Anon., 1988).

Pepsin digestion of lactoferrin

Bovine lactoferrin (LF) (Sigma L4765) was dissolved in 0.05 M KCl/HCl pH 2.0 buffer and porcine pepsin (E.C. 3.4.23.1) (Sigma P-7012) added at a concentration of 3% (w/w of substrate). The original concentration of lactoferrin was determined by spectrophotometry at 280 nm using an extinction coefficient at 1% and 1 cm of 15.1 (Fasman, 1976). The mixture was incubated at 37°C for 45 min, followed by heating at 80°C for 15 min. NaOH (1 M) was added to bring the pH to 7.0 and the digest was filtered through a 0.45 μ cellulose acetate filter (Corning 21053-25) and stored frozen at -20 °C. Digestion was confirmed by SDS-PAGE electrophoresis using a PhastSystem electrophoresis unit (Pharmacia LKB Biotechnology, Uppsala, Sweden); to avoid repetition, the procedure is described in detail in Chapter V. The digest (LFD) was used without further purification. The concentration of lactoferricin was calculated from the original lactoferrin concentration assuming a molecular weight for bovine lactoferricin of 3,126 daltons (Bellamy et al., 1992b) and 83,000 for bovine lactoferrin.

Culture condition

S. enteritidis were grown overnight in 50 ml of trypticase soy broth (TSB), in a 125 ml flask, in a water bath at 37°C, at 80 oscillations per minute. Bacteria were diluted 1:1000 in 1% peptone and further diluted 1:10 in the test medium. To the test medium were also added the lactoferrin digest at a final concentration of 0.8 mg/ml in terms of lactoferrin, unless otherwise stated (30 μg/ml in terms of lactoferricin B). Lysozyme and EDTA were

also added separately or together, at the required concentrations. The final volume was 200 µl. Samples were incubated for 4 hours at 37°C and aliquots removed at the beginning and the end of the incubation period. Serial 1:10 dilutions prepared in 0.1% peptone were plated in duplicate on Difco plate count agar (PCA), or plate count agar to which had been added Difco bile salts #3 at a final concentration of 1.5 g/l (PCA-BS). The drop plate method (ICMSF, 1978) was employed with 10 µl inocula applied to the media. Results of typical experiments are presented.

Calcium and magnesium concentrations were determined by the complexometric method of Ntailianas and Whitney (1964). This is a back titration method where calcium and magnesium in the test sample complex with an excess of EDTA. The excess of EDTA is determined by titration with a solution of calcium of known concentration in the presence of calcein, an indicator that gives a green fluorescent color in the presence of free calcium. Titrations at pH 12 and 13 respectively make it possible to determine amounts of calcium and magnesium in the same sample. An aliquot of the sample to be tested was added to 50 ml of distilled water, to which were added 5 ml of a 0.025 M solution of EDTA (in distilled water with 2 g/l of NaOH. Sufficient potassium hydroxide (8 N) was added to bring the pH to 12, followed by three drops of calcein (0.2 g in 100 ml of 0.025 N NaOH). The solution was back titrated with the standard calcium chloride solution (0.025 M) to obtain the first end point. The combined amount of calcium and magnesium is calculated from this value. The pH is then raised to 13, and the next end point is used to calculate the amount of calcium. Two determinations were performed for each sample, the first to approximate the end point and develop the color; this is used as a reference for the second determination, the results of which are used in the calculations.

B. Results

Digestion of bovine lactoferrin by pepsin was confirmed by SDS-PAGE

electrophoresis. Two preparations were tested. The single band of lactoferrin was no longer present after peptic digestion of lactoferrin (Figure 3) and fast moving bands of small peptides appeared.

The comparative activities against *S. enteritidis* of lactoferrin (LF) and the lactoferrin digest (LFD), added at equivalent molar concentrations, in 1% peptone and trypticase soy broth (TSB) were determined by incubating the bacteria in the respective media for 4 hours at 37 °C followed by plating on plate count agar (PCA) and on plate count agar supplemented with bile salts (PCA-BS). Bile salts have been used as probes to detect increases in permeability of the Gram-negative outer membrane (Vaara, 1992) and for differentiation of injured and uninjured bacteria. Unlike Gram-positive bacteria, Gram-negative bacteria are protected against the effects of bile salts by the LPS of the outer membrane.

Lactoferrin at a concentration of 0.8 mg/ml only slowed the growth of the bacteria in 1% peptone, but caused some injury which was demonstrated when *S. enteritidis* were plated on PCA-bile salts agar, whereas LFD at a concentration of 30 μg/ml in terms of lactoferricin B showed bactericidal activity in 1% peptone (Figure 4). Bactericidal activity is defined as a reduction in the original number of bacteria determined by plating on PCA, and injury as a reduction in the number of bacteria growing on PCA-BS compared to that growing on PCA. Both compounds had little effect on *S. enteritidis* in TSB. The results suggest a permeabilizing effect of both lactoferrin and the lactoferrin hydrolysate in 1% peptone. Ellison *et al.* (1990) could not demonstrate a sensitizing effect of human lactoferrin to desoxycholate against a serotype O26 *E. coli* while they could demonstrate sensitization of a wild type *E. coli* to the antibiotic rifampicin, a rather contradictory finding. In retrospect the study of the effects of human lactoferrin on sensitization to desoxycholate may have been conducted under conditions (high NaCl concentration) where lactoferrin may not have had any effect.

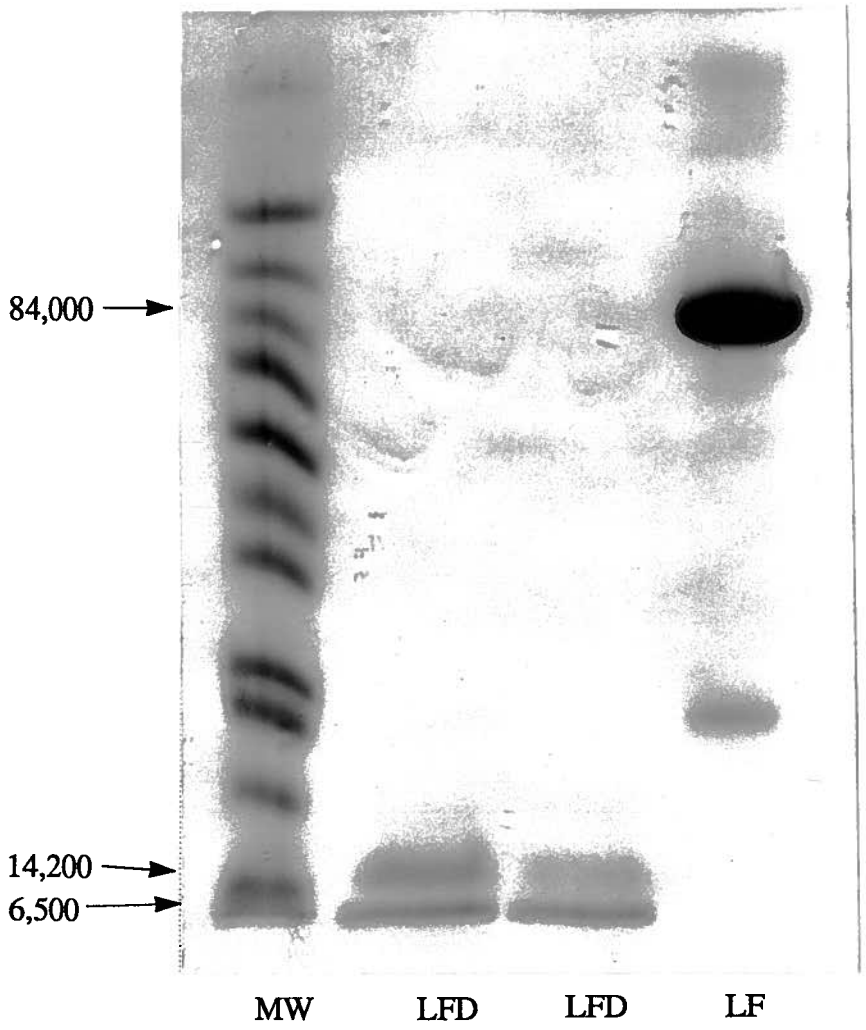


Figure 3. SDS-PAGE profiles of bovine lactoferrin (LF) and of two pepsin digests of bovine lactoferrin (LFD).
MW= Molecular weight markers (Sigma M4038)

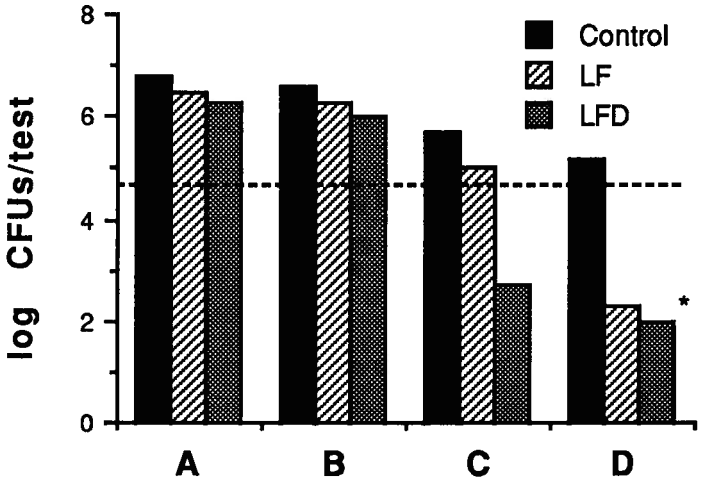


Figure 4. Effect of lactoferrin (LF) and lactoferrin digest (LFD) on growth or survival of *Salmonella enteritidis* in trypticase soy broth (A and B), and in 1% peptone (C and D).
Bacteria were exposed to the various conditions for 4 hr at 37°C.
Counts, expressed as log CFUs in 0.2 ml, obtained by plating on plate count agar (A and C), or on plate count agar + bile salts (B and D). Concentration of lactoferrin: 0.8 mg/ml. Concentration of lactoferrin digest: 30 μg/ml in terms of lactoferricin B.
Log CFUs at start of incubation: 4.7 (dotted line).
Detection limit: 2 log CFUs. *: < 2 log CFUs.

The minimum inhibitory concentration was determined by incubating *S. enteritidis* for 4 hours with different levels of LFD. Two preparations were tested. The lactoferrin digest at a concentration as low as 3 μg/ml in terms of lactoferricin B inhibited the growth of *S . enteritidis* ATCC 13076 in 1% peptone (Table I). This compares with concentrations of 7.8 μg/ml (Jones *et al.*, 1994b) and 12 μg/ml (Bellamy *et al.*, 1992a) determined for other strains of *S. enteritidis* using higher bacterial inocula. By comparing the results in Figure 4 and Table I, it can be seen that in 1% peptone, the minimum inhibitory concentration of lactoferrin was higher than 0.8mg/ml, whereas the minimum inhibitory concentration of the digest was less than 0.08 mg/ml in terms of lactoferrin (3 μg/ml in terms of lactoferricin B), a tenfold improvement or more in potency. This compares to increases in potency of from eight to twentyfold reported by Tomita *et al.* (1991) for various Gram-negative and Gram-positive bacteria.

Given that LFD showed a diminished activity in TSB compared to 1% peptone, and that a synergistic effect of lactoferrin and lysozyme had previously been demonstrated in bacto-peptone or proteose peptone (Ellison and Giehl, 1991; Suzuki *et al.*, 1989), and that activity only in 1% peptone would be of little use in terms of practical application, experiments were carried out to determine whether LFD alone or in combination with lysozyme would have an effect on bacterial growth or survival in some complex foods or media, some of which could be potential candidates for addition of LFD or lysozyme as antibacterial agents.

Table II shows that LFD alone or in combination with lysozyme (80 μg/ml) had no antibacterial action against *S. enteritidis* ATCC 13076 in a milk based and a soy based infant formula and in a chicken skin extract. The concentration of lysozyme in human milk has been reported to be about 65 μg/ ml and in gastric juice about 75 μg/ml (Hankiewicz and Swierczek, 1974).

Bellamy *et al.* (1992a) previously showed that concentrations of calcium and

TABLE I. Effect of various concentrations of LFD on the growth or survival of *S. enteritidis* in 1% peptone[a].

LFD concentration (μg/ml)[b]	Log CFUs	
60	-[c]	2.8
30	2.9	3.4
15	3.5	3.6
6	4.0	4.2
3	4.5	-[c]
0	5.9	6.4

[a]: Bacteria were exposed to the various concentrations of LFD for 4 hr at 37 °C. Two different pepsin digests were tested.
Counts, expressed as log CFUs in 0.2 ml, were obtained by plating serial dilutions on plate count agar. Log CFUs at the start of incubation: 4.8 and 4.5 respectively.

[b]: Calculated in terms of the lactoferricin peptide.

[c]: Data not available

TABLE II. Effect of LFD or LFD plus lysozyme on the growth of *S. enteritidis* in infant formula and chicken skin extract.

	log CFUs/0.2 ml		
	at t=0[a]	at t=4[b]	
Growth medium		plated on PCA	plated on PCA-BS
SIMILAC[c]	4.9	7.0	6.9
SIMILAC + LFD	4.9	7.0	6.5
SIMILAC + LFD + Lys	4.9	6.7	7.0
ISOMIL[d]	5.1	7.4	7.2
ISOMIL + LFD	5.1	7.3	7.1
ISOMIL + LFD + Lys	5.1	7.6	7.4
Chicken skin extract[e]	4.6	7.6	7.6
" " " + LFD	4.6	7.6	7.7
" " + LFD + Lys	4.6	7.6	7.9

[a] : log CFUs/0.2 ml at the beginning of the experiment.

[b] : log CFUs/0.2 ml after 4 hours of exposure in the respective growth media at 37 °C, followed by enumeration on PCA or PCA-BS.

[c] : SIMILAC®: a milk based liquid infant formula.

[d] : ISOMIL®: a soy based liquid infant formula.

[e] : prepared by homogenizing 1 g of chicken skin in 1 ml of distilled H_2O.

magnesium in a range from 2 to 5 mM greatly inhibited the antibacterial activity of lactoferricin B against *Escherichia coli* IID-861, but had only a minimal effect on the activity against *E. coli* O111. Inhibition of the antibacterial activity of lactoferricin B against *E. coli* 10418 by similar concentrations of calcium was also recently reported by Jones *et al.* (1994b).

Concentrations of calcium and magnesium were determined in 1% peptone, TSB, chicken skin and infant formula (Table III). The infant formulas had concentrations of calcium and magnesium substantially higher than those reported to inhibit the action of lactoferricin B against the *E. coli* strains. The foods tested were chosen because they are susceptible to microbial contamination either at the consumer level (infant formula) or at the processing level (chicken).

Addition of calcium (as calcium chloride) to 1% peptone, to a concentration equivalent to that found in milk (25mM), completely inhibited the activity of LFD against *S. enteritidis*. Addition of calcium following 2 hours of incubation with LFD in 1 % peptone also reversed the antibacterial effect of LFD (Figure 5). It was also found that LFD had no bactericidal or bacteriostatic activity against *S. enteritidis* when calcium was added to 1% peptone at a final concentration of 5 mM. The chicken skin extract had concentrations of calcium and magnesium of the same order as those found in TSB. These levels of calcium and magnesium, presumably in conjunction with other factors, therefore appear to be sufficient to inhibit the effect of lactoferricin B against *S. enteritidis* ATCC 13076.

It was of interest to know whether it is possible to extend the range of effectiveness of a mixture of LFD and lysozyme in TSB by increasing the concentration of either independently. TSB was used for convenience and because some preliminary observations indicated that the concentration of inhibitors of lactoferricin in TSB might be just low enough that an improvement in antibacterial activity could be detected by the combined effect of LFD and lysozyme. It can be seen from Figure 6A that increasing the concentration

Table III. Concentrations of calcium and magnesium in some test media.

	Calcium (mM)	Magnesium (mM)
1% Peptone	0.6	0.1
TSB	3.1	0.4
Chicken skin extract[a]	3.3	0.3
SIMILAC	12.3	1.7 (from label)
ISOMIL	17.5	2.0 (from label)

[a]: prepared by homogenizing 1 g of chicken skin in 1 ml of distilled H_2O at room temperature with a tissue homogenizer (Pyrex 7715).

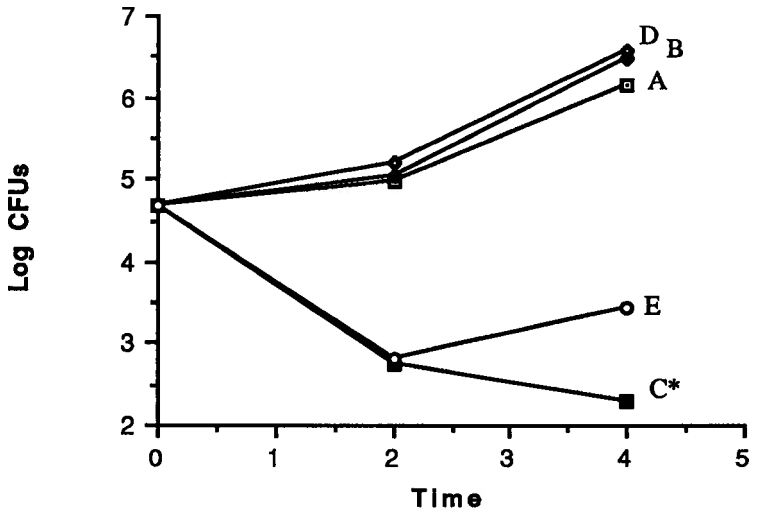


Figure 5: Effect of calcium on the antibacterial activity of LFD against *S. enteritidis* ATCC 13076.

