CHAPTER II

REVIEW OF LITERATURE
2.1 Transferrins: Biological and Phylogenetic Distribution

The transferrin superfamily includes monomeric, non-heme iron binding proteins (Aisen and Listowsky 1980). Other members of the group are serum transferrin (Guard and Wilax 1956), avian ovotransferrin (Wenn and Williams 1968) and melanotransferrin of melanoma cells (Rose et al., 1986). Unlike lactoferrin, which is found only in mammals, serotransferrin is distributed widely throughout the phylogeny, in all the vertebrates and the studied invertebrates. Some of the latter category include the crab Cancer magister (Heubers et al., 1982), insects like cockroach (Jamroz et al., 1993) as well as mosquito (Yozhiga et al., 1997), and a spider (Lee et al., 1997), besides one tunicate (Martin et al., 1984).

Though reported in 1939 (Sorenson and Sorenson), the experimental history of lactoferrin begins from 1960 (Johanson) with "the isolation of an iron containing red protein from human milk", which was latter named lactoferrin. While the abundance of transferrin is confined generally to serum or ovum, lactoferrin enjoys a wide distribution; it has been documented in most of the exocrine secretions like milk (Johanson 1960), tears (Schuchel 1981) and semen (Hekman and Rumke 1968) as well as neutrophils (Masson et al., 1969) and some group of neurons (Fillebeen et al., 2000).

2.2 Physical Structure of Lactoferrin

2.2.1 Primary Structure: Evolutionary Conservation

All the vertebrate components of transferrin family have a molecular weight of approximately 80Kd, except HMTf which is 97Kd. Significant sequence similarity exists between the Lf of different species with highly conserved regions (owing to its inherent evolutionary connotations, this matter has been given an exclusive elaboration in chapter 3). Within a
single molecule itself both N- and C-terminal halves display 30-40% sequence identity. This later view prompts to perceive the occurrence of gene duplication in the evolution of transferrin-proteins (Williams 1982).

Lf from bovine source has 689 residues (Mead and Tweedie 1990), buffalo is with 689 (Karthikeyan et al., 1999b), mare has 689 (Sharma et al., 1999), camel 689 (Kappeler 1999 et al.), goat 689 (Le Provost et al., 1994), human has 691 (Rey et al. 1990), mouse 686 (Pentecost et al., 1987) and pig 686 (Alexander et al., 1992). The isoelectric pH of Lf is >8, while that of STf/OTf is ~ 6.

2.2.2 Tertiary Folding: A Universal Pattern

In conformity with the relative sequence homology of N and C halves, folding of lactoferrin depicts a correspondence in the conformational pattern between the two (Haridas et al., 1995, Moore et al., 1997; Sharma et al. 1999; Karthikeyan et al., 1999b). The molecule is folded into two lobes, each of which is further divided into a pair of domains designated as N1 and N2 in N-lobe and C1 and C2 in C-lobe (Figs: 2.1, 2.2). The bi-domain structure with an interspersed pocket is akin to the "classic binding motif" shared by seen in bacterial periplasmic binding proteins (Quiocho 1990). One insular feature of Lf is the presence of a 3-turn \( \alpha \)-helix connecting the two lobes, which in transferrins is replaced with an irregular non helical proline-rich segment (Kurokawa et al., 1995; Bailey et al., 1988). Within each molecule (and between different species) both halves are at variance showing distinct lobe- as well as domain-orientations.

