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INTERACTIONS OF LACTOFERRIN WITH MONOCYTIC AND MUCOSAL CELLS; ITS ROLE IN IRON UPTAKE AND ABSORPTION

by

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being a thesis submitted for the degree of Doctor of Philosophy in the Faculty of Medicine

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dedicated

to the memory of my mother

departed during the preparation of this thesis

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ABBREVIATIONS

α-	anti
apoLf	apolactoferrin (free of iron)
apoTf	apotransferrin (free of iron)
BSA	bovine serum albumin
Ci	Curie
cpm	counts per minute
d	dalton
DFO	desferrioxamine
ELISA	enzyme-linked immunoabsorbent assay
FCS	fetal calf serum
FeNTA	ferric nitrilotriacetate
Fe-Lf	ferric lactoferrin
Fe-Tf	ferric transferrin
FITC	fluorescein isothiocyanate
HSA	human serum albumin
Lf	lactoferrin
LfR	lactoferrin receptor
PBS	phosphate buffered saline
p	probability of significance
pI	isoelectric point
rpm	revolutions per minute
SD	standard deviation from the mean
SDS	sodium dodecyl phosphate
TX-100	Triton x-100
TCA	tricholoacetic acid
TEMED	N,N,N′,N′-tetramethylethylenediamine

Tf	transferrin
TfR	transferrin receptor
v/v	volume per volume
w/v	weight per volume

PUBLICATIONS

1. **M. Ismail** and J.H. Brock (1993). Binding of lactoferrin and transferrin to the human promonocytic cell line U937: effect on iron uptake and release. *J. Biol. Chem.*, in press.

2. R. Oria, **M. Ismail**, L. Sanchez, M. Calvo and J.H. Brock (1993). The effect of heat treatment and other milk proteins on the interaction of lactoferrin with monocytes. *J. Dairy Res.*, in press.

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SUMMARY

Lactoferrin is a non-haem iron binding glycoprotein present in virtually all mammalian body fluids, especially milk, as well as being secreted from the secondary granules of activated neutrophils. It is structurally similar to the plasma iron transport protein transferrin, and shares with it the ability to bind reversibly two ferric ions per molecule of the protein. This work is undertaken to investigate the possible role of lactoferrin in iron transport and the mechanism of iron transfer involved. The transfer of iron to the cells by transferrin occurs by a mechanism involving binding to a specific receptor followed by endocytosis, during which iron is retained by the cell and the receptor is recycled. Assuming that lactoferrin could deliver its iron utilising a similar pathway, this work has been focussed to study the interactions of lactoferrin with a promonocytic cell line, U937. Binding studies have shown that both lactoferrin and transferrin could bind to these cells. There are however some fundamental differences in the binding pattern. The total binding of lactoferrin to the cells was about ten times greater than total binding of transferrin but most of the lactoferrin binding was non-specific. In contrast most of transferrin binding occurred via a specific receptor. Nevertheless the number of specific binding sites on the cell is similar for both proteins (Lf, 3.0×10^6; Tf, 1.9×10^6) with the dissociation constant for the specific binding of lactoferrin (83 nM) 4-fold lower than that for transferrin (21 nM). Human lactoferrin did not inhibit binding of bovine lactoferrin or human transferrin, and vice versa, suggesting different binding sites exist for each protein. In addition its binding was not inhibited by 30 mM glucose or fucose, nor by incubating the cells with heparinase.

Two main biological effects that follow protein binding have been studied. The first is iron uptake from each protein; while transferrin was internalised, and accumulated intracellularly in the presence of 3 mM primaquine, lactoferrin on the other hand did not release its iron to the cell nor was it internalised. A slow apparent uptake of iron observed from lactoferrin probably resulted from transfer of ^{59}Fe from lactoferrin to unlabelled transferrin during culture. Bound lactoferrin, but not transferrin, was found to release its iron to the extracellular medium. The second effect of lactoferrin binding that was studied is its iron scavenging activity. Lactoferrin inhibited cellular uptake of iron from ferric nitrilotriacetate ($^{59}FeNTA$) but not from transferrin, suggesting that it may regulate uptake of potentially toxic non-transferrin-bound iron.

Another area of controversy surrounding lactoferrin function is its role in iron absorption. As well as the lactoferrin interactions with a monocytic cell line, this work has also included interaction with an enterocyte-like cell line, Caco-2. Transport of lactoferrin-bound iron across differentiated monolayer cultures of these cells grown in bicameral chambers was similar to that of ferric citrate, while transport of transferrin-bound iron was lower. Lactoferrin and transferrin themselves were not transported, although some proteolytically degraded material did cross the monolayer. This study has been reinforced by measurement of plasma lactoferrin levels in newly born infants undergoing different feeding regimes taken as a variable affecting these levels. This work has shown that plasma lactoferrin levels of breast fed infants are not significantly different from those of formula fed, suggesting that in the newly born, when the levels of endogenous iron are adequate, lactoferrin is not itself transported to the circulation, and may instead function as a regulator of iron absorption.

Taken together all these findings, it can be concluded that lactoferrin, unlike transferrin, is not an important iron donor to monocytic cells, nor does it enhance iron transport across the mucosal cells. Lactoferrin may instead serve to regulate uptake of iron in time of adequacy or from other sources which may otherwise be harmful to the cells.

CHAPTER ONE

REVIEW OF LITERATURE

PART ONE: REVIEW OF LITERATURE ON TRANSFERRIN AND LACTOFERRIN

1.1.1 GENERAL ASPECTS OF IRON METABOLISM

Iron is an essential element for all forms of life with the possible exception of lactic acid bacteria (Neilands,1981). It is required for normal cellular functions as iron is involved in numerous key biochemical reactions which govern the cell's metabolism. In man, most of the iron plays an important role as a component of haemoglobin and myoglobin involved in oxygen transport. However the smaller amount that occurs in the enzyme systems which catalyse the electron transfer and respiration reactions are vital for cell viability. Iron is also an essential component of proteins and enzymes that participate in the oxidation-reduction reactions other than those comprising cellular respiration which include those that protect against reactive oxygen species.