A: 1% peptone.
B: 1% peptone + calcium (25 mM).
C: 1% peptone + LFD (30 μg/ml).
D: 1% peptone + calcium + LFD.
E: 1% peptone + LFD; calcium added after two hours of incubation.

Bacteria were incubated at 37 °C in the media indicated. Bacteria counts were determined after 2 hr and 4 hr of incubation, by dilution and plating on plate count agar.
Numbers of *S. enteritidis* expressed as log CFUs/0.2 ml (average of two experiments)
*: < 2.3 log CFUs

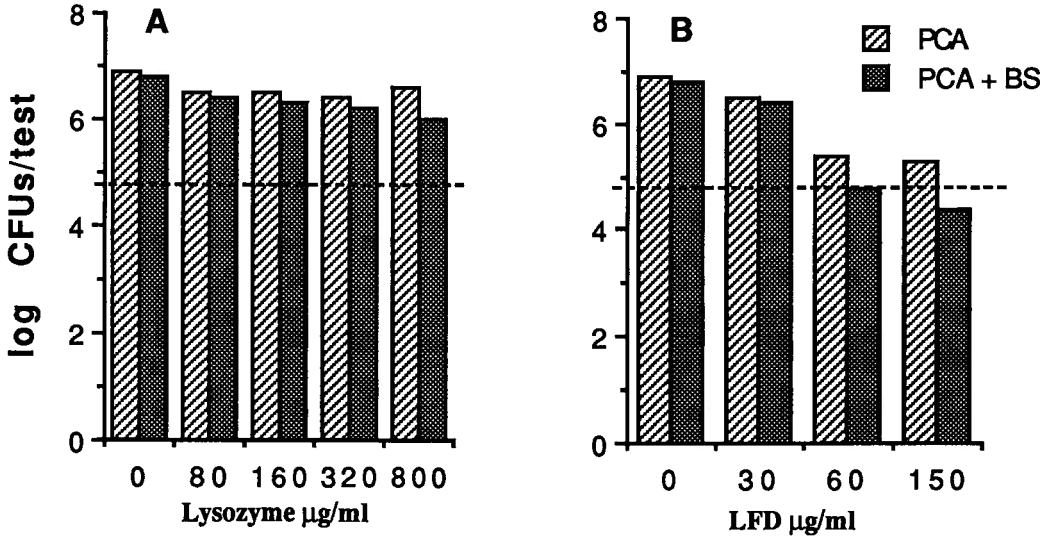


Figure 6. A: Effect of increasing concentration of lysozyme at a constant concentration of lactoferrin digest (LFD) (30 µg/ml in terms of lactoferricin B) on the growth of *Salmonella enteritidis* in TSB. B: Effect of increasing concentration of LFD at a constant concentration of lysozyme (80 µg/ml). Counts of *Salmonella enteritidis*, after 4 hours of exposure in trypticase soy broth at 37°C, were obtained by plating on plate count agar (PCA), or on plate count agar + bile salts (PCA + BS).
Log CFUs at start of incubation: 4.8 (dotted line)

of lysozyme up to 800 μg/ml while maintaining the concentration of LFD (equivalent to 30 μg/ml of lactoferricin) had minimal effect on the potency of the mixture, whereas increasing the LFD concentration above 60 μg/ml while maintaining a constant concentration of lysozyme (80 μg/ml) increased the effectiveness of the mixture, as shown by a smaller increase in numbers and by injury to *S. enteritidis* (as evidenced by the decreased number of CFUs on PCA-BS) (Figure 6B). However, the improvement was not great and even at 150 μg/ml of lactoferricin B, only a bacteriostatic effect could be demonstrated. Moreover the data did not allow for the contribution of lysozyme, if any, to be estimated. The results presented in Figure 6 show that first, lysozyme had little effect on *S. enteritidis* under the conditions of the experiments, and second that any additional effect due to lysozyme will only be evident under conditions where LFD itself has some degree of effectiveness.

To further attempt to counteract the effect of TSB on the activity of LFD, EDTA was added to TSB in increasing amounts, while maintaining the concentrations of LFD and lysozyme. EDTA is an outer membrane permeabilizer that requires the presence of Tris at pH 8.0 to be most effective. It is not as effective in nutrient broth but may be able to chelate the calcium and magnesium in the medium and thereby reduce their effect on lactoferricin. Increasing the concentration of EDTA alone in TSB progressively resulted in a diminished increase in bacteria count, even though the effect was small, while addition of LFD (30 μg/ml) and lysozyme (80 μg/ml) together with EDTA had greater effect. However, EDTA at a concentration of 1.25 mM in combination with LFD and lysozyme was required to prevent an increase in the number of bacteria from the initial inoculum level (Figure 7A). Some injury to *S. enteritidis* could be demonstrated by plating on PCA-BS (Figure 7B).

Considering that TSB at normal concentration appears to inhibit the action of LFD at a concentration equivalent to 30 μg/ml of lactoferricin, the possible synergy of LFD and lysozyme could then be studied either by increasing the concentration of LFD, or by diluting the growth medium to reduce the concentration of inhibitors.

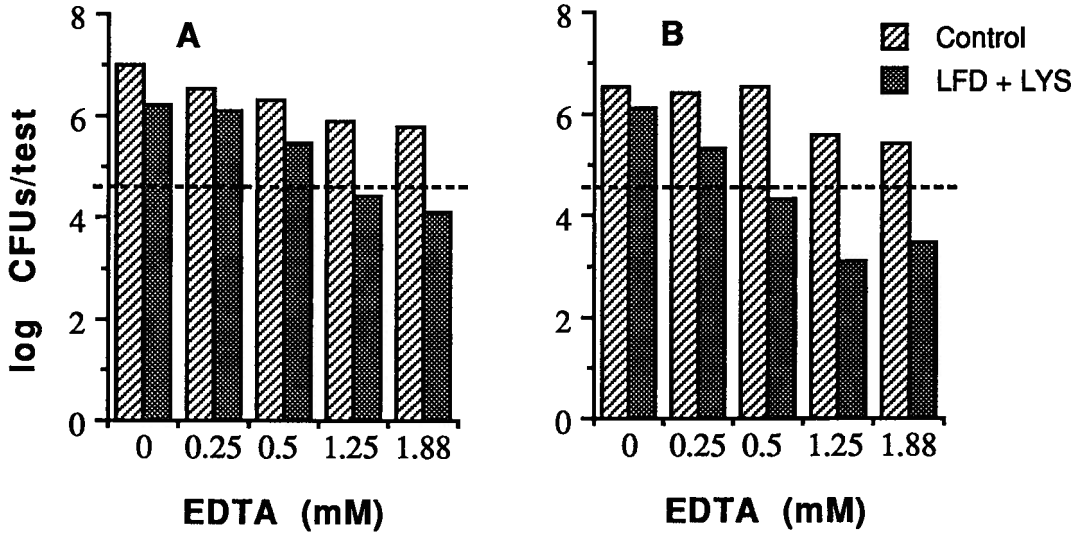


Figure 7. Effect of increasing concentration of EDTA, with or without lactoferrin digest (LFD) and lysozyme (LYS) on growth or survival of *Salmonella enteritidis* in trypticase soy broth after 4 hours of exposure at 37°C.
Counts obtained by plating on plate count agar (A), or on plate count agar + bile salts (B). Lactoferrin digest concentration: 30 µg/ml in terms of lactoferricin B. Lysozyme concentration: 80 µg/ml. Log CFUs at start of incubation: 4.6 (dotted line).

In view of the results in Figure 6, it was felt that the latter would be more productive, and the antibacterial activity of LFD with and without lysozyme was tested in various strengths of TSB.

Table IV shows that *S. enteritidis* grew equally well in TSB or TSB diluted to 1/4 normal concentration, that the effect of LFD increased as the TSB concentration decreased, and that as well an additional effect with lysozyme was apparent as the concentration of TSB decreased. The effect of LFD, lysozyme and EDTA was further studied by reducing the concentration of the growth medium, so that EDTA could be tested at a concentration of 25 mM, which is approved in some foods in Canada (Anon, 1988). Figure 8 shows that lysozyme at 80 μg/ml or EDTA individually had little if any effect on the growth of *S. enteritidis*. On the other hand, addition of lysozyme, or EDTA plus lysozyme, to the medium together with LFD resulted in increased antibacterial action, which again was dependent on the concentration of the growth medium. The results in Figure 8 compare well with the results presented in Table IV and in Figure 7. For instance, the data in the columns LFD or LFD + Lys in Figure 8 are a replicate of a similar test in Table IV, while the data with EDTA are an extension of some of the results presented in Figure 7. Certainly in the combinations where lysozyme or lysozyme and EDTA were added to LFD an increased effect was found compared to the activity of LFD alone. It can also be seen that while in TSB a concentration of EDTA of 1.25 mM was required to achieve a bacteriostatic effect in conjunction with LFD and lysozyme, in 3/4 strength TSB, a bactericidal effect was achieved when EDTA was used in this combination at 0.25 mM.

Considering that exposure to lactoferricin appears to result in some injury to the bacteria, so that they become sensitive to bile salts, the effect of lactoferricin in combination with bile salts was tested, in contrast to the previous experiments where the bacteria where exposed first to lactoferricin, then plated on bile salts agar. If lactoferricin were to be used *in vivo* as suggested to prevent gastrointestinal infection, it would be present in an

TABLE IV. Growth or survival of *S. enteritidis* in various concentrations of TSB with or without lactoferrin digest (LFD) and lysozyme (Lys)[a].

	log CFUs	
Growth medium	on PCA	on PCA-BS
1xTSB	6.8	7.2
0.25xTSB	6.7	6.7
0.75xTSB + LFD	5.7	3.0
0.75xTSB + LFD + Lys	5.0	2.8
0.50xTSB + LFD	5.0	2.3
0.50xTSB + LFD + Lys	4.0	<2.0
0.25xTSB + LFD	3.6	<2.0
0.25xTSB + LFD + Lys	<2.0	<2.0

[a]: bacteria were incubated for 4 hr at 37 °C in the various test media. Bacteria counts, expressed as log CFUs in 0.2 ml, were determined by plating serial dilutions on plate count agar (PCA) and plate count agar plus bile salts (PCA-BS).

Log CFUs at start of incubation: 4.5

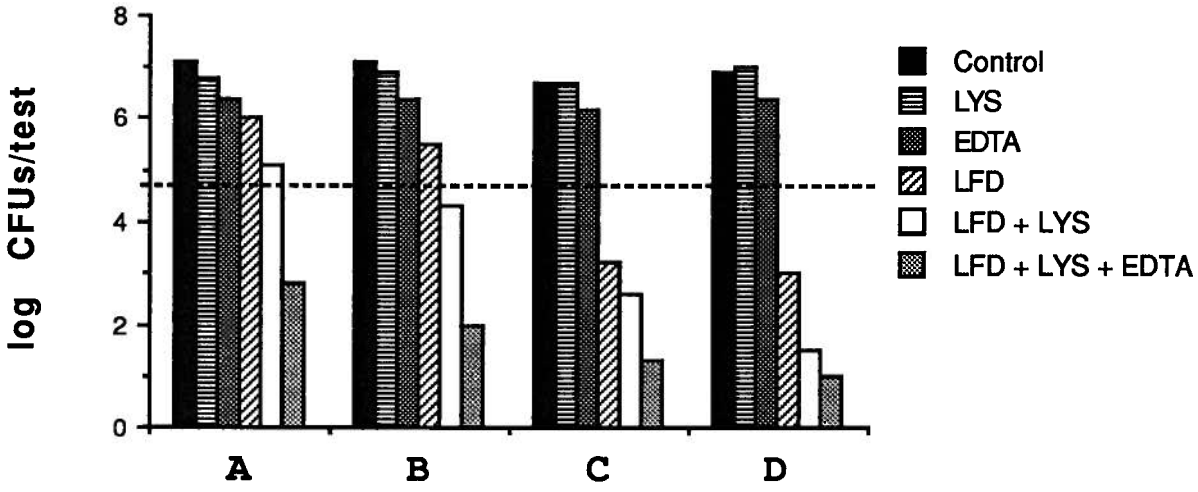


Figure 8. Effect of lactoferrin digest (LFD, 30 μg/ml in terms of lactoferricin B), lysozyme (LYS, 80 μg/ml) and EDTA (0.25 mM), separately or in combination, on growth or survival of *Salmonella enteritidis* ATCC 13076 in three quarter strength trypticase soy broth (A and C), and in half strength trypticase soy broth (B and D), after 4 hours of exposure. Counts, expressed as log CFUs/0.2 ml, obtained by plating on plate count agar (A and B), or on plate count agar + bile salts (C and D). Log CFUs at start of incubation: 4.7 (dotted line). Detection limit: 1 log CFUs.

environment where bile salts are abundant. Some incidental observations had raised doubts about a beneficial effect of bile salts on the antibacterial activity of lactoferricin. The bacteria were incubated for 4 hours at 37 °C in 1% peptone with LFD (30 μg/ml) and bile salts (1.5 mg/ml). While LFD showed the expected bactericidal effect at that concentration, addition of bile salts inhibited the effect of LFD. Bile salts alone only slowed the growth of *S. enteritidis* (Table V).

C. Discussion

The purpose of this work was to determine whether LFD had any antibacterial effect against *S. enteritidis* in some selected foods, and whether a synergistic effect between LFD, lysozyme and EDTA could be demonstrated under conditions approximating those that may be found in foods.

The antibacterial effects can be defined in different ways:

- a bactericidal effect, where a drop in the number of bacteria is observed at some time following the addition of the agent,

- a bacteriostatic effect, where the bacteria are prevented from increasing in number,

- a sensitizing effect, which in the case of Gram-negative bacteria refers to a loss of resistance to agents such as lysozyme, bile salts, or some antibiotics which are effective against Gram-positive bacteria, but from which Gram-negative bacteria are protected by the nature of their outer membrane. The sensitizing agents have been called permeabilizers (Vaara, 1992; Hancock and Wong, 1984), and some of the agents whose entry is facilitated have been used as probes to demonstrate the effects of the permeabilizers. Evidently the different antibacterial effects are not necessarily independent from each other.

A number of experiments were carried out and the results are presented in several tables and figures which when related to each other give a good indication of the potential of lactoferricin as a food additive. For example, data on the effect of lactoferricin in TSB or

TABLE V. Effect of LFD and bile salts on the growth or survival of *S. enteritidis* in 1% peptone.

Medium	Log CFUs[a]
1% peptone	6.4
1% peptone + LFD[b]	2.5
1% peptone + LFD + BS[c]	4.5
1% peptone + BS	5.8

[a]: Log CFUs in 0.2 ml after 4 hours of exposure (average of 2 experiments).
Log CFUs at the start of incubation: 4.8
Counts determined by plating on PCA and overnight incubation at 37 °C.

[b]: 30 µg/ml in terms of lactoferricin B.

[c]: Bile salts at 1.5 mg/ml.

complex media were presented in six different instances; the data on the effect in 1% peptone were presented in five different instances; two different experiments were reported on the effect of lactoferricin in reduced strength TSB; the interaction of LFD and lysozyme was studied in four different experiments; and the interaction with EDTA in two different experiments; finally the effect of LFD on sensitization of *S. enteritidis* to bile salts was examined in six different instances.

The results showed that the pepsin hydrolysate of bovine lactoferrin at a concentration of 30 μg/ml in terms of lactoferricin B had a bactericidal effect against *S. enteritidis* ATCC 13076 in 1% peptone, and that a definite bacteriostatic effect could be demonstrated at a concentration as low as 3 μg/ml. In contrast, bovine lactoferrin at a concentration of 0.8 mg/ml barely showed a bacteriostatic effect in 1% peptone. Therefore it is apparent that at equivalent molar concentration, LFD was approximately 10 times as potent as lactoferrin. Both lactoferrin and LFD sensitized *S. enteritidis* to the bile salts, which suggested that the use of lysozyme in conjunction with LFD might be beneficial. However, neither lactoferrin nor lactoferricin had any detectable effect in TSB, a more complex medium. Testing of LFD alone or with lysozyme in infant formulas or a chicken skin extract showed a complete lack of antibacterial activity at the concentrations tested. It was found that addition of calcium at a concentration of 25mM was sufficient to inhibit the activity of LFD and that the concentrations of calcium in the foods and media tested, with the exception of 1% peptone were at least equal to the level that had been found to inhibit the antibacterial activity of lactoferricin against one strain of *E. coli* (Bellamy *et al.*, 1992a). Addition of calcium to 1% peptone after two hours of exposure to LFD was sufficient to reverse the effect of LFD, so that the surviving bacteria were then able to increase in number.