2.2.3 Domains and Lobes: Rigidity Vs Flexibility

An amazing property of Lf domains is it's remarkable ability to adopt multiple conformations in a ligand (iron) dependent manner (Anderson et al., 1990) (Fig: 2.3. All the iron saturated lactoferrins have the domains in each lobe closer to one another with the metal ion juxtaposed in
2.1: Overall backbone superposition of EALF from crystals grown at 30°C (red) and 4°C (green)
Fig. 2.2 Schematic representation of secondary-structure elements of lactoferrin
Fig: 2.3. Structures of human diferric (left) and apo lactoferrins showing different domain orientations.
between-a state called “closed conformation” (Anderson et.al., 1990; Haridas et.al., 1995; Moore et al. 1997; Sharma et.al., 1999). A classical expectation for the apo-form will be that of an “open conformation” with the components of each domain-pair apart, as observed in ovotransferrins (Kurokawa et.al., 1995). Surprisingly this ceased to be a “universal apo-form” with the identification of closed domains in apo-lactoferrins. Various experiments have proven the higher stability of closed form (Hadden et.al., 1994). This is well exemplified by the difference in the hydrogen bonding pattern between closed and open forms. In the hen OTf diferric form there are 22 contacting residues in the N-lobe, and 16 in C-lobe; while only 13 (N-lobe) and 9 (C-lobe) residues make contact in the apo form (Kurokawa et.al., 1995). A similar difference is seen in the N-lobe of human lactoferrin (HLf) between open and closed forms (Anderson et.al., 1990).

In human, while one form of apo-Lf has both domains in open state (Baker et.al., 1997), another structure has N-lobe open but C-lobe closed (Anderson et al. 1990). Mare (Sharma et al. 1999) apo-Lf has both the lobes closed. More recently camel apo-Lf has been found to possess both N- and C-lobes open (Khan et.al., 2001). The origin of this flexibility has been attributed to the less constrained hinge residues present in two beta strands linking the domains (Gerstein et.al., 1993); a see-saw motion will be originated between the inter-domain interfaces by pivoting about the hinges.

Baker et.al., (2000) has proposed that this irregularity in the conformation of the open form yields the logical interpretation that in apo-form both the lobes are able to adopt any state, and that the observed diversity in apo-domain structures could be the result of a preferential “picking” of one form, out of many, dictated by crystal packing requirements. Additionally, it is also probable that there is very little energy difference between open and closed forms when devoid of a bound metal ion.
More prominent are the tilts in relative lobe orientations between different Lfs, possibly due to the residue substitution in the hydrophobic lobe-interfaces as well as domain conformation. In HLf, the helix with residues 680–691 of C- domain interacts with N- lobe helices of amino acids 315-321 and 321-332, reinforcing the stability of the molecule (Williams and Moreton 1988). Lying at the center of the lobe-interface is a cluster or hydrophobic side chains of Ile 314, Leu 318 as well as the aromatic ring of Tyr 329 (N- lobe), and Ala 685, Phe 688 (C- lobe), flanked by Val 310, Pro 311 and Phe 325 in N- lobe, and Ala 382, Leu 385, Leu 682 and Leu 702 in C- lobe – all contributing to the integrity of the protein. The two lobes of CLf and HLf differ by 11.3Å in their relative orientations (Moore et al., 1997).

2.2.4 Metal Binding: The Synergy

All characterized lactoferrins bind two iron atoms (Fe³⁺ state), one in each lobe, invariably associated with an anion, often carbonate (Baker et al., 2000; Lonnerdal and Iyer 1995). Reported exceptions are HMTf, as well as one insect Tf (Bartfeld and Law 1990) which bind only one iron, leaving the C- lobe empty. The requirement of a synergistic anion is mandatory for iron binding. The binding cleft which lies between the domains in each lobe of Lf is conserved across the species with regard to coordinating ligands and geometry. The anionic ligands which neutralize the +3 charge of metal ion in N- lobe are 2Tyr (92 and 192) and Asp 60, followed by bonding to a neutral histidine (253) in N- lobe; corresponding residues in C lobe are Tyr433, 526, Asp 395 and His 595. Iron coordinates to both the sites in a distorted octahedral geometry. The carbonate is ligated to arginine side chain (+1) and the N-terminus of helix-5 with a charge +0.5. The iron is bonded to carbonate in a bi-dentate manner.