The total quantity of iron in the adult human male is about 4 g. Sixty percent of this iron is found in haemoglobin, 14 % in the myoglobin and tissue enzymes and most of the rest (21%) is stored as ferritin and haemosiderin, principally in the reticuloendothelial cells and hepatocytes (Morris 1987). The total body iron of a healthy mature newborn is in the range of 250-280 mg which is about 6 % of the total iron content of the adult (Heubers and Finch, 1987). In the infant, a higher percentage of iron is found in haemoglobin (75%) with less iron being stored (15 %) as compared to the adult.

Daily loss of iron in the adult male is about 1 mg and this can be reduced to 0.5 mg in the iron-deficient individual. In the infant daily loss is about

0.1 mg (Bothwell *et al.*,1958). Physiological iron losses occur through acute bleeding from the intestine, iron in bile, exfoliation of iron-containing mucosal cells from the intestine, urinary iron and skin desquamation (Bothwell *et al.*,1971). The amount of iron lost is actually small but it must be compensated by intake of dietary iron in similar amount in order to maintain a normal iron balance. An unfavourable balance will eventually lead to iron deficiency resulting in inadequate levels of one or more of the essential iron complexes. Excess of intake over excretion will lead to increasing accumulation of iron which can seriously impair cellular function. It has been estimated that more than 500 million people in the world are iron-deficient and several million are iron-overload (Halliwell and Gutteridge, 1985). Iron overload is especially dangerous since it can lead to the presence of forms of iron that can catalyse free radical formation which has been implicated to be involved in many degenerative diseases. Thus not only must sufficient iron be present for normal metabolic processes, but its uptake, transport and intermediary metabolism must be carefully controlled in order to prevent inappropriate iron loading and cellular and subcellular distribution.

The importance of iron derives from its ability to exist in two stable and interchangeable forms, ferrous (Fe^{2+}) and ferric (Fe^{3+}). Under physiological pH and ionic strength, Fe^{2+} predominates. Since it is a strongly hydrolytic ion with low solubility it tends to polymerise in aqueous environment (Halliwell and Gutteridge, 1985). Free iron also catalyses hydroxyl-radical-mediated oxidative damage whether within cells or in the extracellular fluid. In view of the solubility problems and the danger of free radical formation, free Fe^{3+} is present in exceedingly low concentration in biological fluids. Hence iron is bound by a number of

proteins which convert it to a stable and soluble form. These iron-binding proteins can be divided into two main categories (Brock, 1989):

(1) haem proteins such as haemoglobin and myoglobin, cytochromes and a variety of enzymes

(2) iron transport proteins (transferrins) and iron storage proteins (ferritin and hemosiderin).

This review will only concentrate on transferrins with special emphasis on lactoferrin.

1.1.2 TRANSFERRINS

1.1.2.1 Introduction

Transferrins are a group of iron-binding proteins which share the property of reversibly binding two ferric ions per molecule of protein (Aisen and Listowsky, 1980 ; Morgan, 1981 ; Brock, 1985). Members of the group which are all glycoproteins include the following (de Jong *et al.*, 1990):

(i) transferrin (siderophilin), the iron-transport protein in the blood of vertebrates and some invertebrates

(ii) ovotransferrin (conalbumin) of bird and reptile oviduct secretions and egg whites

(iii) lactoferrin (lactotransferrin), found in mammalian extracellular secretions especially milk and in the secondary granules of neutrophilic leucocytes

(iv) melanotransferrin, an integral membrane protein of human malignant melanoma cells.

The term transferrin is now universally used for the iron-transport protein of plasma, in preference to the earlier name of siderophilin, whereas lactoferrin is now almost always used for the iron-binding protein of milk, other secretions and neutrophil granules, as the alternative name lactotransferrin implies an iron transport function which is at best only doubtfully established (Brock, 1985). Hence in the following review, plasma transferrin will be referred to simply as transferrin and lactoferrin will be used instead of lactotransferrin.

1.1.2.2 Occurence and Synthesis of Transferrin and Lactoferrin

Transferrin was first isolated by Schade and Caroline (1946) from human plasma. On average, the serum level of transferrin is between 2-4 mg/ml at an iron saturation of about 30 %. However, both these figures vary considerably in pathological conditions (Morgan, 1981). Increased plasma transferrin concentrations are found in childhood, pregnancy, iron-deficiency anaemia and after oestrogen administration. Decreased plasma transferrin levels are found in conditions associated with iron overload, protein malnutrition and many other disorders. These changes are probably due to increased and decreased rates of transferrin synthesis respectively.

Lactoferrin was first isolated about thirty years ago by two groups of investigators, Groves (1960) who isolated lactoferrin from bovine milk and Johansson (1960) from human milk. Lactoferrin in contrast to transferrin is scarcely detectable in the serum with an average concentration of less than 1 mg/ml (Bennet and Mohla, 1976). In milk however, it is present at high concentrations ranging from about 7 mg/ml in colostrum to about 1 mg/ml in mature milk though it may rise again towards the end of lactation (Masson and Hereman, 1970; Hennart *et al.*, 1991). The amount of iron in milk on the other hand is only 0.1-0.3 mg/ml which would saturate lactoferrin at most by 20 %. However, it is found that actually only 6-8 % of the iron binding capacity of lactoferrin in human milk is utilised (Lonnerdal, 1990). Possible reasons suggested for this finding were firstly, low molecular weight ligands such as citrate which are able to chelate iron are present at considerably higher concentration than lactoferrin and secondly, most of the iron is present in the lipid fraction and in the casein micelles which is inaccessible for ligand exchange.