Attempts to overcome the inhibition of LFD in TSB by combinations of LFD, lysozyme and EDTA, and by raising their respective concentrations, were generally not successful. Increasing the concentration of lysozyme to 800 μg/ml had no effect even in the

presence of LFD; it was necessary to increase the concentration of LFD to 60 μg/ml in the presence of lysozyme to detect a reduction in the rate of increase of *S. enteritidis* and some sensitization of the bacteria to bile salts; increasing the concentration of EDTA alone had minimal effect, while in combination with LFD and lysozyme a bacteriostatic effect was achieved at a concentration of 1.25 mM EDTA.

To demonstrate a bactericidal effect of LFD with lysozyme, or of LFD, lysozyme and EDTA at a concentration of EDTA that is allowed in foods in Canada, it was necesary to reduce the strength of the medium.

These results raise doubts about the potential for addition of lactoferricin to foods, where calcium is often present at high levels and is an important nutrient. Other inhibitors such as magnesium or citrate are also likely to be present. Suggestions for an *in vivo* use of lactoferricin suffer from the same problem. Moreover, while the sensitizing effect of lactoferricin to bile salts may be thought of as beneficial, it is evidently useful only when bacteria are exposed to lactoferricin prior to being exposed to bile salts, since it was shown that bile salts inhibit lactoferricin when both are present at the same time in the test medium. Bile salts are secreted in large amounts in the intestine, and the conditions in the digestive tract appear to be most favorable to the inhibitors of lactoferrin and lactoferricin. Any effect of lactoferricin *in vivo* remains to be demonstrated, and the fact that lactoferrin or fragments of lactoferrin have been found in stools is no proof of efficacy. Careful experiments by Moreau *et al.* (1983) failed to show any effect of lactoferrin on the intestinal bacterial flora composition in gnotobiotic mice and in infants. It is just as doubtful that increasing the concentration of lactoferricin will help to overcome the inhibition by food components. While a concentration of lactoferricin of about 3 μg/ml is sufficient to show a bacteriostatic effect in 1% peptone against *S. enteritidis*, the concentration of lactoferricin has to go up to at least 150 μg/ml to achieve the same result in TSB, a factor of 50, which does not seem

commensurate with the increase in concentration of the medium. Jones *et al.* (1994b) also observed that addition of milk or infant formula to 1% peptone to a level of only 5% (v/v) was sufficient to increase the minimum inhibitory concentration of lactoferricin against *E . coli* 10418 to over 500 µg/ml. In the case of the two strains of *E. coli* where curves of minimum inhibitory concentrations of lactoferricin versus calcium levels have been produced (Jones *et al.*, 1994b; Bellamy *et al.*, 1992a), the curves rise very steeply at concentrations of calcium above 5 mM. This would suggest that increasing the amounts of lactoferricin in attempts to overcome the inhibitors in the foods is not likely to be productive or economical. A prime requirement of any agent that may be added to foods as a preservative would be the ability to be effective under a wide range of conditions. This does not appear to be the case with lactoferricin.

The results presented here do not provide an answer to the question of whether lactoferricin has potential for an external application, for example in sanitation or, as has been suggested, in cosmetics (Tomita *et al.*, 1991). The use of lactoferrin has been proposed in toothpaste, and in the treatment of pinkeye in cattle (Borgström, 1990). The experiments presented here were not designed to address this question directly, and it remains a possibility that the conditions may be more favorable to the use of lactoferricin, possibly in conjunction with lysozyme and EDTA. Experiments to control *Salmonella* on poultry meat (Samuelson *et al.*, 1985), or coliforms in bladder infections (Goldschmidt *et al.*, 1975), with lysozyme in combination with EDTA used much higher concentrations of either compounds than presented here. The addition of lactoferricin in such experiments could make it possible to reduce the concentrations of the other components. It would be worthwhile to investigate whether lactoferricin has any antibacterial activity at low temperature or under conditions where bacteria may not be actively metabolizing.

A number of experiments reported here showed that under conditions where LFD

was active, it not only had a bactericidal effect, but it also had a permeabilizing effect so that lysozyme, which otherwise had no effect against Gram-negative bacteria, became bactericidal. Ellison and Giehl (1991) using human lactoferrin and human lysozyme, and Yamauchi *et al.* (1993) with lactoferricin B and human lysozyme, reported the same situation, with the difference that lactoferrin was not bactericidal. A permeabilizing effect of a large bacteriostatic molecule like lactoferrin that favors the effect of lysozyme might be viewed as beneficial. On the other hand, the use of lysozyme in conjunction with a small bactericidal molecule like lactoferricin might be of little practical interest, as the same result might be obtained simply by increasing the concentration of lactoferricin itself. It could be argued that a mixture of these two compounds might have a broader effect than individually.

Ellison *et al.*, (1990) reported that bacteria grown in calcium rich medium were more susceptible to the LPS releasing effect of human lactoferrin than bacteria grown in a low calcium medium. Yamauchi *et al.*, (1993) confirmed this finding with bovine lactoferrin and advanced the hypothesis that increasing numbers of cations are incorporated into the outer membrane when bacteria are grown in medium with high concentration of cations, and that this high numbers of cations in the outer membrane would make it more susceptible to damage by agents such as lactoferrin. No mechanism for this effect was offered. However, they also found that lactoferricin releases the same amount of LPS independently of the concentration of cations in the growth medium, and moreover the amount of LPS released by lactoferricin is similar to the maximum amount released by lactoferrin from bacteria grown in a medium with high concentration of cations. This would tend to imply that, at the same molar concentration, lactoferrin and lactoferricin have the same maximum capacity to release LPS, but that when bacteria are grown in medium that is low in cations, the membrane is assembled in such a way that the LPS are not accessible to lactoferrin, whereas they are accessible to lactoferricin. At the same time, Yamauchi *et al.* (1993) found that

under the conditions where both lactoferrin and lactoferricin released the same high proportion of LPS, lactoferrin had no effect on the viability of the bacteria, whereas lactoferricin caused a 99% decrease in CFUs. This may be a reflection of the ability of the smaller lactoferricin molecule to penetrate the bacterial membrane. It would have been interesting to study the effect of probes like bile salts, lysozyme or actinomycin D in such an experiment. This may be an indication that the antibacterial effect is a two steps process (permeabilization with the release of LPS, followed by penetration), and that lactoferricin is able to perform the two steps, but that lactoferrin, presumably restricted by its size, can only effect permeabilization.

Weiss *et al.* (1986) showed that changes in the sensitivity of *E. coli* to the neutrophil bactericidal/permeability increasing factor (BPI) could be significantly influenced by the composition of the growth medium. The BPI is a cationic protein with bactericidal activity against Gram-negative bacteria. Weiss *et al.*, (1986), suggested that the effect of BPI is directly related to its binding to the bacteria, and that the binding is dependent on the chain length of the O-antigenic polysaccharides of the LPS. Longer chain polysaccharides resulting from growth in a richer medium inhibited binding of BPI to the bacteria. It is difficult to reconcile these findings with those of Yamauchi *et al.*(1993). Rana *et al.*, (1991), in a study of the effect of the cationic peptides magainins on *Salmonella typhimurium*, concluded that in addition to the length of the polysaccharides side chains, the charge of the LPS is a factor in the interaction between magainins and LPS.

Whether this situation applies to lactoferricin is not known, but the above observations suggest that the effect of a richer medium may be more complex than simply providing inhibitors of the binding of lactoferricin to the LPS. It may be that the synthesis by the bacteria of longer chain polysaccharides in rich media provides another obstacle to lactoferricin binding. Experiments with isogenic rough mutants that have various lengths of O-polysaccharides side chains may be informative in this respect.

Regarding other future studies that may be undertaken with lactoferricin, it is doubtful that further research with the intention of using it as a general food additive will be productive until the properties of this peptide have been better defined, with respect to the nature of its binding to the bacteria and the nature of its bactericidal effect. It may be worthwhile to investigate a possible application in the areas of sanitation or treatment of surfaces of some food commodities provided that the limitations defined in the above experiments are kept in mind. It may be interesting to investigate applications where permissible levels of EDTA or other chelators would be higher than in foods. Properties of the peptide that may confer an advantage in the area of sanitation are its stability (resistance to heat or to further enzymatic degradation) and its potential for a residual effect. For example, recent experiments in this laboratory point to a bactericidal effect of the lactoferrin digest in water at room temperature at a concentration of about 3 μg/ml in terms of lactoferricin for at least 3 days (C. Chong, unpublished data).

The above discussion applies only to the effect of lactoferricin on *S. enteritidis* and Gram-negative bacteria. A bactericidal effect has also been demonstrated with Gram-positive bacteria (Bellamy *et al.*, 1992a) and particularly on *L. monocytogenes* (Wakabayashi *et al.*, 1992). No information has been published to-date on the effect, if any, of media components on the bactericidal activity of lactoferricin on Gram-positive bacteria.

IV. ANTIBODIES TO THE COLONIZATION FACTOR CFA 1 OF ENTEROTOXIGENIC *ESCHERICHIA COLI* IN BOVINE MILK AND COLOSTRUM

While antibodies to bacterial LPS have been found in the milk of non-vaccinated cows (Li-Chan *et al.*, 1994; Losso *et al.*, 1993; Al-Mashiki *et al.*, 1988), and while immunoglobulin preparations containing antibodies against bacterial LPS have been shown to protect human volunteers against challenge with enteropathogens (Tacket *et al.*, 1988; Tacket *et al.*, 1992), this does not demonstrate that antibodies to LPS are the best or only protection. In the experiment by Tacket *et al.* (1992), the immune concentrates were obtained by vaccinating cows with formalin killed bacteria. Antibodies to structures on the bacteria other than LPS could have been produced, but it is not easy to estimate them. There is evidence that with enterotoxigenic *E. coli*, antibodies against the colonization factors are the most effective. In the case of passive transfer of immunity against ETEC in cattle, it was found that protection was correlated to titers against the colonization factor K99, but not to titers against the O antigen (LPS) (Acres *et al.*, 1979). Similarly, in trials with human ETEC, it has been reported that vaccines against the CFA 1 colonization factor appeared to be more promising than vaccines against other bacterial products, such as LPS or heat labile toxin (Tacket, 1991).

With this in mind, it was thought useful to look for the presence of antibodies to the CFA 1 antigen of human ETEC in bovine colostrum. Some enteric pathogens are able to agglutinate various types of red blood cells (Evans *et al.*, 1979a). This ability correlates with the presence of structures (colonization factors) on the bacteria that are involved in adherence (Evans *et al.* 1979b). It is known that components of human milk (antibodies and oligosaccharides) inhibit agglutination of erythrocytes by the bacteria and also inhibit adherence of the bacteria (Holmgren *et al.*, 1981). Therefore, hemagglutination inhibition

was used to detect anti-CFA 1 antibodies in cows colostrum. Subsequently, a purified preparation of CFA 1 antigen became available and it was then possible to test for anti-CFA 1 antibodies by immunoassays. The results are presented below.

A. Materials and methods

Colostrum samples.

Colostrum samples were obtained from the University of British Columbia Dairy Farm or from Dr L.A. Babiuk, Veterinary Infectious Diseases Organization, Saskatoon, SK. Samples of bovine milk were obtained from Dr. L. Fisher, Agriculture and Agri-Food Canada Research Station, Agazziz, B.C. Human milk samples were obtained from a local hospital.

Wheys were prepared by acidification of the milk to pH 4.6 with 1N HCl in an ice bath. The preparations were then brought to 30°C, centrifuged for 20 min at 10,000 x g and the supernatant adjusted to pH 7 prior to use.

Samples of lyophilized milk immunoglobulin concentrate from cows immunized with a purified CFA 1 preparation, or with a heat killed whole cell preparation of CFA1$^+$ *E . coli* H10407, and from non-immunized cows were obtained from Dr. D. Maneval, Center for Vaccine Development, Baltimore, MD.

Bacteria.

Enterotoxigenic *E. coli* H10407 (a CFA 1 positive strain), was obtained from Dr. M. Levine, Center for Vaccine Development, Baltimore, MD. Enterotoxigenic *E. coli* 2412-91 (a K99 positive strain) was obtained from Dr. M. Schoonderwoerd, Alberta Agriculture, Edmonton, AB. ETEC 2412-91 were maintained at 4 °C on E medium Difco agar slants (Vogel and Bonner, 1956), while ETEC H10407 were maintained at 4 °C on CFA Difco agar slants (Evans *et al.*, 1979b). The bacteria were subcultured monthly. Growth on these media

promotes production of fimbriæ.

Hemagglutination inhibition.

Hemagglutination experiments were done in round bottom 96-well microtiter plates (Corning # 430245) according to the method of Korhonen et al. (1985), in the presence of 0.5% mannose. Briefly, the test proceeded in two steps: titration of the bacterial suspension, followed by the test of inhibition. For titration, serial doubling dilutions of the bacterial suspension in PBS-mannose (0.05 M phosphate buffered saline, pH 7.2, with 0.5% d-mannose) were made in duplicate in wells 1-11 of the microtiter plate. The volume in each well was 25 µl. The last wells in each row were used as negative control and received only PBS-mannose. Then 25 µl of PBS-mannose were added to each well and mixed. This step is required to account for the volume of inhibitor that was later used in the test of inhibition. Then 25 µl of a 2% suspension of erythrocytes in PBS-mannose were added and mixed. The sealed plate was then incubated at 4 °C for 2 hours. Agglutination gives an even covering of red cells, whereas lack of agglutination is shown by a small tight button of red cells at the bottom of the wells. The highest dilution showing agglutination is the titer of the bacterial suspension. This titer is required to get the right proportion of bacteria, red cells and inhibitor in the second step. To test for inhibition, 25 µl of serial doubling dilutions, in PBS-mannose of the colostrum or milk samples were made in wells 1 to 11 of the microtiter plate. The last well was used as positive control. Bacteria (25 µl) were then added at a concentration four times that determined in the first step. Erythrocytes were added and the assay carried out as in the first step.

Samples of human or cow milk whey were used to inhibit the agglutination of indicator red blood cells by human or bovine enterotoxigenic *E coli*. The highest dilution that inhibited agglutination provided a titer and allowed for comparison of inhibitory power of samples of different origins. Sheep red blood cells (obtained from the Animal Care

Centre, UBC) were used to determine the hemagglutination inhibition titer of wheys against the bovine pathogen *E. coli* 2412-91. Human type A blood cells (obtained from a local hospital) were used with the human pathogen *E. coli* H10407.

Gel filtration

A sample of human milk whey and a sample of cow colostrum whey were applied to a 10 cm x 1.5 cm Sephadex G100 column (Pharmacia Biotech, Uppsala, Sweden), eluted with 0.05 M phosphate buffer pH 7.4, to determine whether the inhibitory activity was associated with a large molecular weight compound such as Igs, or a low molecular weight compound, such as oligosaccharides. Exclusion volume and retained volume were determined with Dextran Blue 2000 and potassium dichromate, respectively, and 1 ml fractions were collected.

Determination of total and specific immunoglobulins levels

The following buffers were used in immunoassays: Carbonate coating buffer, pH 9.6 (1.59 g Na_2CO_3; 2.93 g $NaHCO_3$; 2 g NaN_3; 1 l H_2O); PBS, pH 7.4 (8.0 g NaCl; 0.2 g KH_2PO_4; 1.15 g Na_2HPO_4; 0.2 g KCl; 0.2 g NaN_3; 1 l H_2O) with 0.5 ml of Tween 20 added for PBS-Tween; blocking buffer (0.25% ovalbumin in PBS); 10% diethanolamine buffer pH 9.8 (97.0 ml diethanolamine; 0.1 g $MgCl_2.6H_2O$); 0.2 g NaN_3; H_2O to 1 l) (Kummer *et al.*, 1992). The ovalbumin was a gift from Canadian Lysozyme, Abbotsford, B.C.

Amounts of total antibodies in milk and colostrum samples, or in immune concentrate samples were estimated by enzyme linked immunosorbent assay (ELISA). Ninety-six well microtiter plates (Immulon 2, Dynatech Labs, Chantilly, Va) were first coated for one hour at 37 °C with 100 µl of rabbit anti-bovine Ig (Sigma B7265), at a concentration of 1 µg/ml, in carbonate buffer. The plates were then washed 3 times with

PBS, and 250 µl blocking buffer were added to each well. After 30 min of incubation at 37 °C, the plates were washed with PBS, and serial dilutions of the samples in PBS + 0.05% Tween-20 were added in duplicate and incubated for one hr at 37 °C. The plates were then washed with PBS-Tween, and 100 µl of anti-bovine Ig alkaline phosphatase conjugate (Sigma A7914, diluted 1:1000 in PBS-Tween) were added. After one hour of incubation at 37 °C, followed by washing with PBS-Tween and distilled water, 100 µl of substrate (p-nitrophenyl phosphate, Sigma 104) in diethanolamine buffer were added. Following color development, absorbances were read on a Bio Rad 450 microplate reader (BioRad, Richmond, CA) at 405 nm. A titration curve for each test sample was compared to a standard curve obtained from a reference milk sample, the concentration of which had been previously determined by radial immunodiffusion. The values from at least two dilutions in duplicate were used in the calculation.