Apart from the normal iron-carbon complex, Lf has the flexibility to accommodate many other cations and anions (Ainscough et al., 1979;
Aisen et al., 1973; Eaton et al., 1989). In the normal body system this is insignificant; human milk has an iron : manganese ratio of 2000 : 1 (Lonnerdal and Iyer 1985). In some cases this substitution may cause difference in symmetry and bonding as shown in Cu^+Lf (Shongve et al., 1991).

2.2.5 Iron Lodging and Releasing: Intriguing Mechanisms

Despite their overall conformational homogeneity and the extreme conservation of the metal-anion coordinating ligands, sero-ovo-transferrins and lactoferrins vary tremendously in their binding affinity and release. While HSTf releases iron ~pH 6 (Mazurier et al., 1980), HLf retains up to pH 4 (Aisen and Liebman 1972). HLf binds iron 300 times stronger than HSTf (Aisen and Liebman 1972).

The events governing iron lodging and release are still more of speculation. It has been presumed that one of the initial steps in metal release may be the protonation of carbonate anion (to bicarbonate) leading to the moving apart of bicarbonate from iron-site and detachment from helix-5 (in the N-lobe) or may promote a change from bi-dentate to mono-dentate coordination (El Hage Chahine and Pakdaman 1995).

In STf, iron release occur at the low pH of endosomes (Rao et al., 1983). A hydrogen bonded "dilysine - pair" across the domains – viz., Lys 206 and Lys 296 (HSTf) – are present in the N lobe of HSTf and OTf. It is believed that at low pH this bond will get protonated, breaking the linkage, with consequent destabilization of closed-form, and domain opening (Dewan et al., 1993). This dilysine trigger has been mooted as the factor making STf and HSTf more acid labile (Dewan et al., 1993). But the recent manifestation that the mutated (i.e., Arg 210 to Lys) recombinant HLF_N lobe has resulted only in an alteration of binding stability by 0.5 pH units questions the validity of the above view (Peterson et al., 2000). Moreover, CLf, GLf and BLf have dilysine residues but are not bonding. The current view holds that the origin of this paradoxical binding nature may lie in the
contributions of the second shell residues surrounding the actual bonding residues (Baker et. al., 2000). A co-operativity in the form of an interaction of C-terminal helix to N-lobe, stabilizing the closed domain of latter, has also been proposed (Baker et.al., 2000).

Recently it has been deduced that many low molecular weight chelators/non synergistic anions bind to specific sites of Stf and Otf and modulate the iron release (Mizutani et.al., 2000, Zak et.al., 1997, He et.al., 1997). In vitro chelator mediated iron release studies have shown that two pathways exist for release: a mechanistic model which is hyperbolic in nature and a kinetic first-order version (Bailey et.al., 1997). The model proposed by Cowart et.al., (1986) involving a mixed ligand intermediate between domain closed holo-form and domain opened apo-form is widely accepted where the rate limiting step is the conformational change due to (nonsynergistic) anion binding followed by rapid removal of bound iron. The prerequisite of nonsynergistic anion binding for domain opening and subsequent iron release was clearly shown in the case of HSTfN (Kretchmar et.al., 1988), and diferric-HSTfN (Nguyen et.al., 1993).

Substantiating the above view, recent structural report of the sulphate-Nlobe-Otf complex identifies two sites for nonsynergistic anion binding ("regulatory anion binding sites" 1 and 2) in the interdomain cleft, and is shown to play key role in domain opening and synergistic anion release mechanism (Mizutani et.al., 2000, Muralidhara and Hirose 2000).