Lactoferrin levels in the cow are also highest in the colostrum (0.83 mg/ml) but the concentration in mid-lactation milk is very much lower, only about 0.1 mg/ml (Sanchez *et al.*, 1988). Apart from milk, lactoferrin is also found in a wide range of external secretions such as seminal fluid, tears, sweat, nasal and genital secretions (Masson *et al.*, 1966) as well as in the secondary granules of neutrophils (Masson *et al.*, 1969). In most cases lactoferrin appears to be largely devoid of iron as in the case of lactoferrin in milk.

The principal site of transferrin synthesis is the liver where it is produced by hepatocytes (Morgan, 1981), as shown in studies of isolated rat hepatocyte suspension using immunological techniques (Jeejebhoy *et al.*,

1975) and by the presence of specific mRNA in chick hepatocytes (McKnight *et al.*, 1980). Synthesis also occurs in other tissues such as placenta, Sertoli cells and the central nervous system (Skinner *et al.*, 1984). In the fetus, transferrin synthesis may occur in the yolk sac (Gitlin and Perricelli, 1970). The rate of synthesis varies considerably in pathological states. The major factors which are known to influence transferrin synthesis are the storage iron levels, certain hormones (especially oestrogen) and nutritional status.

Lactoferrin is synthesised in many of the secretory organs in which it is found (Masson and Heremans 1970). The iron nutritional status of the mother has no direct influence on milk content of lactoferrin (Hennart *et al.*, 1991; Prentice *et al.*, 1989). However, lactoferrin levels decreased in conditions of overall malnutrition (Houghton *et al.*, 1985), which suggests that protein energy malnutrition rather than iron defeciency influences lactoferrin synthesis in the mammary gland.

1.1.3 STRUCTURE OF TRANSFERRIN AND LACTOFERRIN

1.1.3.1 General Aspects

Transferrin and lactoferrin are both monomeric glycoproteins with 59% homology in their amino acid sequence (Metz-Boutigue *et al.*, 1984); (**Fig.1**). The single polypeptide chain of human serum transferrin consists of 679 amino acid residues, which together with two oligosaccharide chains gives a calculated molecular weight of 79,570 (MacGillivray *et al.*, 1983). The polypeptide chain of human lactoferrin consists of 692 amino acid residues with a molecular weight of 80,600 (Rey *et al.*, 1990; Powell and Odgen, 1990).

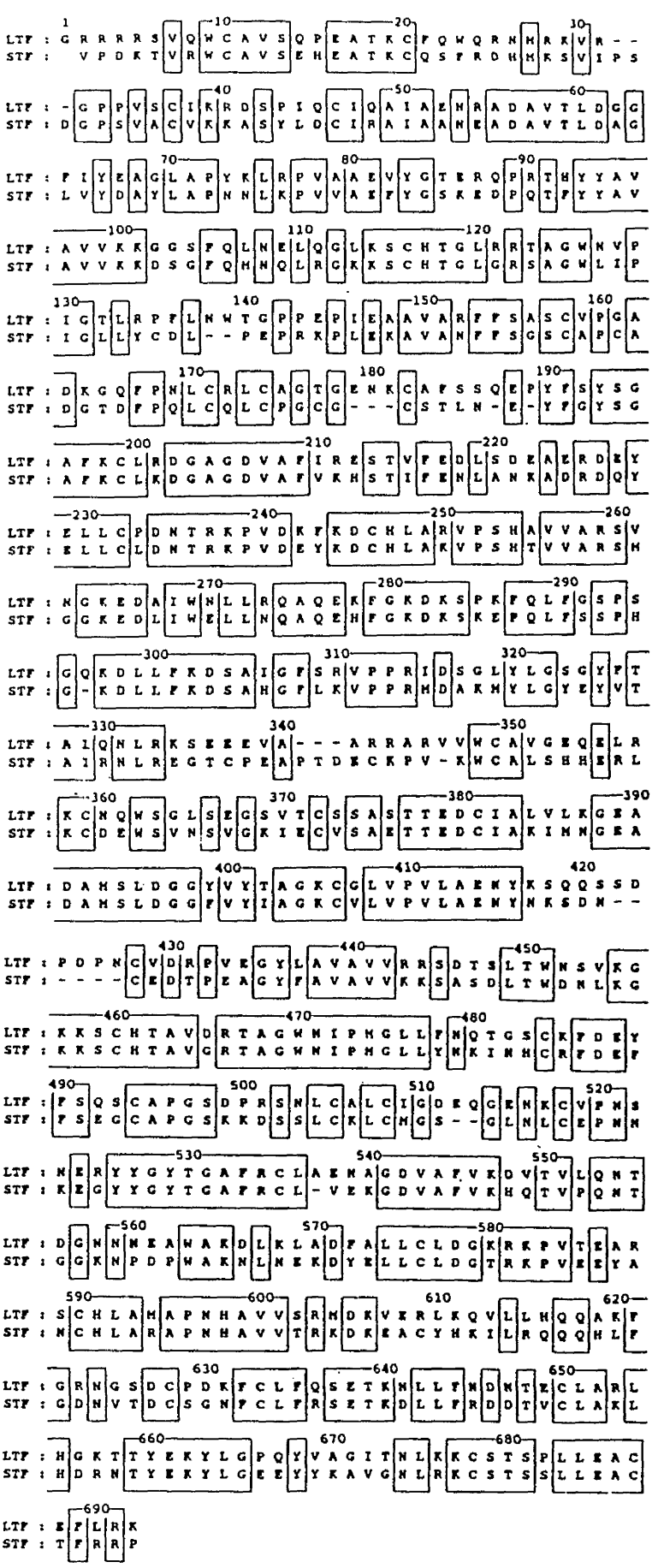

FIG. 1: Amino acid sequence of human lactoferrin(LTF)(Rey *et al.*, 1990) and human transferrin(STF)(MacGillivray *et al.*, 1983)

Transferrin has an isoelectric point in the range of 5.4-5.9 with diferric transferrin having a slightly higher pI than apotransferrin (Keller and Pennel, 1959; Hovanessian and Awdeh, 1976). Lactoferrin has a much higher pI than transferrin with a value in the range 8.4-9.0 (Van Snick and Masson, 1976). This is due to the presence of a large number of basic residues at the surface of the lactoferrin molecule (Anderson *et al.*, 1989). Because of its high isoelectric point, lactoferrin displays a marked tendency to complex with other molecules.