Amounts of anti-CFA 1 antibodies in cows milk wheys and milk immune concentrates were estimated by enzyme linked immunosorbent assay (ELISA), using a purified CFA 1 preparation (obtained from Dr D. Maneval, Center for Vaccine Development, Baltimore, MD) as coating antigen at a concentration of 2.5 µg/ml. Otherwise, the assay was identical to the above. Since no standard for specific anti-CFA 1 Igs was available, the absorbance values were compared to a standard curve for total Igs, obtained as above, in the same microtiter plate.

B. Results

Experiments were conducted to determine the extent to which cows milk or colostrum whey and human milk whey inhibit agglutination of sheep red blood cells by enterotoxigenic *E. coli* 2412-91, and of human type A red blood cells by enterotoxigenic *E . coli* H10407.

Results of an initial experiment indicated that both a human milk whey sample and a

cow colostrum whey sample inhibited agglutination of red cells by human (H10407 CFA 1^+) and bovine (2412-91 $K99^+$) *E. coli* strains. The inhibition titers against H10407 were 128 for the human milk whey and 64 for the bovine colostrum whey, while the titers against 2412-91 were 64 for the human milk whey and 32 for the bovine colostrum whey. Dialysis of the wheys, using a membrane with a molecular weight cutoff of 6,000 to 8,000, did not result in a decrease in agglutination inhibition titers.

Fractionation of the wheys on a Sephadex G100 column showed that all the detectable agglutination inhibition activity was associated with the high molecular weight fractions. The immunoglobulins, as determined by ELISA, eluted in the exclusion volume of the column. No activity was detected in the late fractions (Figure 9).

Further experiments were conducted to determine whether this activity could be found in samples of different origins. Serial dilutions of several samples of human or cows milk whey were tested for inhibition of agglutination of red blood cells by human or bovine pathogenic strains of *E. coli.*

Nine samples of cows milk or colostrum whey, as well as four samples of human whey, were tested. The titers of the preparations are shown in Table VI.

The human whey samples as well as the bovine whey samples inhibited red blood cell agglutination by both the human and bovine strains of *E. coli*; the titers of the bovine colostrum samples were comparable to the titers of the human whey samples, while the titers of the bovine milk whey samples were lower.

At that time, a sample of purified CFA 1 antigen was donated by Dr. D. Maneval, as well as samples of milk immune concentrates from cows immunized with CFA 1 or whole cell heat killed *E. coli* H10407. It then became possible to estimate the specific antibody activities of the bovine whey samples against the human CFA 1 antigen by using an immunoassay technique (ELISA). The specific activities of lyophilized samples of milk immune concentrates from cows immunized with this antigen or with a whole cell *E. coli*

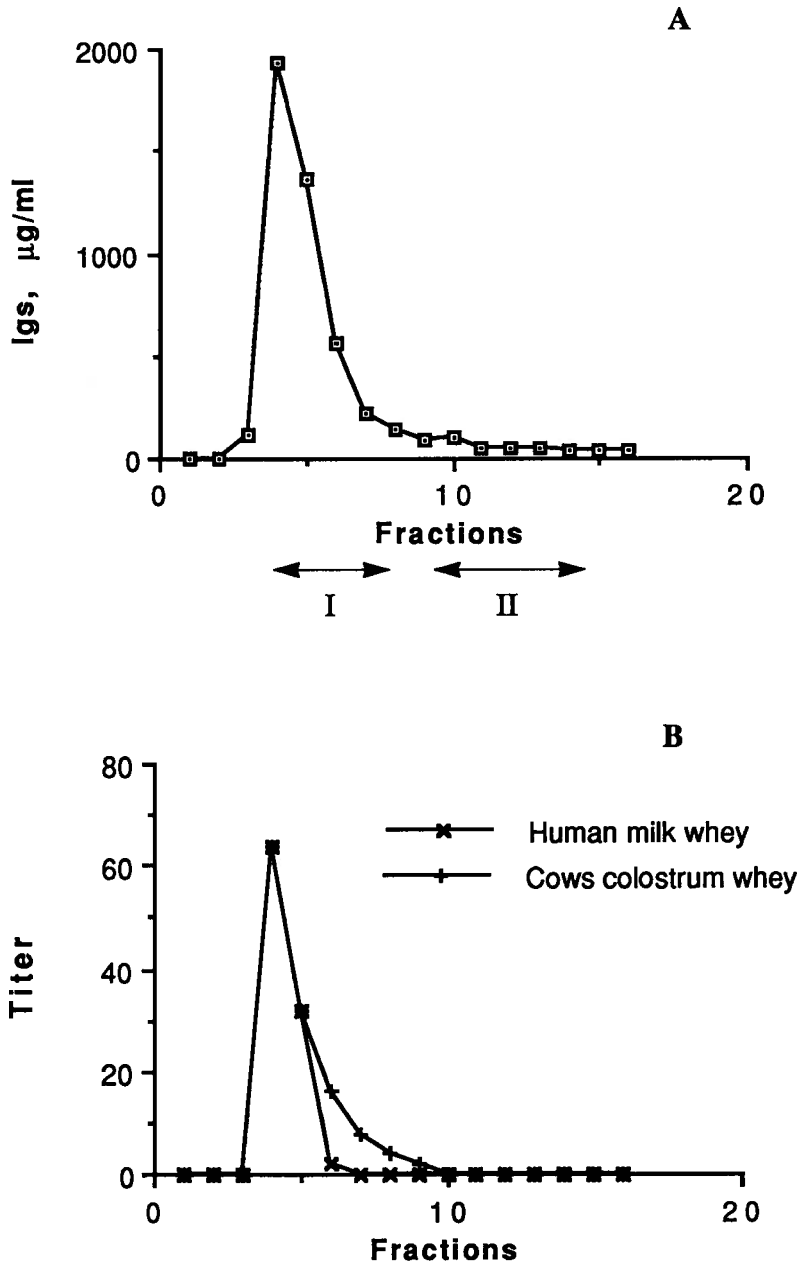


Figure 9. A. Immunoglobulin G concentration of fractions of bovine colostrum whey obtained by chromatography on a G100 Sephadex column. B. Agglutination inhibition titers against *E. coli* H10407 of fractions of bovine colostrum whey and human milk whey obtained by chromatography on the same G100 Sephadex column.
Exclusion volume (I) and retained volume (II) are shown.

Table VI. Agglutination inhibition titers of various whey preparations against bovine (2412-91) and human (H10407) *E. coli* strains.

Samples	2412-91 titers	H10407 titers
J...[a]	256	128
H...[a]	1024	512
W...[a]	1024	512
Z...[a]	64	128
579-5[b]	256	256
731-7[b]	128	128
8522[c]	64	32
8917[c]	64	128
8617[c]	64	64
8831[c]	128	128
8240[c]	8	64
8550[c]	64	128
UBC Col[d]	128	128

a: Human milk samples
b: Colostrums obtained from Dr. L. Babiuk, Veterinary Infectious Diseases Organization, Saskatoon, SK.
c: Cows milk samples obtained from Dr. L. Fisher, Agazziz Research Station, Agriculture and Agri-Food Canada, Agazziz, B.C.
d: Colostrum from the UBC dairy herd.

preparation were also estimated. Since no preparation of purified, specific and standardized bovine antibodies to the CFA 1 antigen was available to us, it was necessary to compare absorbances obtained from the titration of anti-CFA 1 antibodies to a standard curve obtained from the titration of total IgGs of a milk sample whose concentration had been determined by immunodiffusion.

The amounts of specific antibody to the colonization factor antigen 1 (CFA 1), in samples of colostrum from cows that had not been vaccinated, ranged from 0.55 to 5.2 μg/ml (Table VII). The amount in milk was lower and barely detectable. Total immunoglobulins levels were also estimated and a ratio of specific anti-CFA 1 antibodies to total immunoglobulins was calculated. Similar immunoassays of the reconstituted milk immune concentrates showed that vaccination increased the ratio of specific antibodies to total Igs in milk by factors of about 10 to 25 (Table VIII). For comparison, the amount of anti-CFA 1 antibodies in human serum (from asymptomatic individuals), was reported to average about 6 μg/ml (Clegg *et al.*, 1980). This amount increased on average by a factor of 40 in the serum of patients infected with a CFA 1 positive enterotoxigenic *E. coli*, but very large differences in responses were found between patients, with some individuals having no detectable specific anti-CFA 1 antibodies, even after infection (Clegg *et al.*, 1980).

Table VII. Estimates of total IgG concentration and of specific anti-CFA 1 IgG concentration in various samples of cows colostrum and milk wheys.

Samples	Total IgGs mg/ml	Anti-CFA IgGs µg/ml	% anti-CFA 1 IgGs
112-7[a]	>80	1.0	>0.001
579-5[a]	79	5.2	0.007
95-8[a]	60	1.4	0.002
8-1[a]	43	0.8	0.002
731-7[a]	40	1.0	0.003
83-8[a]	33	1.1	0.004
345-8[a]	14	0.6	0.004
UBC Col[b]	18	1.3	0.007
8738[c]	0.86	0.1	0.012

[a] : Colostrums obtained from Dr L. Babiuk, Veterinary Infectious Diseases Organization, Saskatoon, SK.

[b] : Colostrum from the UBC dairy herd.

[c] : Milk obtained from Dr L. Fisher, Agazziz Research Station, Agriculture and Agri-Food Canada, Agazziz, B.C.

Table VIII. Estimates of total IgG concentration and of specific anti-CFA 1 IgG concentration in samples of bovine milk immune concentrates.

Samples	Total Igs mg/ml	Anti-CFA 1 Igs µg/ml	% anti-CFA 1 Igs
WC MIC[a]	30	25.0	0.083
CFA MIC[b]	31	12.0	0.039
Control MIC[c]	31	0.93	0.003

[a] : A sample of milk immune concentrate, lyophilized and reconstituted to 1 ml in PBS, from cows vaccinated with a whole cell preparation of *E. coli* H10407.

[b] : A sample of milk immune concentrate, lyophilized and reconstituted to 1 ml, from cows vaccinated with the CFA-1 antigen.

[c] : A sample of milk immune concentrate, lyophilized and reconstituted to 1 ml, from non vaccinated cows.

The above samples were a generous gift from Dr. D. Maneval, Center for Vaccine Development, Baltimore, MD.

C. Discussion

The purpose of the experiments reported above was to detect and estimate specific antibodies to the colonization factor CFA 1 of human ETEC in bovine milk or colostrum.

Anti-CFA 1 antibodies were detected in bovine milk and colostrum wheys, and in human milk wheys, by inhibition of hemagglutination. Anti-K99 antibodies were also detected. Testing for anti-K99 antibodies provided a check of the method and of the hypothesis: if antibodies to CFA 1 were to be found in cows milk or colostrum, conversely it would be reasonable to look for antibodies to K99 in human milk. No low molecular weight factors were found that inhibited hemagglutination.

Determination of amounts of total IgGs and particularly of specific Igs in milk by immunoassays can be a rather difficult task. For determination of total IgGs, the dilutions required are sufficiently high that background levels due to non-specific binding are not much of a problem. This is not the case for the determination of specific antibodies and at times high background may make this work impossible. Other difficulties arise from the heterogeneous nature of the immunoglobulins to be estimated. This heterogeneity may cause problems with the use of the standard curve method of data analysis, where the titration curve of the test sample is compared with that of the reference sample (Peterman and Butler, 1989). There may be some difficulty in finding a suitable standard, as there is some evidence that purified IgGs do not necessarily behave in the ELISA in the same way as IgGs in milk; one solution to this problem has been to use a sample of milk whose IgG concentration has been determined by another method (Kummer *et al.*, 1992). This was the method adopted here. Obviously this relies on the accuracy of the original measurement.

The problem is more difficult in the case of determination of specific antibody concentration. Generally, a sample of specific antibody of known concentration, from which to construct a standard curve, is not available. Various methods of expressing the results have been used. For instance, the optical density at a given dilution can be stated (Li-Chan *et al.*,

1995). The results can be expressed as titers, the inverse of the dilution giving a minimum arbitrary optical density. Results have been expressed as relative fluorescence at a given dilution in Particle Concentration Fluorescence Immunoassays (PCFIA) assays (Losso *et al.*, 1993). Results have been expressed as relative values of a reference sample; in one example a range of concentration of specific Igs for the reference sample was estimated to be 7.5 to 60 ng/ml by determining dilutions that would give comparable absorbance values in the specific ELISA and in the total ELISA (Li-Chan *et al.*, 1994). A similar method has been used in this presentation except that rather than expressing the results as a percentage of the reference sample that would give the same aborbance as the test sample, the calculation of concentration has been done for every sample tested rather than just for the reference sample and the results expressed in μg/ml rather than as a percentage. This is not ideal but does provide an easier way to visualize a range of values for comparison purposes. Expressed in this manner, it was found that the ratio of specific anti-CFA 1 antibodies to total IgGs in colostrum of non-vaccinated cows was at least 1:15000. Li-Chan *et al.* (1994) found ratios around 1:5000 for specific anti-LPS antibodies in cows milk.

It may be surprising to find antibodies to a human pathogen in cows milk, or antibodies to a bovine pathogen in human milk, but recently published work suggests a possible explanation: it has been found that colonization factor antigens of human ETEC can prime and boost immune response against heterologous, serologically distinct (by immunoassay) colonization factor antigens (Rudin and Svennerholm, 1994). Even though the CFAs are distinct antigenically, some homology has been demonstrated at the level of amino acid sequence. A similar situation has been found with rotavirus (Taniguchi *et al*, 1991). Yolken *et al.* (1985) also found antibodies to human rotavirus in cows' milk.

It appears, from this and from other work cited above, that antibodies to human pathogens are commonly found at low levels in cows milk. A survey of milk samples from various areas of the Province of British Columbia was conducted to determine the total

immunoglobulin G content and the levels of specific antibodies to the LPS of *E. coli* O111:B4, O128:B12, *Shigella flexnerii* 1A, *Salmonella enteritidis* and *S. typhimurium* (Li-Chan *et al.*, 1994). Specific antibodies against the same bacterial LPS were also estimated in samples of human milk (Losso *et al.*, 1993). It could be argued from these results that antibodies will be found in milk or colostrum against any bacterial antigen that one tests for.

Fukumoto (1992) discussed in detail the proposition that immunoglobulins could be separated from whey obtained from the milk of non-immunized cows: even though the IgG concentration is very low, the volumes are so high that considerable amounts could be obtained. Of course specific antibodies need to be present, and the studies cited in the previous paragraph, as well as the results presented in this chapter, show this to be the case. This proposal has had some experimental support. Stott and Lucas (1989) described a process to concentrate whey obtained from the milk of non-immunized cows, and presented data showing that feeding approximately 1 g/kg bodyweight to colostrum deprived calves immediately after birth resulted in performance equal to or better than that of calves receiving colostrum.

While the findings of specific antibodies to human pathogens in bovine milk are useful and interesting, the limitations of this approach are also becoming evident. Certainly, antibodies to the above bacterial LPS and CFA 1 were found first because the corresponding antigens were available for immunoassays, not necessarily because these antigens were the most significant; specific antibodies cannot be found by this method unless the corresponding antigen is known and available, or a workable immunoassay exists. For example, attempts at detecting antibodies to the enterotoxin (LT) of *E. coli* in bovine colostrum were unsuccessful because while a system is available to capture the antibodies, using the GM1 ganglioside and cholera toxin (CT), problems of high background, and cross-reactions with the commercially available antisera made it impossible to develop a working immunoassay.

Immunoassays are useful for the detection and estimation of levels of antibodies, but they provide no information on their biological activity or on the relative efficacies of specific antibodies. For example, Apter *et al.* (1993) showed by challenge of neonatal mice that anti-LPS antibodies are much more effective at preventing *V. cholerae* induced diarrhea than anti-cholera toxin antibodies. On the other hand, a study of breast-fed Mexican infants found that the milk concentration of sIgA against *Shigella* virulence plasmid-associated antigen was a better predictor of symptom status than the milk concentration of anti-*Shigella* antibodies (Hayani *et al.*, 1992). Other factors with antibacterial activity that may be present in milk or colostrum may not lend themselves to detection by this method.

The next chapter will report on the use of a cell culture method to estimate the antibacterial activity of bovine colostrum.

V. EFFECT OF BOVINE COLOSTRUM ON THE INVASIVENESS OF *SALMONELLA ENTERITIDIS, S. TYPHIMURIUM* AND *ESCHERICHIA COLI* IN HeLa CELLS.