Muralidhara et.al., (2000) have proposed a model based on the three available structural forms of Otfn (viz., holo [Dewan et.al., 1993], an intermediate [Mizutani et.al., 1999] and the sulphate anion bound apo [Mizutani et.al., 2000]), explaining the origin of the dual pathway of iron release. The closed holo "X" form is converted into CO3²⁻ – iron loaded but open "Y" form with an anion (A) binding to each regulatory anion-binding site, yielding a mixed ligand intermediate of A• Otfn•Fe3⁺ – CO3²⁻ as proposed by (Cowart et.al., 1986). "Y" will then become non-coordinating
intermediate “Y1” by carbonate ion release; similarly “X” will transform to “X1” by binding of non-synergistic anion to site 1 and direct coordination to iron with concomitant release of carbonate, producing an \( \text{A} \cdot \text{OTf}_n \cdot \text{Fe} \) intermediate. In the Y1 state both Arg 121 and Ser 122 of site 2 are occupied by anion without coordinating iron. The structural state “X1” cannot receive anion at site 2; similarly the state Y1 can not have direct iron coordination by another anion. These states then undergo iron release by the attack of water molecules (Mizutani et.al., 2000) to Tyr 92-OH and Tyr 191-OH yielding a common apo-form “Z”. The structural transitions from X to Z and Y to Z are kinetically single step processes with respective to first order rate constants \( k_1 \) and \( k_2 \); and the intermediate states X1 and Y1 are assigned to the mechanistic model only.

2.3 Folding Stability

See chapter 7.

2.4 Glycosyl Moieties of Lactoferrins

All the reported lactoferrins are glycosylated with a uniqueness dictated by the differential possession of the number and location of potential and actual glycosylation sites, and the kind of glycan monomers; this feature could be extended to all members of the transferrin family except some piscine transferrins which lack carbohydrate attachments (Dautry-Varsat et.al., 1986). Glycoconjugation to Lf is N-linked and exists in N-acetyl lactosaminic and oligo mannosidic types (Montreuil et.al., 1980).

Glycosylation of lactoferrins is a well explored area (Legrand et.al., 1984) (Table: 2.1). Human, murine, caprine, bovine and porcine Lfs contain N-acetyl lactosaminic type biantennary glycans with \( \alpha 1,6 \) fucosylation on the basal N-acetyl glucosamine residue; respectively they have 2,1,4,4, and 1 glycosyl chains (Spik et.al., 1994).
While HLf has an additional peripheral α 1, 3 fucosylation, caprine, bovine and porcine have oligomannosidic type also. Presence of polynacetyllactosaminic type is exclusive to HLf. Human neutrophilic Lf has been found to be devoid of fucosylation (Derisbuorg et al., 1990).

Studies on the functional implications of Lf-glycosylation is far from complete. Comparative structural analysis of the differentially glycosylated native and recombinant (expressed in Aspergillus awamori) human milk Lf reiterate that attached oligosaccharides do not have any directive influence on the folding of Lf (Sun et al., 1999). Further observations on the interglobally located glycan chain of residue 545 of bovine Lf in comparison with HLf prompts to draw the conclusion that glycans tend to constrain the folding flexibility rather than affecting polypeptide conformation (Baker et al., 2000). Invariability in the secondary structure or solvent exposure of aromatic amino acids of deglycosylated apo-CLf substantiates this notion (Antonini et al., 2000); they further report that deglycosylation markedly reduces the resistance to denaturants in apo-CLf, but not in holo-Lf. While deglycosylated tryptic N-terminal fragment of HLf showed diminished affinity towards iron (Legrand et al., 1990), van Berkel et al., (1995) have observed that both forms of intact molecule is completely saturated with iron. The efficacy of partially deglycosylated CLf over untreated one in inhibiting Rotavirus-SA-11 suggests that in fact glycan chains hinder the exposure of respective recognition site (Antonini et al., 2000). This, along with the explicit demonstration of the irrelevance of the glycan oligomer in Lf-bacterial LPS interaction (van Berkel et al., 1995) as well as the independence of the binding of Lf to hepatic p45 Lf receptor/RHL-1 to the presence of galactose on Lf' (Bennat et al., 1997) adds to the preponderating evidences questioning the general notion of glycan's global involvement in molecular recognition, at least with respect to Lf.
Lf has been found to resist remarkably the action of proteases when glycosylated (van Berkel et al., 1995). This may be relevant in ensuring the functional intactness of Lf in the gastrointestinal tract (Kuwata et al., 1998; Spik et al., 1982).