1.1.3.2 Overall Molecular Organisation

The overall molecular organisation of lactoferrin molecule as described by Anderson *et al.* (1987; 1989; **Fig. 2**) is essentially the same as that of rabbit transferrin (Bailey *et al.*, 1988). The three-dimensional structure of human milk lactoferrin as determined by X-ray crystallography at 3.2 Å resolution (Anderson *et al.*, 1989) revealed that the single polypeptide chain is folded into two lobes. The two lobes are referred to as the N-lobe, comprising the N-terminal half of the molecule and the C-lobe consisting the C-terminal half. The N-lobe is made up of residues 1-332 and the C-lobe constitutes residues 344-703.

The shape of each lobe can be described as a prolate ellipsoid (Taylor *et al.*, 1983) of approximate dimensions 55 x 35 x 35 Å . Their axes run roughly anti-parallel to each other at an angle of 150° between them. One lobe is superimposed on the other by a rotation of about 180°, followed by a translation of 25 Å but the equivalent parts of the two lobes do not superimpose on each other. The two lobes are connected by a three turn α-helix consisting of residues 333-343.

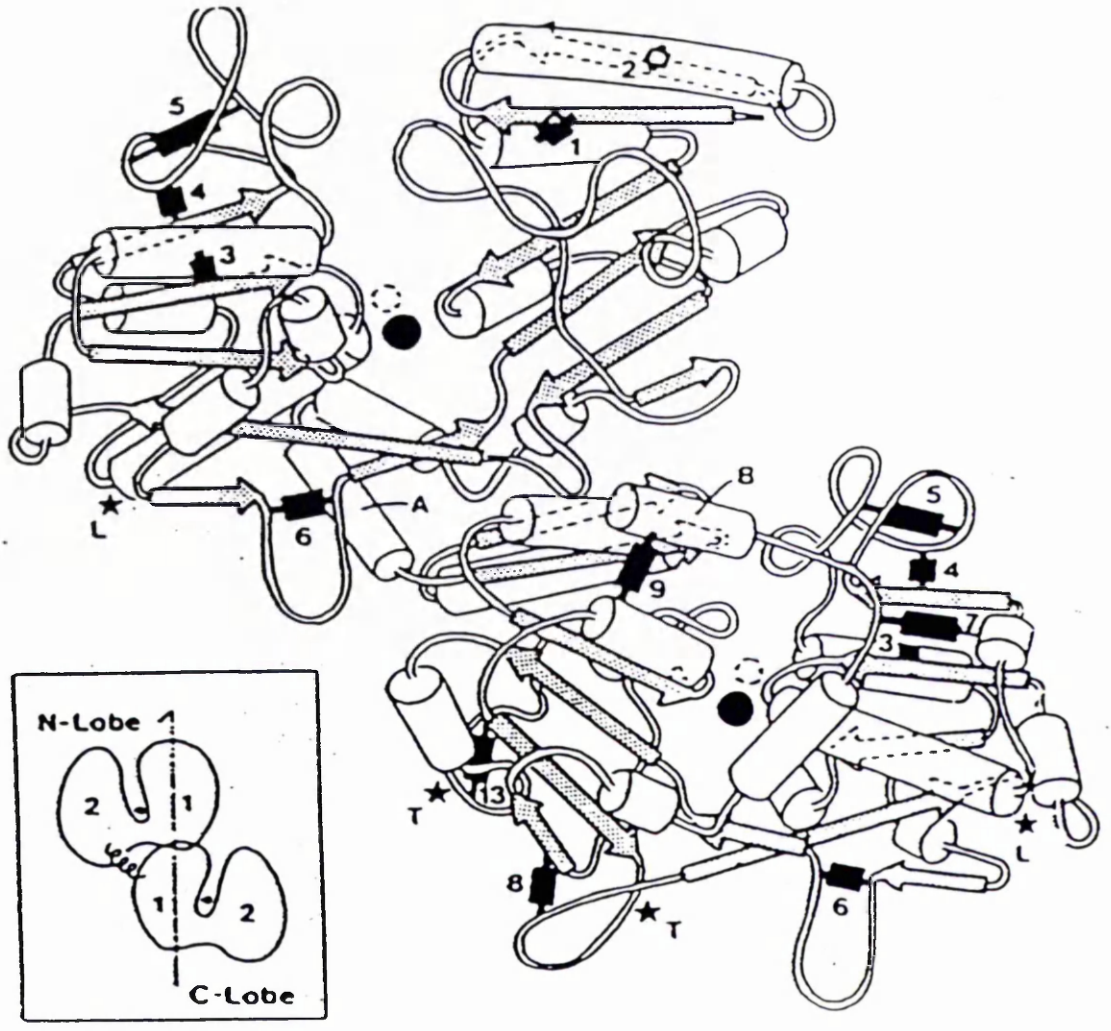

FIG. 2: Schematic diagram of the complete human lactoferrin molecule. The positions of carbohydrate attachment are marked: T, human transferrin; L, human lactoferrin. The disulphide bridges are indicated by solid lines, and the iron- and bicarbonate- (or carbonate) binding sites are indicated by filled or open circles, respectively (From Baker *et al.*, 1987).