Model systems are required to study the biological activity of antibacterial factors that may be isolated from milk or colostrum. In the case of antibodies to enteropathogens, studies of bactericidal activity *in vitro* are not likely to be informative given indications that protection by milk antibodies in the intestine may not be the result of agglutination or reduced viability of the bacteria, but is rather the result of inhibition of adherence of the bacteria to the intestinal epithelium. Some of the problems associated with immunoassays have been presented in the previous chapter. Animal models may be available, but suffer from many drawbacks, such as cost, reproducibility or low productivity. The alternative was therefore to use cell cultures.

The purpose of the experiments presented below was to determine whether bovine colostrum could inhibit invasion of HeLa cells by invasive enteropathogens. Results of experiments with *Salmonella enteritidis, S. typhimurium* and enteropathogenic *Escherichia coli* will be presented.

While cell culture systems have been used to test for anti-adherent activity of human milk or human milk components against *E. coli* strains, to the author's knowledge no such studies have been reported with bovine milk or colostrum. In addition no reports of anti-invasive activity of human or bovine milk have been found in the literature.

Antibodies to the colonization factor antigen 1 of ETEC have been found in cows colostrum (previous chapter), and it would have been logical to test these samples of colostrum for anti-adherent activity in a cell culture system. However, it was deemed preferable to postpone this type of experiment in favor of anti-invasion assays for the following reasons: 1) ETEC adherence has been demonstrated only in Caco-2 cells that are

fully differentiated, requiring lengthy cultures and slower turnover of experiments. 2) From a methodological point of view, the assessment of adherence or lack of it appeared to be subject to uncertainties that are not present in assessment of invasion. 3) A wider range of cell lines are available to test for invasion with a greater range of bacterial strains. While we have no interest in the fate of the bacteria following invasion, the method simply appeared better suited to initial experiments of this kind with bovine colostrum.

HeLa cells were therefore chosen to test the anti-invasive ability of bovine colostrum; colostrum rather than milk was chosen to make the study more efficient: any antibacterial factor in milk would be expected to be present in colostrum at a much higher concentration.

A. Materials and methods

Colostrum samples.

Colostrum samples from individual cows were obtained from the University of British Columbia Dairy Farm (UBC Col) or from Dr L.A. Babiuk, Veterinary Infectious Diseases Organization, Saskatoon, SK (Samples # 112-7, 83-8, 8-1, 95-8, 579-5, 731-7).

Cottage cheeese whey was obtained from Dairyworld Foods, Burnaby, B.C.

A whey protein concentrate (WPC) in powder form was obtained from Stolle Biologicals, Cincinnati, OH. The milk from which this concentrate was obtained had been produced by cows vaccinated against a variety of pathogens (Stolle and Beck, 1987).

Bacteria.

Salmonella enteritidis ATCC 13076 was obtained from the American Type Culture Collection, Rockville, MD. *S. enteritidis* CD5, *S. typhimurium* SL1344 and enteropathogenic *Escherichia coli* E2348/69 were obtained from Dr. B. Finlay, Biotechnology Laboratory, UBC. The bacteria were maintained at 4 °C on tryptic soy agar and subcultured monthly.

Cell cultures

HeLa cells were obtained from Dr. B. Finlay, Biotechnology Laboratory, UBC and used between passages 12 and 65. The cells were maintained in Minimum Essential Medium with Earle's salts, L-glutamine and non-essential amino-acids (Gibco 410-1500EB), supplemented with sodium bicarbonate (2.2 g/l), penicillin G (100 U/ml), streptomycin (100 µg/ml) (Gibco 600-5140AG) and 10% fetal calf serum (Gibco 200-6140AJ). The cells were passaged as needed by trypsinization with trypsin-EDTA (Gibco 610-5300AJ), seeded at a 1 to 5 dilution in 75 cm^2 flasks (Falcon 3023) and grown in a 5% CO_2 environment at 37 C°.

In all experiments, except immunoassays, Dulbecco's PBS (pH 7.4) was used (0.1 g $CaCl_2$; 0.2 g KCl; 0.2 g KH_2PO_4; 0.1 g $MgCl_2.6H_2O$; 2.16 g $Na_2HPO_4.7H_2O$; 8.0 g NaCl; to 1.0 l H_2O).

Invasion assays.

Invasion assays were done according to Betts and Finlay (1992). HeLa cells were harvested by trypsinization and the cell density adjusted to $1x10^5$/ml. One ml of cell suspension was added to each well of a 24 well microplate and incubated overnight as above. The monolayers were then washed twice with penicillin and streptomycin free medium, and 300 µl of this medium were added to each well, followed by 30 µl of colostrum or other test sample, and finally 10 µl of an overnight standing culture of bacteria in trypticase soy broth (TSB) (*Salmonella*) or LB broth (*E. coli*). After one hour of incubation as above, the cells exposed to *Salmonella* were washed twice with PBS, and 500 µl of culture medium containing 100 µg/ml of gentamicin (Sigma G1272) were added. The condition of the cell monolayer was checked by light microscopy at every step of the experiment. After one hour of incubation at 37 °C in a 5% CO_2 incubator the cells were washed twice with PBS, and 100 µl of 1% Triton X100 in PBS were added. After 5 min of incubation, 400 µl of TSB were added; following mixing, 10 µl of serial dilutions were drop plated in duplicate on plate

count agar which was then incubated overnight at 37 °C.

In the case of the cells exposed to *E. coli*, the wells were washed twice with PBS one hour after addition of colostrum and bacteria, and medium and test colostrum replenished for a further period of incubation. After two hours, the cells were treated with gentamicin as above. In any given experiment, each control or colostrum sample was tested in quadruplicate, unless otherwise noted.

Immunofluorescence.

Hela cells were seeded in a 24-well microplate as described above, on microscope cover slips. After overnight incubation, the cells were washed as above and medium without antibiotics was added. Following addition of 10 µl of an overnight culture of bacteria (SL1344 or E2348/69) the plates were incubated for one hour at 37°C in 5% CO2. The cells were then washed twice with PBS and exposed to 2% paraformaldehyde in PBS for 30 min. Following washing, the cells were permeabilized with 0.1% (v/v) Triton X100 for 5 min. During subsequent steps, the cells were exposed for 60 min at room temperature first to the test colostrum samples, then to a fluorescein isothiocyanate (FITC) anti-bovine IgG affinity purified antibody (Jackson Immunoresearch 301-095-003). The cover slips were then mounted on slides on a drop of mounting fluid, sealed with nail polish and examined with an epifluorescent Zeiss Axioskop microscope. Photographs were taken with Kodak T-Max 400 film.

Immunoassays.

The following buffers were used in immunoassays: Carbonate coating buffer, pH 9.6 (1.59 g Na_2CO_3; 2.93 g $NaHCO_3$; 2 g NaN_3; 1 l H_2O); PBS, pH 7.4 (8.0 g NaCl; 0.2 g KH_2PO_4; 1.15 g Na_2HPO_4; 0.2 g KCl; 0.2 g NaN_3; 1 l H_2O) with 0.5 ml of Tween 20 added for PBS-Tween; blocking buffer (0.25% ovalbumin in PBS); 10% diethanolamine

buffer pH 9.8 (97.0 ml diethanolamine; 0.1 g $MgCl_2.6H_2O$); 0.2 g NaN_3; H_2O to 1 l) (Kummer *et al.*, 1992). Ovalbumin was a gift from Canadian Lysozyme, Abbotsford, B.C.

a) Determination of bovine IgGs:

An enzyme linked immunoassay (ELISA) to estimate the relative concentration of bovine Igs in milk or colostrum or colostrum fractions was conducted in 96 well microtiter plates (Immulon II, Dynatech Labs, Chantilly, Va.). The plates were first coated with an affinity purified rabbit antibody to bovine IgGs (Sigma B7265) in 100 µl of carbonate buffer. Following one hr incubation at 37 °C, the plates were washed with PBS and 250 µl of blocking solution were added. After 30 min of incubation the plates were washed with PBS-Tween, and 100 µl of serial dilutions of colostrum or other test samples in PBS-Tween were added. Following one hr incubation, the plates were washed and 100 µl of a rabbit anti-bovine IgG alkaline phosphatase conjugate (Sigma A7914, 1:1000 dilution) was added to each well, and the plates incubated for another hour. The plates were then washed with PBS and distilled water, and 100 µl of substrate (p-nitrophenyl phosphate, Sigma 104) in diethanolamine buffer were added. Following color development, the plates were read in a BioRad 450 microplate reader at 405 nm.

b) Determination of $F(ab')_2$ fragments:

An ELISA to estimate bovine $F(ab')_2$ was carried out as above using a rabbit antibody to $F(ab')_2$ as coating (Jackson Immunoresearch 301-005-006), and the corresponding conjugate (Jackson Immunoresearch 301-055-006).

The assays were done in duplicate. A reference sample in any one assay was used to establish a standard curve. The absorbance values of at least two dilutions in duplicate for each sample were used to determine the IgG concentration relative to the reference sample.

SDS-PAGE electrophoresis.

To each sample were added 20 µl of 10% SDS, 2 µl of mercaptoethanol and 5 µl of 0.05% bromophenol blue, to a total volume of 100 µl in 10 mM Tris/HCl, 1 mM EDTA. The samples were then heated for 5 min in a boiling water bath. Electrophoresis of fully reduced samples was performed on a PhastSystem (Pharmacia LKB Biotechnology AB, Uppsala, Sweden), with PhastGel gradient 10-15 acrylamide gels. The gels were run at 250 V, 10 mA, 15 °C for 63 volt hours. The gels were stained with Coomassie blue (0.1% PhastGel Blue R solution in 30% methanol and 10% acetic acid). The gels were destained with 30% methanol and 10% acetic acid, and preserved in 10% acetic acid and 5% glycerol.

Immunoblotting.

Colostrum fractions were analysed by SDS-PAGE in 12% gels on a Mini PROTEAN II Electrophoresis System (BioRad 165-2940) at 100 volts according to the method of Læmmli (1970) and the bands transferred to nitrocellulose for 1 hr at 100 volts with a Mini Trans-Blot Electophoretic Transfer Cell (BioRad 170-3930) according to the method of Towbin *et al.* (1979). Blots were blocked for 1 hr with 0.25% ovalbumin in PBS and exposed using rabbit alkaline phosphatase antibody conjugates to bovine IgG and F(ab')2 fragments (1:1000 in PBS-ovalbumin). The blots were developed with a 5-bromo-4-chloro-indolyl phosphate (0.2 mg/ml) and nitroblue tetrazolium (0.4 mg/ml) (BCIP/NBT) substrate (McGadey, 1970) in diethanolamine buffer..

Affinity chromatography.

Colostrum (250 µl) was applied to a 1.5 ml column of immobilized protein G (Pierce 20398) in 0.2 M acetate buffer pH 5.0. Following washing of the unbound fractions, the bound material was eluted with 0.05 M glycine buffer, pH 2.8. Fractions were monitored by UV spectrophotometry at 280 nm and by ELISA.

Following chromatography, Centricon concentrators (Amicon Inc. Beverly, MA, USA) were used, according to manufacturer's directions, in a refrigerated centrifuge, to concentrate the fractions and to exchange buffers prior to the invasion assay. In some experiments, fractions were separated sequentially according to size on Centricon 100, 30 and 10, with respective cut-off sizes of 100, 30, and 10 kDa. The respective retentates or filtrates wereused in the invasion assay.

Enzyme digestion.

Colostrum or colostrum fractions were digested with pepsin (Sigma P7012) (1:25 w:w) at pH 2.8. Following digestion for 4 hrs at 37°C, the pH was raised to neutrality with 1 N NaOH and the samples dialysed overnight in PBS at 4 °C with a Spectrapor membrane (Spectrum Medical Industries, Los Angeles, CA) with a cut-off of 6,000-8,000, or fractionated on Centricon C30. Colostrum or colostrum fractions dialysed in PBS were digested with papain (Sigma P4762) (1:50 w:w) in the presence of mercaptoethanol (0.015M) and EDTA (0.01M). Following digestion for 4 hours at 37°C, iodoacetamide was added to a final concentration of 0.03M. The samples were dialysed in PBS as above prior to use.

B. Results

Inhibitory effect of colostrum

The inhibitory capacity of various bovine colostrum samples was determined by challenging monolayers of HeLa cells with invasive bacteria in the presence of colostrum. Approximately 1 x 10^7 to 2 x 10^7 CFUs were added to each well. A normal level of invasion was determined by control wells to which no colostrum was added. The extracellular bacteria were eliminated by treatment with the antibiotic gentamicin. Following lysis of the monolayer, the intracellular bacteria were diluted as needed and plated for determination of colony forming units. A number of initial experiments were done with *S. enteritidis* ATCC

13076. Even though colostrum was found to effectively reduce the levels of invasion compared to control, it was observed that the invasiveness of ATCC 13076 varied greatly from day to day. One experiment with *S. enteritidis* CD5 gave a very low level of invasion. A number of experiments with *S. typhimurium* SL1344 gave consistently acceptable levels of invasion, in the order of 5×10^4 to 2×10^5 colony forming units per well, and therefore most subsequent experiments with *Salmonella* were done with SL1344. Results will be presented as percent invasion in test wells compared to invasion in control wells.

Five experiments to test different colostrum samples were done with *S. enteritidis* ATCC 13076, one with *S. enteritidis* CD5, and four with *S. typhimurium* SL1344. Inhibition ranged from 97.7% to 87.3%. The results of typical challenges with the three strains of *Salmonella* are presented in Table IX.

The results of two challenge experiments to determine the inhibitory ability of colostrum samples against *E. coli* E2348/69, performed on two different days, are presented in Table X. Inhibition ranged from 99.6% to 73%. All samples of colostrum tested inhibited invasion of the HeLa cells by the bacteria. One sample (UBC col) which was a good inhibitor of invasion by *Salmonella*, was a weaker inhibitor of *E. coli* E2348/69 than the other samples. This may be a reflection of the origin of this particular sample, or of the time post partum at which the various samples of colostrum were obtained. Visual examination of the samples would lead to the conclusion that the samples originating from Saskatoon were obtained from the first milking after calving, while the sample originating from UBC appeared to have been obtained from a later milking. The concentration of colostrum rapidly declines over the first 24 hours of lactation.

To determine whether the presence of colostrum had an effect on HeLa cells that could make them less susceptible to invasion, or whether the inhibitory factors from colostrum could bind to the mammalian cells, HeLa monolayers were exposed to colostrum for 30 min, then washed with medium three times prior to challenge with the bacteria. The

Table IX. Effect of bovine colostrum on the invasion of HeLa cell monolayers by *Salmonella*.

Samples	*S. enteritidis* ATCC 13076	*S. enteritidis* CD 5	*S. typhimurium* SL1344
Control	100.0[a] (48.9)	100.0 (35.9)	100.0 (24.4)
UBC Col.	6.9 (2.3)	2.3 (1.6)	12.7 (11.2)
112-7		3.5 (1.9)	5.0 (2.0)
83-8	4.2 (2.2)		
8-1	9.5 (1.6)		
95-8	6.1 (3.3)		

[a]: Percent of internalyzed bacteria relative to control, averages of 4 wells, standard deviations in parentheses.

Table X. Effect of bovine colostrum on the invasion of HeLa cells monolayers by *E. coli* E2348/69.

Samples	*Expt 1*		*Expt 2*	
Control	100.0[a]	(12.2)	100.0	(38.3)
UBC Col.			26.8	(9.5)
345-8			1.2	(0.8)
8-1			1.5	(0.5)
112-7	0.8	(0.4)		
579-5	0.4	(0.3)		
731-7	2.1	(0.8)		

[a]: Percent of internalyzed bacteria relative to control, averages of 4 wells, standard deviations in parentheses.

Experiments 1 and 2 were conducted on different days.

results of such a test are presented in Table XI. An effect of bovine colostrum on the HeLa cells could not be detected under such conditions, therefore the inhibiting agents of colostrum do not appear to bind strongly to the mammalian cells or to have an effect on their ability to be invaded by the bacteria.

To establish whether the reduced invasiveness was a reflection of reduced viability or agglutination of the bacteria, aliquots of medium were taken from wells with and without colostrum after one hour of incubation, and bacterial counts determined by serial dilution and drop plating. Counts of internalized bacteria were also obtained. As a further control, some wells were treated with a rabbit antiserum specific for the LPS of SL1344 (Difco 2948-47-6). The results (Table XII) show that addition of colostrum did not result in a decrease in the number of colony forming units recovered from the medium even though it caused a significant reduction in invasiveness, whereas the rabbit antiserum, which was very effective at preventing invasion, caused a reduction in the number of colony forming units in the medium. All samples tested were subsequently checked for their effect on the viability of SL 1344, and no instances of reduced viability were detected. However, this does not preclude the involvement of immunoglobulins in this phenomenon. Immunoglobulins in colostrum could be inhibiting adherence to the mammalian cells without having a bactericidal effect or agglutinating the bacteria.

Are low molecular weight fractions responsible for the inhibition of invasion?