<table>
<thead>
<tr>
<th>Species</th>
<th>Sugar content (%)</th>
<th>Number of glycans</th>
<th>Glycosylation sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equine</td>
<td>unknown</td>
<td>3</td>
<td>137, 281, 476</td>
</tr>
<tr>
<td>Buffalo</td>
<td>unknown</td>
<td>4</td>
<td>233, 281, 476, 545</td>
</tr>
<tr>
<td>Camel</td>
<td>unknown</td>
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<tr>
<td>Human</td>
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<td>2</td>
<td>137, 476</td>
</tr>
<tr>
<td>Bovine</td>
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<td>5</td>
<td>233, 281, 368, 476, 545</td>
</tr>
<tr>
<td>Murine</td>
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<td>1</td>
<td>476</td>
</tr>
<tr>
<td>Caprine</td>
<td>11.00</td>
<td>5</td>
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</tr>
<tr>
<td>Porcine</td>
<td>3.40</td>
<td>3</td>
<td>370, 476, 575</td>
</tr>
</tbody>
</table>

Table: 2.1. Characteristics of Lactoferrin Glycans from Different Species

2.5 Functions of Lactoferrin

A wide array of functions have been attributed to lactoferrins, although none could yet be branded as its primary role(s), and more confounding, many of which are independent of iron binding-nature (Brock et al., 1997; Lonnerdal and Iyer 1995). The accumulating reports on this aspect of Lf could be categorized as follows:-

2.5.1 Iron Absorption

Despite the identification of a receptor for lactoferrin in the small intestine (Hu et al., 1990), no proof is available to demonstrate the direct involvement of Lf in iron uptake across the mucosal membrane (Davidson and Lonnerdal 1988; Lonnerdal and Iyer 1995). The classical notion of ascribing an iron supply role to Lf (like that of STf) is still controversial (Davidsson et al., 1994; Kawakami et al. 1990; Schulz-Iell et al., 1991).
A very recent study (Hagivara et.al., 2000) in fact hints that in weanling mice Lf tends to inhibit iron uptake from the digestive tract (to protect from iron toxicity) when fed with excess iron-rich diet. The mechanism of regulation is unclear; however, this effect is absent from young mice. This points to the existence of a more dynamically balancing physiological equilibrium.

2.5.2 Anti-inflammatory Effects

During inflammation, iron accumulates at local inflammatory sites, with accentuated catalytic conversion of reactive oxygen species into toxic reactive hydroxyl radicals. While STf may be incapable of iron binding at the low pH of inflammatory lesions, Lf can do so (Lonnerdal and lyer 1995). Additionally, unlike STf which can donate iron to cell (in an endosome dependent way), Lf generally abstains from being an iron donor to cells so that it can detoxify and remove iron from the site of inflammation. Antiinflammatory effect of lactoferrins has been well proven in mouse models of arthritis (Guillen et.al., 2000); ingested apo-HLf has significantly diminished the extent of the disease in both rheumatoid and septic, as well as microbial counts. More startling is the lowered (10%) incidence of arthritis in HLf-transgenic mice compared to congenic normal littermates (29%).

2.5.3 Immunomodulatory Roles

HLf and CLf have been proved to be involved in the maturation of both immature B- and T- cells (Legrand et.al., 1997; Mazurier et.al., 1989). HLf and CLf up-regulate the expression of CD4 antigen in a cell cycle arrest-independent manner and is believed to be through the activation of MAP-Kinase pathway (Dhennin-Duthille et.al., 2000).