In transferrin, the connecting peptide is two residues longer and includes three prolines. Both lobes have very similar structures with at least 40 of their amino acid sequence being homologous. Each lobe is further organised into two dissimilar domains, having about 160 residues each. Thus the total number of domains in the molecule is four; N1, N2, C1 and C2.

The basic folding pattern of secondary structures is illustrated in **Fig.3** (Anderson *et al.*, 1989, Bailey *et al.*, 1988). A similar folding pattern is observed within the N- and C- lobes. Each domain consists of a central core of five or six irregular twisted ß-sheets, connected to each other by helices and loops. Helices and mixed ß-sheet make up about 41% and 24% respectively of the secondary structures. The chain starts in domain N1 and initially folds to form the first four strands in a ß-sheet; a, b, c and d which are connected through helices 1,2 and 3 respectively. From strand d, the chain traverses into the lower domain N2, where it folds into ß-sheet consisting of strands e, f, g, h and i. These strands are connected to each other by helices 4, 5, 6, 7, 8 and 8a. After strand i, the chain returns to domain N1 through strand j where the folding in domain N1 is completed by helix 9, β-sheet k and terminate as helices 10 and 11. Helix 12 then connects the N-lobe to the C-lobe.

Strands of β-sheet e, i and j together with helices 10 and 11 form an important part of the lobe. These structures not only connect the two domains but they provide a flexible hinge which allows movements of the two domains with respect to one another. This will result in the opening and closing of the iron binding cavity.

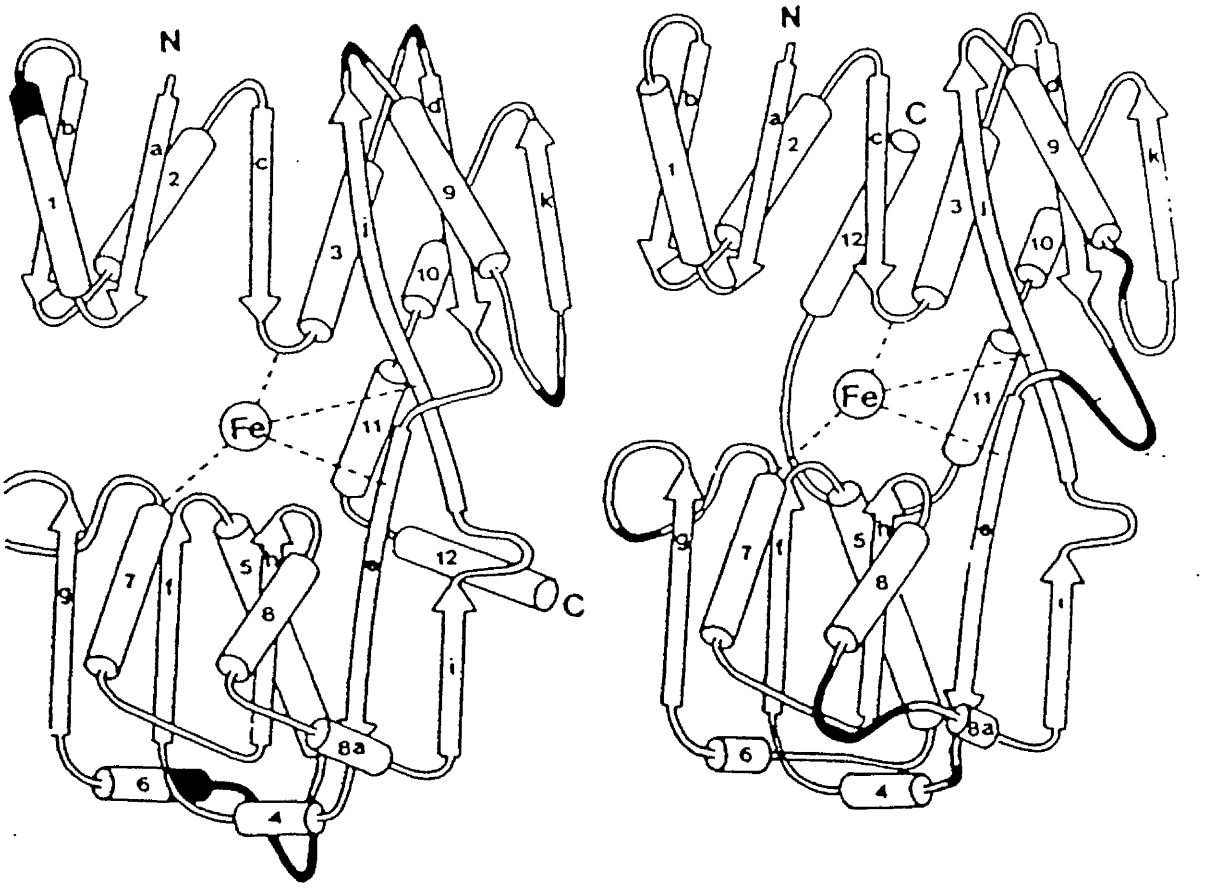

FIG. 3: Schematic representation of the folding pattern for the N-lobe and C-lobe of human lactoferrin. Helices are shown as cylinders; β-strands as arrows. (From Anderson *et al.*, 1989).

1.1.3.3 Disulphide Bridges

There are 16 disulphide bridges in lactoferrin and 19 in transferrin (Williams, 1982). The disulphide bridges in both proteins are located in quite homologous positions. In lactoferrin six disulphide bridges are located in the N-lobe and ten in the C-lobe. There are no disulphide bridge linking the two lobes. The fact that the C-lobe contains 4 additional disulphide bridges could explain the stability towards denaturation and proteolysis.