Both immunoglobulins (sIgAs) and oligosaccharides in human colostrum and breast milk have been reported to inhibit adhesion of some EPEC strains to HEp-2 cells (Cravioto *et al.*, 1991), while in a similar system using HeLa cells, only an effect of sIgAs could be demonstrated (Camara *et al.*, 1994; Silva and Giampaglia, 1992). In a study of inhibition of attachment by human milk of *Streptococcus pneumoniæ* and *Hæmophilus influenzæ*, the inhibitory activity was attributed to a non-immunoglobulin high molecular weight fraction,

Table XI. Invasion of HeLa cells monolayers by *S. enteritidis* ATCC 13076 or *E. coli* E2348/69 following preincubation of the cells with bovine colostrum[a].

Samples	*S. enteritidis* ATCC 13076	*E. coli* E2348/69
Control	100.0[b] (48.9)	100.0 (28.1)
UBC Col.	141.2 (51.9)	
112-7		86.1 (11.7)

[a]: HeLa cell monolayers were exposed to colostrum for 30 min, then washed 3x with medium prior to challenge with the bacteria.

[b]: Percent of internalyzed bacteria relative to control, averages of 4 wells, standard deviations in parentheses.

Table XII. Effect of colostrum on the viability of *S. typhimurium* SL1344.

Sample	*Internal CFUs*[a]	*S*	*External CFUs*	*S*
Control	6.8×10^4	2.3×10^4	1.32×10^7	0.32×10^7
Difco[b] 1/10	<25		0.27×10^7	0.14×10^7
UBC Colostrum	1.2×10^3	2.9×10^2	1.16×10^7	0.28×10^7

[a]: colony forming units per well. Averages and standard deviations (S) of quadruplicate determinations.

[b]: a specific rabbit antiserum.

and the hypothesis was put forward that oligosaccharides on glycoproteins could be the factors involved in the inhibition (Andersson *et al.*, 1986). In the same study, inhibition of attachment of *S. pneumoniæ* was also attributed to a low molecular weight fraction, tentatively identified as a glycolipid. Holmgren *et al.* (1981) and Ashkenazi and Mirelman (1987) similarly observed that a non-immunoglobulin fraction of human milk inhibited adherence of enterotoxigenic *E. coli.*

To test whether a low molecular weight fraction of bovine colostrum was responsible for inhibition of invasion, samples of colostrum were separated into fractions of various molecular weight either by dialysis at 4°C in PBS with a Spectrapor membrane with a cut-off of 6000-8000, or by sequential filtration through Centricon filters. The respective fractions were then tested by the gentamicin resistance assay.

Table XIII shows that dialysis did not reduce the inhibitory activity of the samples tested. To confirm this finding, fractions of high or low molecular weight were obtained by filtration with Centricon separators. Centricon filters separate fractions on the basis of size by centrifugation so that the filtrate is collected and changes in volume are minimized. This process yielded a retentate (C30R) of fractions above 30 kDa and a filtrate after filtration with Centricon C30. This filtrate was again run through Centricon C10 yielding a retentate (C10R) of fractions between 10 kDa and 30 kDa, and a filtrate (C10F) of fractions below 10 kDa. Volumes of retentate were adjusted to account for the increase in concentration of the retained colostrum components. The respective fractions were tested for inhibition of invasion by SL1344 and E2348/69. All the inhibitory activity was found in the high molecular weight fractions (Table XIV).

To determine whether a low molecular weight fraction with inhibitory activity could be generated from colostrum by enzymatic digestion, colostrum was digested with pepsin. A parallel control was run with no pepsin added. Both samples were fractionated with Centricon C30. The retentates and filtrates were tested for inhibitory activity against *S* .

Table XIII. Inhibition of invasion of HeLa cells monolayers by bovine colostrum or dialysed colostrum.

Samples	*S. typhimurium* SL1344	*E. coli* E2348/69
Control	100.0[a] (24.4)	100 (12.2)
UBC Col.	8.8 (2.0)	
UBC Col dialysed	3.2 (2.4)	
112-7		0.8 (0.4)
112-7 dialysed		1.6 (0.7)

[a]: percent of internalyzed bacteria relative to control, averages of 4 wells, standard deviations in parentheses.

Table XIV. Inhibition of invasion of HeLa cells monolayers by *S. typhimurium* SL1344 or *E. coli* E2348/69 by bovine colostrum or colostrum fractions separated on the basis of size.

Samples	*S. typhimurium* SL1344		*E. coli* E2348/69	
Control	100.0[a]	(21.4)	100.0[a]	(31.0)
UBC Col	2.4	(1.1)		
UBC Col C30R[b]	1.4	(0.4)		
UBC Col C10R[c]	142.4	(18.7)		
UBC Col C10F[d]	150.6	(34.1)		
8-1			9.0	(3.4)
8-1 C30R[b]			12.6	(3.5)
8-1 C10R[c]			162.7	(82.7)
8-1 C10F[d]			125.4	(47.5)

[a]: percent of internalyzed bacteria relative to control, averages of 4 wells, standard deviations in parentheses.

[b]: retentate after filtration with Centricon 30.

[c]: retentate after filtration of the Centricon 30 filtrate with Centricon 10.

[d]: filtrate after filtration with Centricon 10.

typhimurium SL1344. Following pepsin digestion, the inhibitory activity of the high molecular weight fraction was reduced compared to the original sample and to a control that was treated in the same manner but without pepsin. No anti-invasion activity appeared in the C30 filtrate (Table XV).

To determine whether the inhibitory activity was sensitive to heat, colostrum was heated at 80°C for 30 min. Heating resulted in extensive coagulation of the sample, but the soluble fraction following heat treatment was extracted and tested for inhibitory activity (Table XVI). Heat treatment reduced the activity of colostrum.

Inhibitory effect of immunoglobulin and non-immunoglobulin fractions

Chromatography on Protein A or protein G agarose is a convenient method to purify immunoglobulins. Protein A and protein G are bacterial proteins, obtained from some strains of *Staphylococcus* and *Streptococcus*, which have the ability to bind specifically to IgGs of various animal species. Protein G binds more strongly to bovine IgGs than protein A and therefore was used to obtain purified bovine colostrum IgGs (Björck and Kronvall, 1984).

Samples of colostrum (UBC Col) were applied to a protein G agarose column. Unbound fractions of colostrum were eluted at pH 5.0 while IgGs were eluted by lowering the pH to 2.8. Fractions were monitored by spectrophotometry at 280 nm. Levels of immunoglobulin G in the respective peaks were determined by ELISA in order to establish the volume of sample that could be applied without exceeding the column capacity for binding immunoglobulins, in order to achieve a good separation of the IgGs. A typical elution pattern is presented. Immunoglobulins were retained on the column and appeared in the second peak (Figure 10). A relatively low level of immunoglobulins was detected in the first peak. Recycling, on protein G agarose, of the pooled first peaks from several columns showed that only about 4% of the protein was then recovered in the second peak. Pooled fractions of each peak were concentrated with Centricon 30 concentrators, washed with PBS,

Table XV. Invasion of HeLa cells monolayers by SL1344 in the presence of fractions from a pepsin digest of bovine colostrum.

Samples	*% Invasion*
Control	100.0[a] (26.8)
UBC Col	2.8 (1.4)
Pepsin digest C30R[b]	33.8 (5.8)
Pepsin digest C30F[c]	85.6 (17.7)
Control no pepsin C30R[b]	3.1 (2.3)
Control no pepsin C30F[c]	84.5 (34.7)

[a]: Percent of internalyzed bacteria relative to control, averages of 4 wells, standard deviations in parentheses.

[b]: Retentate after filtration with Centricon 30.

[c]: Filtrate after filtration with Centricon 30.

Table XVI. Invasion of HeLa cells monolayers by SL1344 in the presence of heat treated colostrum.

Samples	*% Invasion*	
Control	100.0[a]	(28.1)
UBC Col	10.1	(5.8)
Heat treated colostrum[b]	79.8	(15.0)

[a]: Percent of internalyzed bacteria relative to control, averages of 4 wells, standard deviations in parentheses.

[b]: Colostrum heated at 80 °C for 30 min.

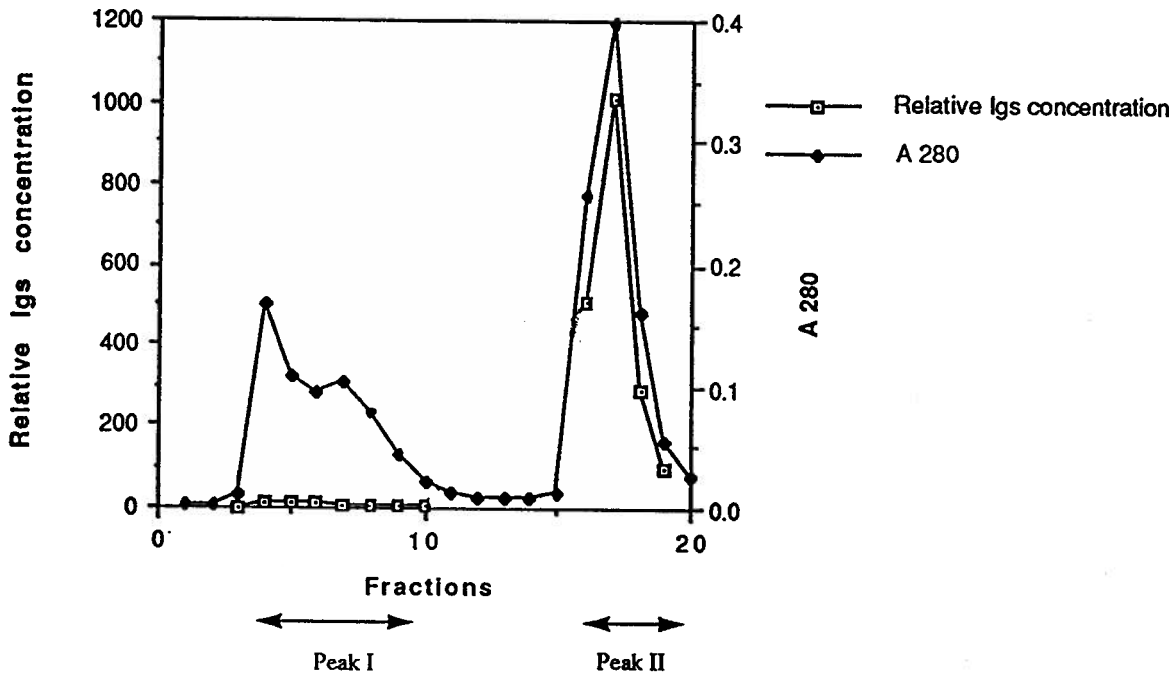


Figure 10. Absorbance and relative IgG concentration of colostrum fractions obtained by chromatography on a protein G-agarose column.

Column volume: 1.5 ml; sample volume: 0.25 ml; fraction size: 0.25 ml.

and their IgG concentration relative to the original colostrum sample estimated by ELISA. SDS PAGE electrophoresis showed minimal contamination of the respective peaks by the other (Figure 11). However, some of the strongest bands in Peak I had a mobility similar to what would be expected of Fab fragments of immunoglobulins in their reduced form. Peak I and Peak II were then tested for their ability to inhibit invasion of HeLa cells by SL1344 (Table XVII).

Table XVII shows that while inhibition of invasion (about 13% of control) was associated with the IgG containing peak, a high level of inhibitory activity (about 9% of control) was also found in the first peak, where the IgG concentration was approximately 1% of that of the second peak. These results of this experiment were confirmed by three other independent fractionations of this sample of colostrum.

This test was repeated with a sample of cottage cheese whey, obtained from the pooled milk of a large number of cows. Since protein concentration in whey is quite low, a total volume of 90 ml of whey was used, and the fractions obtained following chromatography on protein G-agarose required extensive concentration with Centricon 30 separators. Approximately 20 mg of IgG were recovered. Table XVIII shows the results of a challenge of HeLa cells with *S. typhimurium* SL1344 in the presence of the Peak I and Peak II obtained from this cottage cheese whey. Both Peak I and Peak II inhibited invasion. Peak I had not been concentrated to the same extent as Peak II and therefore the effect was smaller, but nevertheless substantial. Therefore an inhibiting effect of the immunoglobulin fraction and the non-immunoglobulin fraction was found in samples of very different origins.

Longhi *et al.* (1993) reported an anti-invasive effect of human lactoferrin when HeLa cells were challenged with *E. coli* HB101(pR1203), which is capable of invading cultured epithelial cells. Lactoferrin would be expected to elute in Peak I on the protein G-agarose column. To check on the activity of bovine lactoferrin in this system, HeLa cells were challenged with *S. typhimurium* SL1344 in the presence of bovine lactoferrin at a final

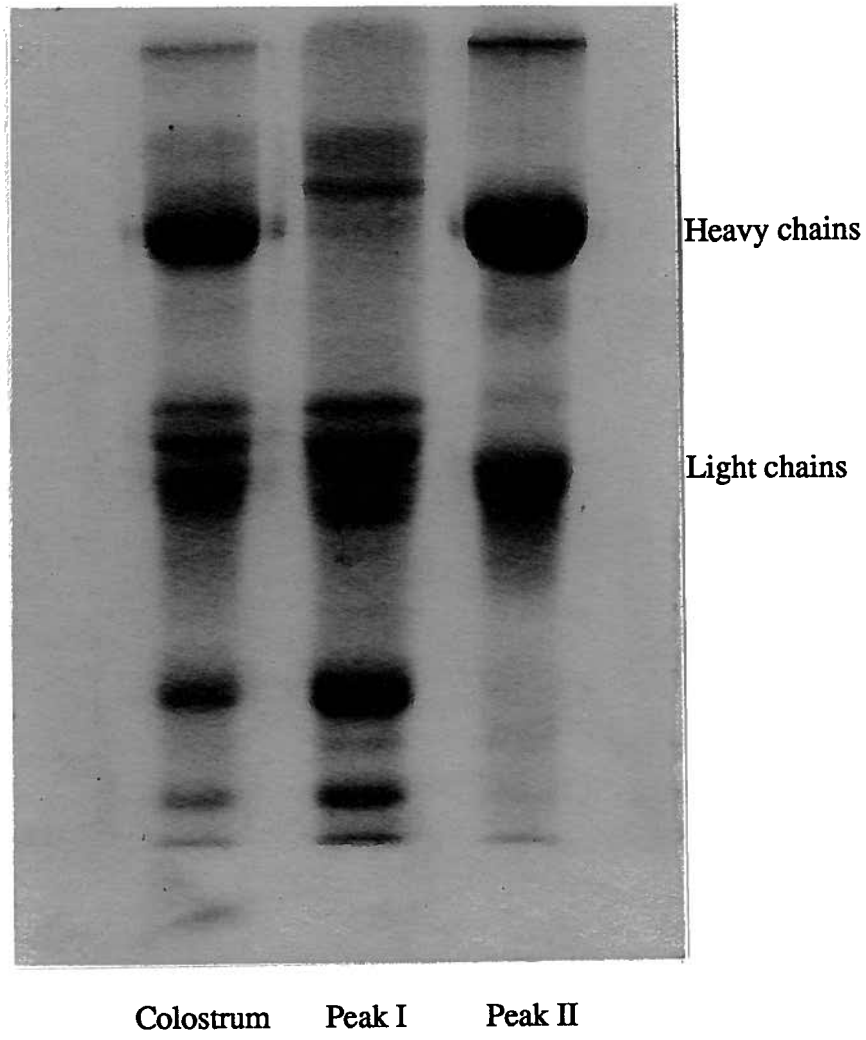


Figure 11. SDS PAGE profiles of colostrum (UBC Col), and of Peak I and Peak II protein G agarose fractions.

Table XVII. Invasiveness of SL1344 in the presence of concentrated pooled fractions following separation of colostrum on a protein G-agarose column.

Sample	*IgGs*[a]	*% invasion*[b]
Control		100.0 (44.2)
UBC Colostrum	100.0	8.3 (3.2)
Protein G Peak 1	0.44	9.6 (2.8)
Protein G Peak II	53.6	13.3 (2.4)

[a]: relative concentration of IgGs in Peaks I and II as % of the original colostrum sample (UBC Col). Peak I and Peak II reconstituted in PBS to approximately the original volume.

[b]: percent of internalyzed bacteria relative to control, averages of 4 wells, standard deviations in parentheses.

Table XVIII. Invasiveness of SL1344 in the presence of concentrated pooled fractions following separation of cottage cheese whey on a protein G-agarose column.

Sample	*IgGs*[a]	*% invasion*[b]	*Concentration factor*[c]
Control		100 (18.0)	
UBC Colostrum	100	4.1 (1.6)	
Whey Peak I	0.2	16.5 (2.9)	30
Whey Peak II	57.9	6.9 (1.7)	300

[a]: relative concentration of IgGs as % of the UBC colostrum sample. Approximately 20 mg of IgG were recovered in Peak II, as determined by spectrophotometry, from 90 ml of whey.

[b]: percent of internalyzed bacteria relative to control, averages of 4 wells, standard deviations in parentheses.

[c]: factor by which the volume of the whey sample was reduced following concentration.

concentration of 2.5 mg/ml. The results presented in Table XIX do not support an anti-invasive effect for bovine lactoferrin at that concentration. A similar finding with *E. coli* has also been reported by Longhi *et al.* (1994), who also found that lactoferrin had no bactericidal or cytotoxic effect at that concentration.