Lf has got modulatory impact on the activity of many cells and release of many cytokines. Cytotoxicity of NK cells has been found to be enhanced by Lf, against hematopoietic K562 and MDA-MB-231 breast epithelial cancer cells (Dhennin-Duthille et.al., 2000). HLf is reported to
elevate the random motility of human neutrophils, the production of Superoxide dismutase by these cells and the release of IL-8 (Gahr et al., 1991); in the same way CLf also induces IL-8 from human neutrophils (Shinod et al., 1996). Unlike the low-affinity binding of HLF to human neutrophils (Spik et al., 1994), CLf ligands more tightly to the same (Maneva et al., 1994). Lactoferricin (see section 2.4.10) has been reported as the active neutrophil enhancing region of CLf (Miyauchi et al., 2000).

Another exciting finding is the G1-arrest during apoptosis induction in human monocytic THp-1 cells by Lactoferricin-B (Yoo et al., 2000). All the regulatory molecules involved in G1-transition like cyclin H2, cyclin E, CDK2 and CDK4 are down regulated in Lactoferricin-B treated THP-1 cells, and is suspected to be controlled by ROS at a point upstream of the apoptosis cascade.

2.5.4 Antiviral Properties

Experimental evidences are accumulating proving the inhibitory effect of Lf on viral infections. Lf-sensitive viruses include enveloped ones like human cytomegalovirus (Harmsen et al., 1995), herpes simplex virus type 1 and 2 (Hasegawa 1994; Marchetti et al., 1996; Marchetti et al., 1998) human immuno-deficiency virus (Harmsen et al., 1995; Puddu et al., 1998) and hepatitis C virus (Ikeda 1998); those in the naked category include simian rotavirus SA-11 and poliovirus (Superti et al., 1997, Marchetti et al., 1999).

A generalisation drawn out is that Lf acts in the early phase of viral infection and is poorly influenced by saturation with different metal ions (Marchetti et al., 1998). In some case like HSV-1 and HIV, the Lf-mediated inhibition is presumed to arise from competition between both for the cell receptors (Wu et al., 1995; Rodriguez et al., 1995). Conversely a direct Lf-viral interaction is required for inhibition in others like HCV (Ikeda et al. 1998). Initial studies by Valenti et al. (2000) with Lf digests hints at the restriction of antiviral activity to intra-Lf segments. But in at least
one case intact molecule has been found to be more potent in inhibiting viral replication compared with lactoferricin-B (Hammer et al., 2000).

2.5.5 Antimicrobial Efficiency and Infant Food-formula

Plenty of reports are available indicating the antibacterial property of Lf beginning with the major report of Oram and Reiter (1968) (Arnold et al., 1977, 1980). Though the initial studies hinged on the iron-binding role of lactoferrin for its mechanism of action (Bullen et al., 1972; Reiter et al., 1975), Arnold et al, 1982 later proved that this can occur in an independent way also. These experiments, along with the observed greater resistance of breast-fed infants over formula-fed ones to intestinal infections (Cunningham et al., 1991; Dewey et al., 1995), strengthened the suggested protective role of breast-milk lactoferrin in infants.

Many of the in vivo studies in this area involve analyses of the effect of oral application of CLf and Lactoferricin B (Miyazaki et al., 1991; Nakasone et al., 1995; Bhimari et al., 1999). All these experiments paved way for the idea of development of the commercial "lactoferrin-enriched infant food formula. But most of the clinical observations on the lactoferrin supplemented formula-food fed infants conducted so far turned out to be rather ambiguous and flustering, failing to display any explicit effect on either the microflora (Balmer et al, 1989; Roberts et al., 1992), or iron uptake (Schulz-lell et al., 1991).

2.5.6 Antifungal Potencies

in vitro studies with Candida sp. have testified the potential of CLf and HLf as a preservative agent against fungal infection (van der Borg et al., 2000). Lf shows strain and pH dependent variations in the requirement of the minimum inhibitory concentration against Candida sp. Taking this lead, a clinical pilot study on the effect of lactoferrin against candidiasis in HIV patients is ongoing (van der Borg et al., 2000).
More significant is the protective effect of Lf against Trichophyton spp., the causative pathogen of one of the most common skin diseases dermatophytosis (Artis et.al., 1983).