1.1.3.4 Iron and Anion Binding Sites

There are two iron-binding sites per molecule of transferrin or lactoferrin. The iron site is found in each lobe of the protein molecule. They are located in the interdomain cleft about 4.2 nm apart. The iron atoms are deeply buried in the cleft at least 1 nm from the exterior of the molecule. They are surrounded by a hydrophilic environment with numerous polar side chains in the vicinity.

The coordination sphere of the iron atom is the same for both lobes. Each iron atom is coordinated to four protein ligands and two non-protein ligands. The protein ligands are provided by one carboxylate oxygen, two phenolate oxygens and one imidazole nitrogen. This has resulted in the iron site being highly anionic and easily attracts cations with a high positive charge. Therefore Fe^{3+} ions which favour anionic oxygen ligands bind more strongly (binding constant: 10^{20} M^{-1}) than Fe^{2+} (binding constant: 10^{3} M^{-1}) (Baker *et al.*, 1987).

In the N-lobe of lactoferrin (**Fig.4**), the carboxylate oxygen is provided by Asp 60, two phenolate oxygens are side chains of Tyr 92 and Tyr 192, and His 253 provides the imidazole nitrogen. Asp 60 and Tyr 192 are trans to one another while Tyr 92 and His 253 occupy cis positions. In the C-lobe, the corresponding residues are Asp 395, Tyr 528, Tyr 435 and His 597. All these ligands are widely spaced along the polypeptide chain.

The carboxylate group of Asp 60 in the N-lobe and Asp 395 in the C-lobe has a dual role. One oxygen is bound to iron while the other oxygen forms two hydrogen bonds linking domain 1 to domain 2. The other three protein ligands do not seem to be constrained by the surrounding protein structure and this has resulted in greater flexibility in the iron binding site.

Beside the four protein ligands, there are two remaining octahedral positions surrounding the iron atom which are filled by non-protein ligands. Binding of iron to lactoferrin and transferrin occurs with synergistic binding of a bicarbonate (or carbonate) (Bates and Schlabach, 1975; Anderson *et al.* 1989). Kinetic and spectroscopic data indicate that binding of bicarbonate precedes iron binding. The role of bicarbonate in binding is probably two fold (Baker and Lindley, 1992). First, it neutralises positive charges which might otherwise repel ferric ion. Second, it partially prepares the iron binding site of the apoprotein by adding two more potential ligands (two carbonate oxygens) to the four protein ligands already present in the site.

Crystallographic refinement of lactoferrin structure at 2.8 Å (Anderson *et al.*, 1989) has revealed that the carbonate fits perfectly in a pocket between the iron and two positively charged groups, an arginine side

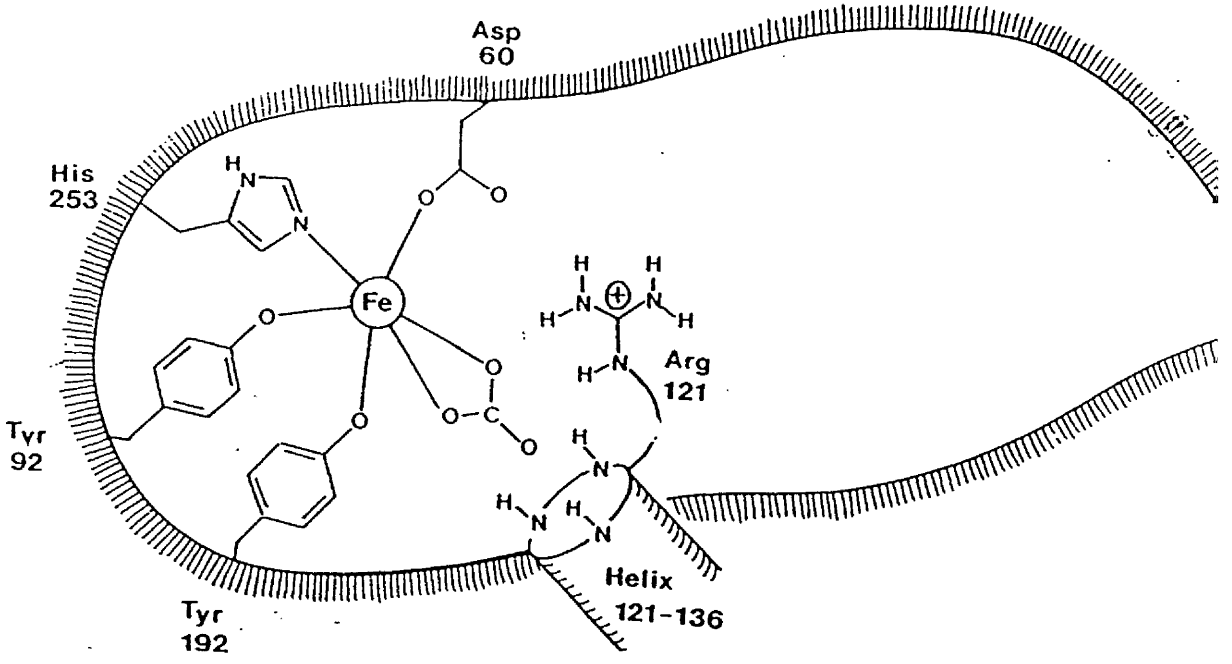

Fig. 4: Schematic representation of the iron and anion-binding sites in lactoferrin (N-lobe) (from Anderson *et al.*, 1989)

and the N-terminus of helix 5. One oxygen of the carbonate is bound to iron and also forms a hydrogen bond with Arg 121. The second oxygen also binds to iron and forms a hydrogen bond with the peptide bond NH of residue 123. The third oxygen makes two hydrogen bonds with the NH of residue 124 and the side chain of Thr 117 (see **Fig. 3**).