A number of tests were then done to characterize the inhibiting factors in each peak, and particularly to determine whether the activity in Peak I was associated with contaminating immunoglobulins or with immunoglobulin fragments that could have been generated by proteolytic activity in the colostrum. Following fractionation on a protein G-agarose column, the pooled fractions from each of Peak 1 and Peak 2 were sequentially separated with Centricon concentrators C100, C30 and C10. The respective fractions were assayed for IgG and $(Fab')_2$ content by ELISA. It was speculated that if immunoglobulin fragments such as Fab were somehow present in colostrum, it might be possible to detect them in Peak I with an anti-$F(ab')_2$ antibody. HeLa cells were challenged with SL1344 as previously described. The results (Table XX) complement the data from Tables XVII and XVIII and in addition show that most if not all the activity in both Peak 1 and Peak 2 was contained in the fraction above 100 kDa. No great differences were found in the immunoassays for IgGs and for $F(ab')_2$ fragments.

A sample of whey protein concentrate (WPC) (200 mg) was reconstituted and treated as above (fractionation on protein G-agarose, separation on the basis of size with Centricon concentrators). HeLa cells were challenged with SL1344 in the presence or absence of the fractions which were also assayed for their IgG and $F(ab')_2$ relative concentrations by ELISA (Table XXI). Results similar to those presented in Table XX were obtained. The results in Tables XX and XXI do not totally preclude activity in the lower MW fractions; however most of the activity was recovered in the high MW fraction, in agreement with the results presented in Table XIV.

From the results presented in Tables XVII, XVIII, XX and XXI, it can be concluded

Table XIX. Invasiveness of SL1344 in the presence of bovine lactoferrin.

Samples	*% invasion*[a]	
Control	100.0	(31.4)
UBC Colostrum	3.4	(2.1)
Lactoferrin (2.5 mg/ml)	76.0	(15.2)

[a]: percent of internalyzed bacteria relative to control, averages of 4 wells, standard deviations in parentheses.

Table XX. Invasiveness of SL1344 in the presence of colostrum fractions obtained by chromatography on a protein G-agarose column, followed by separation on the basis of size.

	Concentration[a]		*% invasion*[b]	
Samples	*IgGs*	*F(ab')$_2$*		
Control			100.0	(41.6)
UBC Col	100.0	100.0	2.5	(0.8)
Peak I C100R[c]	6.6	5.6	4.7	(3.2)
Peak I C30R	0.065	0.073	48.6	(15.4)
Peak I C10R	<0.018	<0.019	27.6	(6.6)
Peak II C100R	133.0	97.0	4.4	(2.3)
Peak II C30R	0.157	0.112	46.5	(26.6)
Peak II C10R	<0.018	0.023	109.6	(53.0)

[a]: relative concentration of IgGs or F(ab')2 fragments in the various fractions as % of the original colostrum sample (UBC Col). Approximately 20 mg of IgG were recovered in Peak II, as determined by spectrophotometry.

[b]: percent of internalyzed bacteria relative to control, averages of 3 wells, standard deviations in parentheses.

[c]: R: retentates following concentration with the respective Centricons, to approximately 50% of the original volume.

Table XXI. Invasiveness of SL1344 in the presence of whey protein concentrate fractions obtained by chromatography on a protein G agarose column, followed by separation on the basis of size.

	Concentration[a]		*% Invasion*[b]	
Samples	*IgGs*	*F(ab')$_2$*		
Control			100.0	(57.0)
UBC Col	100.0	100.0	4.3	(2.6)
Peak I C100R[c]	1.0	2.9	6.3	(2.6)
Peak I C30R	0.06	0.05	40.2	(10.5)
Peak I C10R	<0.014	<0.028	36.6	(4.4)
Peak II C100R	22.7	20.7	3.4	(1.0)
Peak II C30R	0.09	0.075	39.7	(15.2)
Peak II C10R	<0.016	0.029	69.1	(19.2)

[a]: relative concentration of IgGs or F(ab')2 fragments in the various fractions as % of the reference colostrum sample (UBC Col). Approximately 8 mg of IgG were recovered in Peak II, as determined by spectrophotometry.

[b]: percent of internalyzed bacteria relative to control, averages of 3 wells, standard deviations in parentheses.

[c]: R: retentates following concentration with the respective Centricons, to approximately 50% of the original volume.

that inhibiting activity was associated with both the immunoglobulin rich fraction, and with the non-immunoglobulin fraction obtained by chromatography on the protein G agarose column.The inhibitory activity of the IgG fraction isolated from the whey protein concentrate appears to be relatively higher than that of the IgG fraction isolated from the colostrum. The cows that produced the milk from which the whey concentrate was obtained had been vaccinated with a proprietary mixture of bacteria, among which were *S. enteritidis* ATCC 13076 and *S. typhimurium* ATCC 13311 (Stolle and Beck, 1987).

The results presented in Tables XX and XXI do not support the hypothesis that the activity in Peak I is due to immunoglobulin fragments such as F(ab')2 or similar types of fragments. In a situation where both whole immunoglobulins and $F(ab')_2$ fragments may be present, immunoassays using affinity purified antibodies to the whole immunoglobulin would be expected to be more efficient at detecting whole immunoglobulins than $F(ab')_2$ fragments, because of the high immunogenicity of the Fc fragment. The opposite would be true of antibodies to $F(ab')_2$, which would be more efficient at detecting immunoglobulin fragments containing light chains, and quantitative ELISAs done side by side using such antibodies for capture and detection would be expected to give widely differing results if one sample (Peak I) happened to contain an amount of fragments sufficient to cause inhibition of invasion of the same magnitude as Peak II. This was not found to be the case.

To determine whether the inhibitory activity of Peak I could be due to contamination by IgGs, in spite of their low level as determined by ELISA, a sample of pooled Peak I, concentrated with Centricon separators, was recycled on a protein G agarose column. The recycled Peak I fraction was concentrated again in the same manner, assayed for Igs level and tested for inhibitory activity. Even though about 75% of the contaminating Igs were removed by the second passage on the column, the inhibitory activity of peak I was not reduced. Conversely, dilution of the Peak II fraction to bring the IgG concentration to a level

similar to that of Peak I prior to recycling resulted in a decrease in inhibitory activity (Table XXII).

The data presented in Tables XVII, XVIII, XX, XXI and XXII do not support the hypothesis that the activity in Peak I is related to the presence of contaminating whole immunoglobulins or even of immunoglobulin fragments. This last point was further examined by immunoblotting of colostrum, Peak I, Peak II, and a papain digest of Peak II, using affinity purified antibodies to whole bovine IgG or to bovine $F(ab')_2$ fragments for detection. In neither type of blot were bands in Peak I stained with the respective antibodies beyond barely visible indication of slight contamination with Peak II, while colostrum, Peak II and the papain digest of Peak II showed all the bands corresponding to reduced immunoglobulins or their fragments (Figures 12A, 12B).

Incidentally, the intensity of the bands in the blots respectively exposed to the anti-bovine IgG or the anti-$F(ab')_2$ antibodies confirms the operating hypothesis on which the experiments presented in Tables XX and XXI were based.

Finally, to further characterize Peak I, pooled concentrated Peak I was digested with pepsin at pH 2.8 for 4 hours at 37°C, followed by dialysis in PBS. A control was done in parallel without pepsin. The results of an invasion assay show that pepsin digestion reduced the activity of Peak I (Table XXIII).

To confirm the presence in colostrum of antibody to the bacteria, HeLa cells seeded on cover slips were infected with SL1344 or E2348/69. Following incubation in medium for one hour, the cells were washed, fixed and permeabilized as described in the method section. Colostrum was added to each cover slip, and following incubation for one hour and washing of the test samples, bovine antibodies bound to bacteria were detected with an affinity purified anti-bovine IgG fluorescent antibody. A control where HeLa cells were not infected with bacteria was included to detect any non specific binding of bovine antibody to the cells,

Table XXII. Invasion of HeLa cells monolayers by SL1344 in the presence or absence of bovine colostrum, Peak I, Peak I recycled or diluted Peak II.

Samples	*IgGs*[a]	*% invasion*[b]	
Control		100.0	(26.2)
UBC Col	100.0	2.1	(1.8)
Peak I	7.8	1.0	(0.7)
Peak I recycled	1.9	0.5	(0.2)
Peak II	136.9	1.8	(0.5)
Peak II 1/20	6.8	18.0	(2.8)

[a]: relative concentration of IgGs in the various fractions compared to the original colostrum sample (UBC Col).

[b]: percent of internalyzed bacteria relative to control, averages of 4 wells, standard deviations in parentheses.

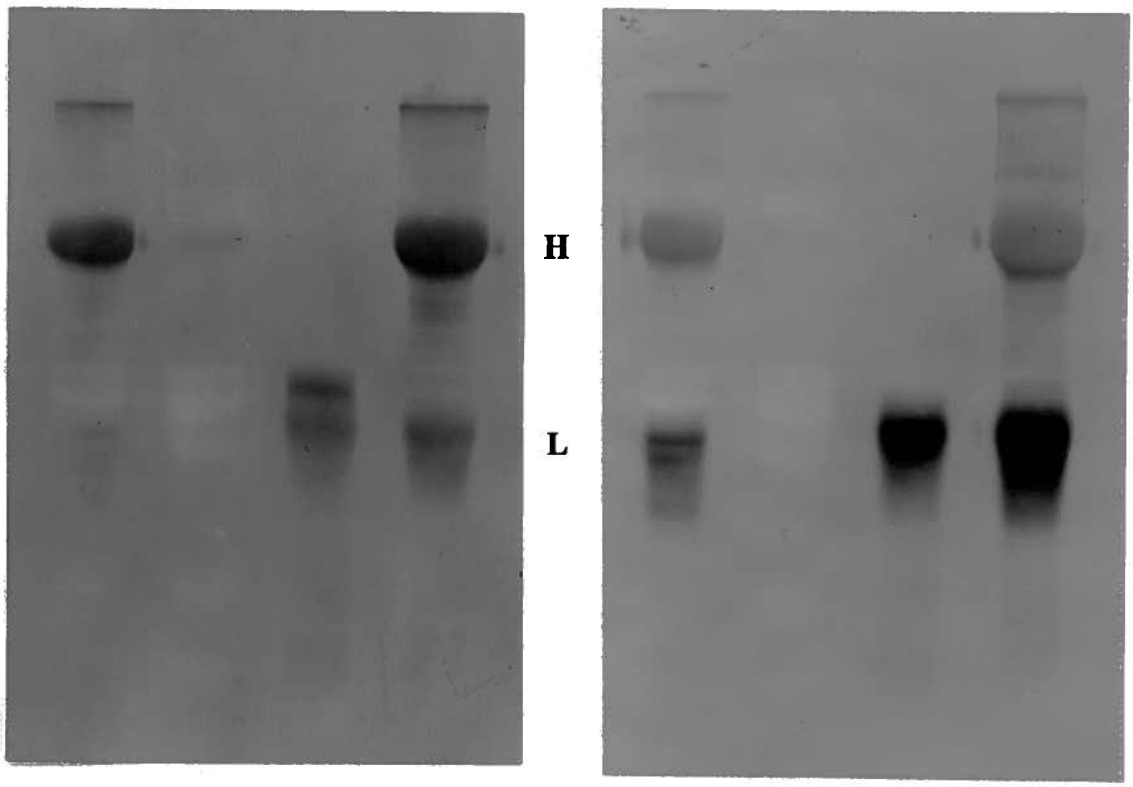


Figure 12. Immunoblots of bovine colostrum (UBC Col), Peak I, Peak II obtained by chromatography on a protein G agarose column, and a papain digest of Peak II.
A: blot detected with an affinity purified antibody to bovine IgG.
B: blot detected with an affinity purified antibody to bovine $F(ab')_2$ fragments.
H= Heavy chains; L= Light chains.

Table XXIII. Invasion of HeLa cells monolayers by SL1344 in the presence or absence of bovine colostrum, Peak I or Peak I digested with pepsin.

Samples	*% Invasion*	
Control	100.0[a]	(19.1)
UBC Col	2.4	(0.4)
Peak I control[b]	0.7	(0.2)
Peak I pepsin digest	54.8	(8.7)

[a]: percent of internalyzed bacteria relative to control, averages of 4 wells, standard deviations in parentheses.

[b]: IgG concentration in Peak I was determined by ELISA to be about 3.2% of that of UBC Col prior to pepsin digestion. Peak I control was treated identically to Peak I pepsin digest, in the absence of pepsin.

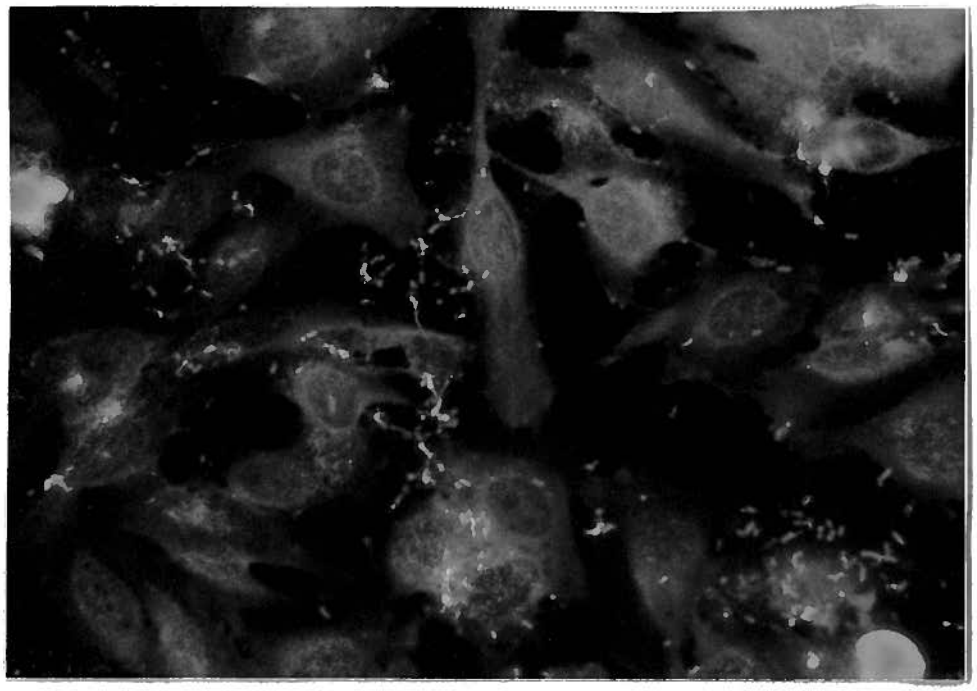
as well as a control (in the case of SL1344) where the bacteria were detected with a specific anti-LPS typing antiserum. Examination of the control cover slips showed that SL1344 were associated with the cells, and that no non-specific binding of bovine antibody could be detected (Figures 13A, 13B). Fluorescence associated with the bacteria was evident on the cover slips exposed to colostrum. However, while fluorescence was found evenly distributed around the bacteria exposed to the specific anti-LPS antiserum, fluorescence on the bacteria exposed to colostrum or colostrum fractions was not so evenly distributed around the bacteria or appeared to be directed at fimbriæ. On the other hand, in the case of E2348/69, the fluorescence was associated strictly with the bacteria, which themselves appeared as clumps, or microcolonies (Figures 14A, 14B). No specific antiserum was available to us to independently detect the bacteria, but the microcolonies were clearly visible by phase contrast microscopy. Microcolonies are a typical adherence pattern to HeLa cells or to HEp-2 cells for this type of EPEC (Levine *et al.*, 1985).

A

B

Figure 13. A: Detection by TRITC immunofluorescence of *S . typhimurium* SL1344 on cover slips seeded with HeLa cells.
B: Absence of non-specific binding of bovine immunoglobulins to cover slips seeded with HeLa cells.
FITC immunofluorescence with affinity purified antibody to bovine IgG. Scale: ⊢——⊣ = 10 µm.