2.5.7 Antitumour Activity

Evidences are available for the action of CLf against tumours of colon (Sekine et.al., 1997; Tsuda et.al., 1998), oesophagus and lung (Ushida et.al., 1999) besides the inhibitory effect of CLf on intestinal polyposis in Apc-min mice (Ushida et.al., 1998). Yet another promising result is the ability of CLf to inhibit the lung metastasis of colon carcinoma 26 cells. This latter effect is believed to be accomplished through concomitantly increased populations of cytotoxic GM1+ and CD8+ cells. There are no report yet indicating that Lf has got an antitumourigenic role in liver, kidney and thyroid. Abnormal expression of Lf gene in endometrial adenocarcinoma (Walmer et.al., 1995) as well as breast carcinoma (Charpin et.al., 1985) and leukemic myelopoiesis (Rado et.al., 1987) suggests an alteration in Lf gene regulation in malignant tissue (Teng et.al., 2000).

2.5.8 Lactoferrin in Pathology and Clinical Applications

In polymorphonuclear leukocytes of uremic patients, Lf granules shift to a perinuclear position unlike the normal even cytoplasmic distribution, as well as abnormalities in the release of granular Lf (Deicher et.al., 2000). Lf concentration in the tears of chronic Hepatitis C patients is found to be less (Abe et.al., 1999). In the localised amyloidosis of the seminal vesicle, lactoferrin has been identified as a major component (Tsutsumi et.al., 1996). The enhanced Lf secretion by goblet cells, epithelial cells and newly formed atypical glands of human sinus mucosa during chronic sinusitis may be to impart more protection to mucosa (Fukami et.al., 1993). Entry of Lf into enteric circulation has been experimentally deduced, strengthening the theory that the linkage
between primary sclerosing cholangitis and inflammatory bowel disease could be due to entry of released neutrophil-Lf to Lf enteric circulation (Pereira et al., 1998). Lf has also been reportedly associated with neurodegenerative disorders (Levengte et al., 1994; Kawamata et al., 1993).

Attempts have been made to explore the possibility of utilising the measurement of the level of lactoferrin as a diagnostic test for pathologic conditions. Although the use of stool test for foecal lactoferrin as a diagnostic test of invasive-inflammatory diarrhoea has been documented as effective (Fine et al., 1998), a recent study did not yield any standard values (Ruiz-Pelaezjg and Mattar 1999). Another pilot study suggests that foecal Lf-measurement is very useful in detecting colorectal diseases (Saitah et al., 2000). Trubnikov et al., (1998) have shown that tests for ferritin and Lf are useful in evaluation of bronchopulmonary inflammation activity, early detection of pulmonary and intrapleural suppuration, differential diagnosis of chronic nonspecific pulmonary diseases and lung cancer.

2.5.9 Recombinant Lactoferrin and Mass Production

This includes expression of mutant Lfs for finding its underlying structural mechanisms of functioning (Faber et al., 1996; Peterson et al., 2000), as well as many aiming at commercial applications. One of the notable results of the latter category is the creation of transgenic rice and tomato with an iron content double the amount of a Lf-deficient control (Anzai et al., 2000). This experiment offers great potential for the development of transgenic Lf food materials, to compensate iron deficiency, at least. A highthroughput scale Lf preparation has been achieved by fermentation of recombinant Aspergillus niger var awamori (Headen et al., 2000); safety and toxicity testing for oral and ophthalmological applications, besides clinical studies in gastroenterology and dermatology have shown no adverse effects. Phase III clinical trials
in the areas of gastroenterology, dermatology and ophthalmology are underway.