1.1.3.5 Carbohydrate Moiety

Human transferrin and lactoferrin contain approximately 6 % carbohydrate. They both have biantennary glycans of the N-acetyllactosaminic type (Spik, *et al.* 1982; Spik *et al.,* 1985) (**Fig. 5**). However transferrin in addition possesses triantennary glycans, but the biantennary glycans are more prominent (**Fig. 6**). In transferrin, the glycans are located in the C-lobe at residues 413 and 611. Both glycans are devoid of fucose residues. The glycans of lactoferrin from human milk on the other hand are fucosylated. They are located in each lobe in homologous positions at residues 137 and 490 (Montreuil *et al.*, 1985). However a later study by Derisbourg *et al.* (1990), revealed that both glycans in neutrophilic lactoferrin are devoid of fucose residues just like in transferrin.

1.3.6 Metal Binding to Lactoferrin and Transferrin

In vivo, iron is the most important metal bound by transferrins. As mentioned earlier transferrin and lactoferrin are both capable of binding two ferric ions per molecule of protein. Hence transferrin and lactoferrin may exist in the apo-, mono- (with either the N- or C-terminal binding sites occupied) and diferric forms.

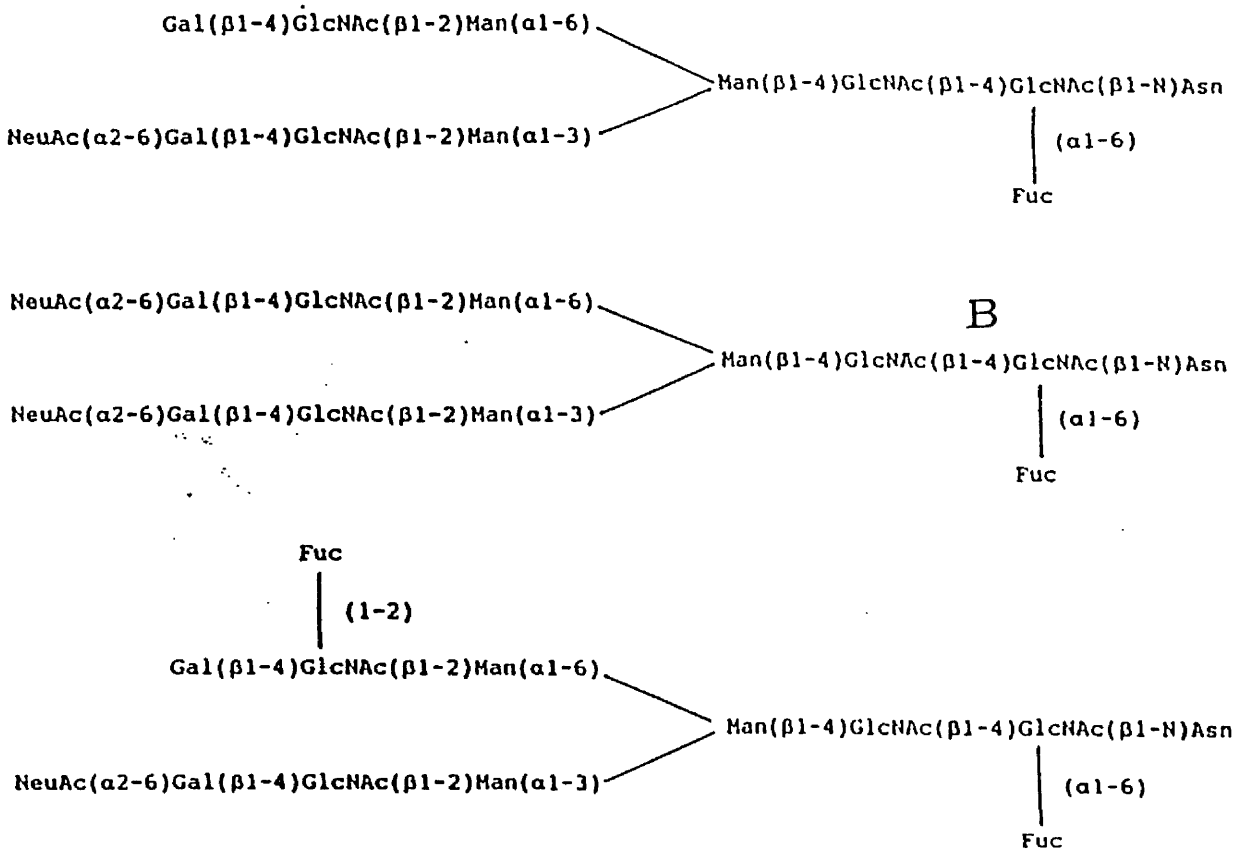

Fig. 5: Structure of glycans of human lactoferrin with B being the principle glycan (Spik *et al.*, 1985)