A



B

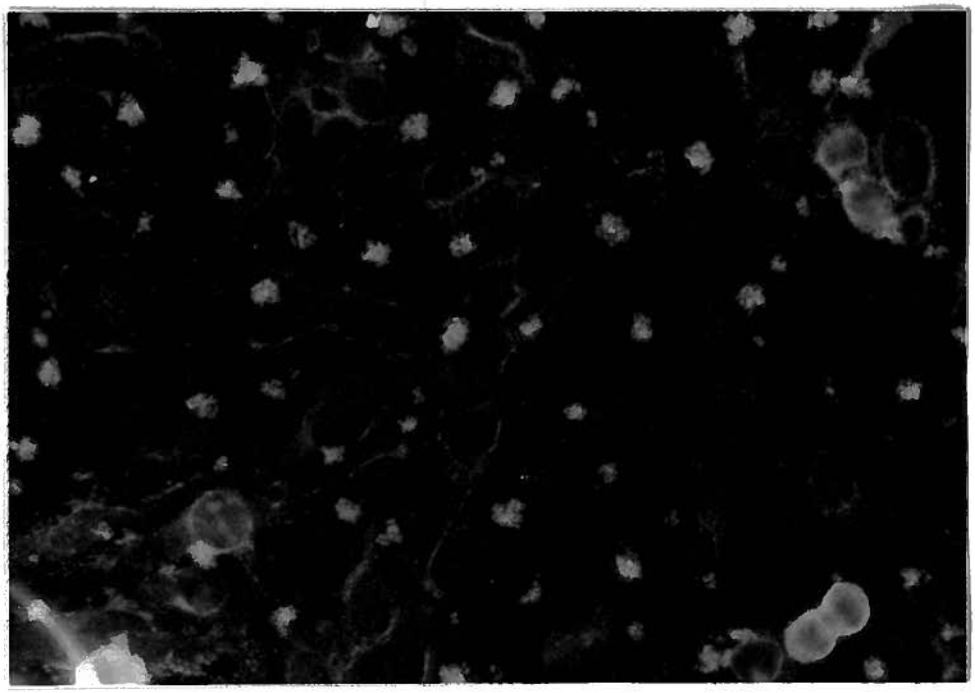


Figure 14. A: FITC immunofluorescence with affinity purified antibody to bovine IgG of *S. typhimurium* SL1344 exposed to bovine colostrum on cover slips seeded with HeLa cells.
B: FITC immunofluorescence with affinity purified antibody to bovine IgG of *E. coli* E2348/69 exposed to bovine colostrum on cover slips seeded with HeLa cells. Scale: ⊢——⊣ = 10 µm.

C. Discussion

The purposes of the experiments reported above were: 1) to evaluate the suitability of cell cultures methods for *in vitro* testing of the antibacterial activity of fractions that may in the future be purified from cows milk or whey, and: 2) to determine whether any antibacterial effect that may be found could be attributed to immunoglobulins. In these experiments, the anti-invasive properties of bovine colostrum or whey against *S. enteritidis, S. typhimurium* and *E. coli* were tested. Colostrum was tested first in this investigation because of its very high content of antibacterial factors, thereby alleviating the need for extensive purification and concentration.

The results presented here showed that the fraction of bovine colostrum remaining after removal of fat or insoluble matter inhibited the invasion of HeLa cells by *S. enteritidis* ATCC13076 and CD5, *S. typhimurium* SL1344 and *E. coli* E2348/69. All samples of colostrum tested showed inhibitory activity. One sample was from B.C. and the others from Saskatchewan; all were from individual cows. To the best of this author's knowledge, it is the first time that an anti-invasive effect of bovine colostrum, or milk or whey has been reported. So far only reports of anti-adherent effects of human milk against some strains of *E. coli* have been found in the literature.

The inhibitory activity was not reduced by dialysis and was not found in low molecular weight fractions. The inhibition did not seem to be related to reduced viability of the bacteria. A similar finding with human milk IgAs was reported by Cravioto *et al.*, 1991. The colostrum factors did not appear to become associated with the HeLa cells. The low molecular weight fractions of colostrum, able to pass through Centricon 30 and 10 membranes, had no activity. Similarly, no low molecular weight fractions with anti-invasive properties were generated following digestion of the colostrum with pepsin. Digestion with pepsin and heat treatment both reduced the anti-invasive ability of the colostrum.

Fractionation of one colostrum sample, one cottage cheese whey sample and one

reconstituted whey protein concentrate sample on a protein G-agarose column showed that both the immunoglobulin fraction (Peak II) and the IgG depleted fraction (Peak I) were able to inhibit invasion of HeLa cells by *S. typhimurium* SL1344. Separation on the basis of size showed that the inhibitory activity of both fractions was retained on Centricon 100.

The immunofluorescence studies showed that bovine IgGs bound to *S. typhimurium* SL1344 and *E. coli* E2348/69, but the pattern of binding was different. In the case of SL1344, bovine IgGs bound to the bacteria and to filamentous structures (fimbriæ), while with E2348/69, bovine IgGs appeared to be associated strictly with the bacteria. Human subjects infected with this type of EPEC are known to produce antibodies specific for a 94 kD bacterial outer membrane protein associated with localized adherence to epithelial cells (Levine *et al.*, 1985), and human milk antibodies have also been shown by immunoblotting to react with the 94 kD outer membrane protein, and to inhibit adherence to HEp-2 cells (Cravioto *et al.*, 1991; Camara *et al.*, 1994). Whether bovine immunoglobulins react with the same outer membrane protein has not been determined.

With regard to the effect against *Salmonella,* the gene products responsible for adherence and invasion of *S. typhimurium* are not known (Finlay, 1994). Peralta *et al.* (1994) showed that oral administration of egg antibodies specific for a 14-kDa fimbriæ protected mice against experimental salmonellosis with *S. enteritidis*, and also that the same antibodies reduced adhesion of *S. enteritidis* to mouse intestinal epithelial cells *in vitro*.

The factor(s) in Peak I responsible for its anti-invasive properties have not been identified. It is not known whether the inhibitory activity is caused by more than one compound. The findings presented here point to the factor(s) being predominantly protein(s) of a large size. The question was whether the factors were fragments of immunoglobulins resulting from enzymatic degradation by milk proteinases. The Fc fragments of bovine IgGs are known to be particularly susceptible to enzymatic action with resulting production of fragments made up of various portions of heavy and light chains (Wie *et al.*, 1978;

Heyermann and Butler, 1987). SDS PAGE electrophoresis showed some major components of Peak I to be found approximately where one would expect to see bands for the reduced Fab fragment of IgGs, and association of four chains or parts of chains (similar to $F(ab')_2$) would be large enough to be retained on Centricon 100. It is known that Fab fragments from specific antibodies are able to block adherence of enterotoxigenic *E. coli* to Caco-2 cells (Darfeuille-Michaud *et al.*, 1990) or to block invasion of HEp-2 cells by invasin-producing bacteria (Leong *et al.*, 1990). The evidence presented above, obtained by immunoassays and immunoblots, does not support the hypothesis that the activity of Peak I is due to the presence of immunoglobulin fragments. Other incidental evidence derives from attempts to separate Fab fragments, obtained by papain digestion of Peak II, by protein G-agarose chromatography: it was found that a considerable proportion of the fragments bind to the column and therefore are not likely to be efficiently recovered in the first peak. While it is difficult at this point to speculate on the nature of the factor(s) involved, studies by Longhi *et al.* (1994) as well as results presented above, showed that bovine lactoferrin at concentration of 2 mg/ml did not prevent invasion of HeLa cells by *E. coli*.

There are several examples in the literature of non-immunoglobulin fractions of human milk having anti-adherent activity. The anti-adherent activities have been attributed to glycoproteins, glycolipids or oligosaccharides (Andersson *et al.*, 1986; Ashkenazi and Mirelman, 1987; Holmgren *et al.*, 1981). Cravioto *et al.* (1991) showed that an oligosaccharide fraction of human milk inhibited adherence of *E. coli* E2348/69 to HEP-2 cells. However Camara *et al.* (1994) were not able to confirm this finding. To this writer's knowledge, no example of a non-immunoglobulin fraction of bovine milk or colostrum that inhibits invasion of mammalian cells by *Salmonella* has been published.

Adherence, the interaction between an adhesin and a receptor, could be inhibited in several ways: by blocking of the adhesin on the bacteria with antibodies or with receptor analogs; by flooding the receptors on the host cells with an adhesin analog; and last by

blocking the receptor with antibodies. Adhesins analogs have been shown to block invasion experimentally (Leong *et al.*, 1990). But blocking of receptors does not appear to be a likely scenario in nature, considering that they probably have other more important functions than binding to bacteria. Blocking of adhesins is a more desirable strategy, and the results presented above suggest that the effect of bovine colostrum is probably on the bacteria, not on the host cells. Antibodies against adhesins of course have been found in milk or serum as a result of experimental or natural infections (Tacket *et al.*, 1988; Tacket, 1991).

The presence of specific oligosaccharide receptor analogs has been demonstrated in milk. For example the GM1 gangliosides, the cell membrane receptors for CT (cholera toxin) and LT (heat labile toxin), are present in human and bovine milk and have been shown to block enterotoxin binding. Milk gangliosides are derived from mammary gland cell membranes (Lægrid *et al.*, 1986). Whether the non-immunoglobulin fraction with anti-invasive activity reported above, which is probably a protein, is also a receptor analog, remains to be demonstrated. The receptor for the invasin of *Yersinia pseudotuberculosis* is known to be part of the protein family of integrins (Leong *et al.*. 1990). Could protein cell receptors analogs, derived from mammary gland cell membranes, also be found in milk? Somatic cells are present in significant numbers in milk and colostrum (Tizard, 1992).

The work reported in this chapter opens many avenues for further research on the antibacterial factors of cows milk. With regard to the unidentified factor in Peak I obtained by protein G-agarose chromatography, the work is still at the stage where confimation of its presence in a greater number of samples of milk, cheese whey or colostrum is required. The fact that it has been detected in three samples (one colostrum and two whey samples) of widely different origins is encouraging. Moreover, it is relatively easy to separate from the immunoglobulins, and the gentamicin assay provides a test system to track this factor through the further purification steps that will be required to identify it. However, it would be quite useful to have some other more sensitive means to track this factor, which would

facilitate its recovery from the wheys where it is present at low concentration, or to have some purification protocol which would efficiently separate it from most of the other whey proteins. A confirmation of its size would be the first step, so that preliminary fractionation by membrane filtration would achieve that goal and increase its concentration at the same time. Scaling up the chromatography on protein G-agarose would greatly increase the efficiency of the process, which, while satisfactory so far, will require much larger amounts of separated Peak I and Peak II in any attempt to further characterize their activity. The effect of the Peak I factor(s) on bacteria other than *S. typhymurium* SL1344, or in other cell systems, also remains to be investigated. Irrespective of the results of such experiments, questions will be raised about the specificity, the homogeneity and the mode of action of the factor(s).

The anti-invasive activity of bovine immunoglobulins also needs to be investigated further. Samples of different origins should be tested, against other enteropathogens, and using other cell lines, to demonstrate the generality of the phenomenon. Of particular interest would be experiments to compare the anti-invasive ability of bovine colostrum with that of human milk. It is rather puzzling that only an anti-adherence effect against *E. coli* has been demonstrated with human milk to-date, and that no results of experiments to investigate an anti-invasive effect of human milk, for instance against *Salmonella*, have been reported. It would also be useful to identify the targets of the colostrum antibodies, with particular attention to the bacterial components that are known to either induce antibody production in the course of natural infection, or that are associated with adherence and invasion, for instance: the 94 kDa outer membrane protein of EPEC; the *S. enteritidis* fimbriæ identified by Peralta *et al.* (1994); or, in the case of bacteria for which the effect of bovine colostrum in cell cultures remains to be tested, the outer membrane proteins of *Shigella* identified by Oaks *et al.* (1986); the *Yersinia* invasin, or the outer membrane proteins of *E. coli* O157:H7 identified by Sherman *et al.* (1991). The finding of antibodies to the CFA 1 of ETEC has

been reported in this thesis, and these could also be tested for anti-adherence activity with the cell culture system used by Darfeuille-Michaud *et al.* (1990), who demonstrated that specific rabbit antisera to the colonization factors antigens of human ETEC prevented adherence to Caco-2 cells by the homologous bacteria.

In future work it will also be of interest to determine whether colostrum antibodies or other factors inhibit adherence, or do not affect adherence but inhibit invasion. This may depend on whether invasion follows adherence automatically, or whether adherence and invasion are separate steps in the process of entry into the mammalian cell. In *E. coli* E2348/69 and related strains, there is evidence that invasion is a two-step process (Francis *et al.*, 1991) and it may be possible to test for both an anti-adherence effect and an anti-invasion effect of colostrum. Francis *et al.* (1991) found that the localized adherence pattern of this type of EPEC was mediated by the EPEC adherence factor (EAF), while another factor (*eae*) was required for effacement of microvilli and entry into mammalian cells. This *eae* factor has been correlated with the presence of the 94 kDa outer membrane protein referred to above (Jerse and Kaper, 1991). Efficacy *in vivo* could be tested with the ligated loop model, which was used by Jones *et al.* (1994a) to demonstrate invasion of the intestinal Peyer's patches by *S. typhimurium.*

To end with a more general and speculative discussion, it may be that the question of the specificity of the milk inhibitors will be of great interest. Recent studies of bacterial pathogenesis have uncovered an intriguing conservation of structures and functions of proteins involved in transport of virulence determinants in a variety of pathogens, among which are *Salmonella, Shigella* and *Yersinia* (Ginocchio and Galan, 1995; Collazo *et al.*, 1995). However, the determinants exported by these systems appear to be very specific to the various pathogens. If the role of milk in the protection of infants against enteropathogens is accepted, then the question arises whether this protection is directed only at the vast number of antigenic determinants present on the various species of bacteria, or wheher there

also exists some general mechanism that would interfere with the export system mentioned above. If this export system is activated or enhanced by contact of the bacteria with the host cell, as may be the case with *Yersinia* (Rosqvist *et al.*, 1994), then a prime target of the milk inhibitors, besides preventing contact between the bacteria and host cells, could be whatever structure on the bacteria generates a signal to activate the secretion sytem. There is no evidence for such a mechanism at this point, but this should be kept in mind when investigating the identity of the immunoglobulin or non-immunoglobulin milk inhibitors or their targets on the bacteria.

And finally, to return to the original question of whey utilization, more samples of a variety of cheese wheys need to be studied to determine their antibacterial properties by the above methods, and in particular to investigate the influence of various manufacturing processes on their antibacterial activity. The suitability of cell cultures to study the antibacterial effect of cows milk and whey was demonstrated by the experiments in the present study, particularly in this case by providing a test system for anti-invasive activity by a non-immunoglobulin fraction which could not have been detected by immunoassays.

It may be that continuation of this work will provide results that would make further processing of cheese whey a more attractive proposition. In any case it should provide added understanding of the mechanisms by which milk contributes to protection against enteropathogens.

VI. CONCLUSION.

The results of a study of the antibacterial activity of a pepsin digest of bovine lactoferrin (LFD) were reported. It was found that LFD is bactericidal against *Salmonella enteritidis* ATCC 13076 in 1% peptone at a concentration of 30 μg/ml, and bacteriostatic at a concentration as low as 3 μg/ml. It was also found that some bacteria were injured by the effect of LFD and became susceptible to killing by bile salts. LFD had no activity in trypticase soy broth (TSB) or in milk-based or soy-based infant formulæ. Calcium at a concentration of approximately 5 mM was sufficient to inhibit the activity of LFD in 1% peptone.

Addition of lysozyme or EDTA to LFD in TSB was not beneficial unless the concentration of EDTA was significantly higher than that permissible in foods. Even though lysozyme (80 μg/ml) or EDTA (0.25 mM) alone had no inhibitory effect on *S. enteritidis*, they showed a synergistic effect with LFD provided that the concentration of TSB was reduced to 75% of its recommended strength. It was also found that bile salts inhibited the activity of LFD in 1% peptone.

It can be concluded that while the bovine lactoferrin digest showed some considerable antibacterial activity under some limited conditions, there is no evidence that it had any effect against *S. enteritidis ATCC* 13076 in the foods tested. These findings raise doubts about the application of lactoferricin in foods.

Antibodies to the Colonization Factor Antigen 1 of enterotoxigenic *Escherichia coli* H10407 were detected in cows milk and colostrum by hemagglutination inhibition and immunoassays. The concentration of antibodies was determined by ELISA and found to range approximately from 0.5 to 5 μg/ml in colostrum samples of non-vaccinated cows.

Testing of milk immune concentrates showed that vaccination with a purified antigen or a whole cell preparation increased the concentration of specific antibodies relative to the total immunoglobulin G concentration of the samples.

Finally, it was found that bovine colostrum inhibited invasion of HeLa cells by *S. enteritidis* ATCC 13076 and CD5, *S. typhimurium* SL1344, and *E. coli* E2348/69. Inhibition levels ranged from 73% to over 99% when colostrum was added to a final concentration of 10% of the culture medium. Seven samples of colostrum were tested.

An effect of colostrum on the number of viable bacteria in the medium or on the mammalian cells could not be detected. The inhibitory activity of colostrum was diminished by heat treatment above 80 °C or by digestion with pepsin, and was not found in low molecular weight fractions. The immunoglobulin-containing fraction, isolated from colostrum by affinity chromatography on a protein G-agarose column, inhibited invasion by SL1344. An unidentified high molecular weight factor in the non-immunoglobulin fraction also inhibited invasion of HeLa cells by SL1344. The inhibition by the non-immunoglobulin fraction did not appear to be caused by contaminating immunoglobulins or immunoglobulin fragments. A sample of cottage cheese whey and a sample of whey protein concentrate were also tested. In both cases it was found that the immunoglobulin fraction and the non-immunoglobulin fraction had an inhibitory effect on the invasion of HeLa cells by *S. typhimurium* SL1344.

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