### 2.6 Functional Fragments of Lactoferrin

Further studies with pepsin digestion of CLf have identified a 25-residue peptide of CLf from the N-terminal as the antimicrobial-property conferring segment (Bellamy et.al., 1992). This peptide, termed Lactoferricin B (Lfcin B), extends from residues 27–41 and is effective against gram-positive as well as gram-negative bacteria (Bellamy et.al., 1992), fungi (Bellamy et.al., 1992) apart from the recently shown anti-metastasis role (Yoo et.al., 1997). Lfcin from bovine source is more potent than its human, murine or caprine counterparts (Vorland et.al., 1998).

More exploratory dissection of the Lfcin lead to the singling out of a core hexapeptide of residues 4–9 (Lfcin B₄₋₉) as the antimicrobial center of Lfcin B (Tomita et.al., 1994) with equal effectiveness as intact Lfcin B. Another fragment of Lfcin B, called Lfcin₄₋₁₄, also has the same but reduced activity while the N-acylated nonamer of Lfcin₄₋₁₂ has an improved efficiency (Wakabayashi et.al., 1998).

Intact Lf is highly basic with 5 Arg and 3 Lysines. Lfcin B has 3 Try and 3 Arg, which is similar to a group of Try and Arg containing hexapeptides formed by combinatorial chemistry (Blondelle and Houghten 1996). NMR analysis shows that the Lfcin B forms an amphipathic strained beta sheet with a polarization in the arrangement of the basic and hydrophobic residues (Hwang et.al., 1998). It is believed that Lfcin B attaches to membrane due to it's cationic nature, and will insert to the membrane forming an amphipathic structure, increasing the porosity of the membrane. Structure of Lfcin B₄₋₉ in SDS micelles is of an amphipathic nature with Trp residues inserted in the hydrophobic core (Schibli et.al., 1999). Another remarkable observation is the identification of a deca peptide capable of
inhibiting the fungal enzyme Proteinase K; this may have implications in the antifungal activity of Lf (Singh et al., 1998).

### 2.7 Porcine Lactoferrin: Studies Done So Far

The studies done so far on porcine lactoferrin are mainly in conjunction with its physiological attributes. No serious attempts have been reported analyzing the biochemical or structural features of PLf.

The first major attempt to characterize PLf was done by Roberts and Boursnell (1975) who reported the existence of at least four isoforms due to differential glycosylation; the pl ranges from 9.3 to 10, which on deglycosylation showed 9.65. The PLf glycosyl moieties have been reviewed by Spik et al., (1994). It has 3.4% glycosylation, the lowest among all reported lactoferrins (Spik et al., 1994). The only sugar chain of PLf is biantennary, N-acetyllactosaminic type with an α 1-6 fucosylation and N-acetylgalactose. The aminoacid sequence of PLf has been determined to be 686 (Alexander et al., 1992; Lydon et al., 1992).

A detailed analysis of the promoter of PLf gene has also been reported (Wang et al., 1998). The transcription start site was localised to residue G, 41 nucleotides upstream from ATG start codon. '5' flanking region of PLf gene possesses several putative cis-acting regulatory elements found in both housekeeping and inducible genes. The region upto -150 sufficed for basic promoter activity, whereas -780 was required for maximal promoter activity. More detailed analyses have indicated that the ubiquitous factors SP1, AP1 and mammary gland specific factor (MGF) might play significant roles in regulating the transcription of the PLf gene. Wang et al. also expressed the cloned PLf gene (1997).

Besides these, plenty of reports are available on the functional roles played, and distribution of PLf in pigs. Yang et al., (2000) has observed an increased serum and milk concentration of lactoferrin in the
lactating sows having inflammation. Transport of PLf across the intestinal epithelial cells to bile through blood has been established (Harada et.al., 1999). The low digestibility of PLf in the intestine of piglets has been noted by Drescher et.al., (1999), who correlated it with the increased immunity role played by lactoferrin in piglets. The interaction with lactoferrin, of a receptor specific for it has been identified in piglet intestine (Gislason et.al., 1994).