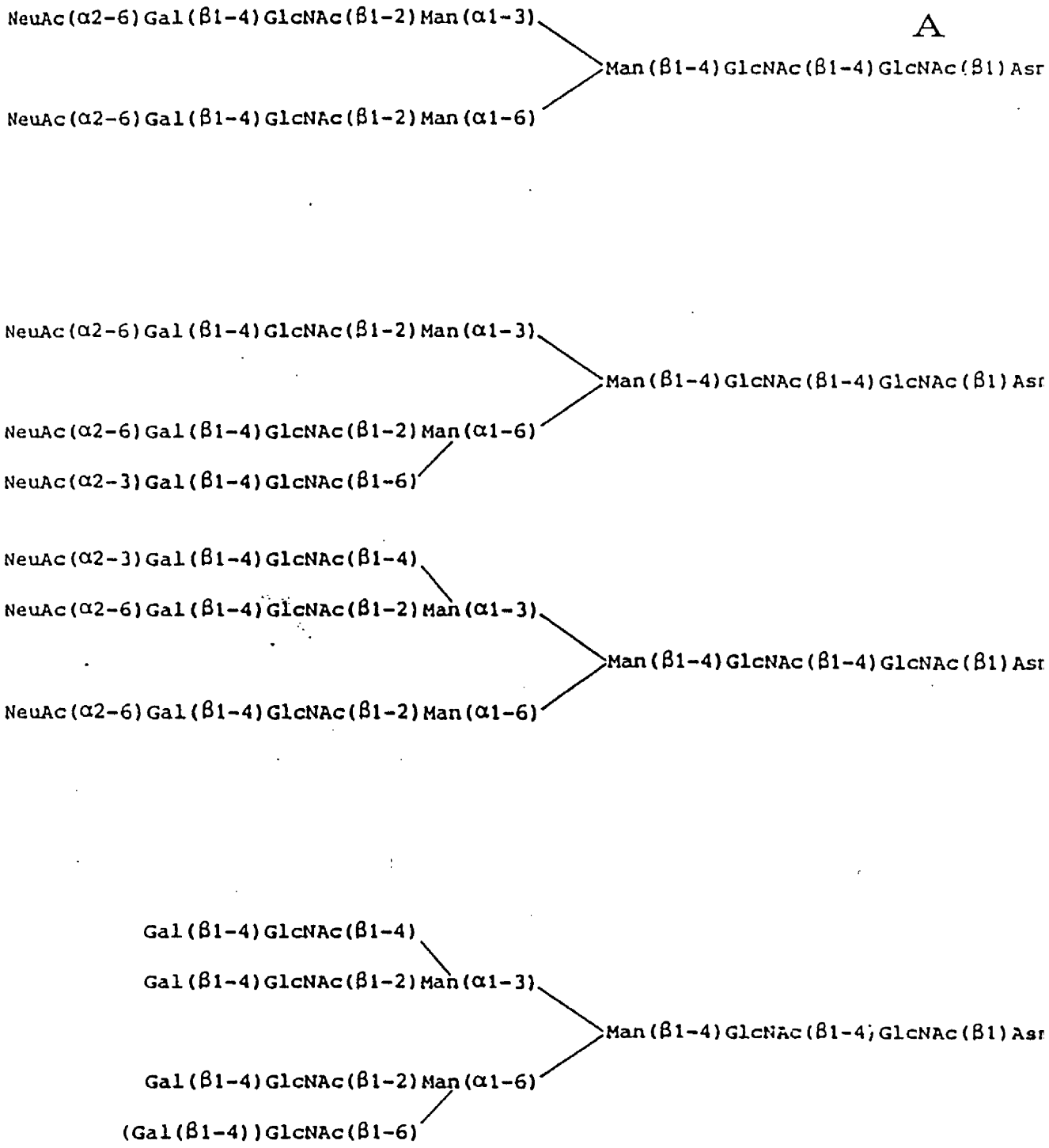

FIG. 6: Structure of glycans of human transferrin with A being the principle glycan (Spik *et al.*, 1985).

Apotransferrin and apolactoferrin are colourless but develop a red brown colour when iron is bound, giving λmax at 465-470 nm.

Iron binding by transferrins varies with pH (Morgan, 1981). It is maximal at about pH 7.0. As the pH is reduced, the iron starts to dissociate from the molecule at about pH 6.5 and is completely dissociated by pH 4.5 for transferrin. Lactoferrin, by virtue of its higher binding constant, retains its iron until the pH is reduced to about 2 (Masson and Heremans, 1968). Hence, under physiological conditions, the affinity constant for diferric transferrin is of the order 10^{24} M^{-1}, but this value decreases to less than 10^{10} M^{-1} at pH 4.5. The affinity constant of lactoferrin on the other hand is 26 times greater than that of transferrin at pH 6.4 (Aisen and Leibman, 1972) Therefore, despite their high sequence identity and generally similar binding properties, lactoferrin binds iron more strongly than transferrin and at distinctly lower pH values. This difference in iron binding may be critical to the different roles played by these two proteins.

Princiotto and Zapolski (1975) observed a difference in binding affinities for iron between two sites of both proteins. Factors which influence preferential binding to either site depend not only on the binding affinities of the sites but also on pH and the nature of the iron donor (Brock, 1985). Thus, when nitrilotriacetate is used as an iron donor to transferrin, the C-terminal site is preferentially occupied. With other donors including ferric citrate, ferric oxalate, ferrous ammonium sulphate or ferric chloride, iron binding favours the N-terminal site even though the C-terminal site has a higher affinity constant (Zapolski and Princiotto, 1977; Aisen *et al.*, 1978; Evans and Williams, 1978).

Recent work on the crystal structure of human apolactoferrin at 2.8 Å resolution by Anderson *et al.,* (1990), has shown that a substantial conformational change accompanies iron binding and release. In apolactoferrin, the N-lobe is in an open configuration, whereas the C-lobe is a closed configuration as in the iron-bound structure. The open structure is due to the opening of the interdomain cleft. This is brought about by a rotation of the N2 domain about a hinge at the back of the iron binding site (**Fig. 7**). The rotation axis lies almost exactly along the axis of helix 11. N2 domain rotates about 54 relative to domain N1 causing the binding cleft to open wide.

The opening of the cleft exposes three basic side chains previously buried within it (Arg 121, Arg 210 and Lys 301). These side chains tend to attract the bicarbonate anion which is then bound to the N-terminus of helix 5. Four of the six metal ligands (Tyr 92, Tyr 192 and the two carbonate oxygens) are then in a position to bind the ferric ion to the N2 domain. Binding is completed by rotation of N2, closing the cleft with Asp 60 and His 263 completing the coordination. Asp 60 further links the two domains by hydrogen bonding.

Upon iron binding the isoelectric point increases (Keller and Pennell, 1959; Hovanessian and Awdeh, 1976). This causes molecules of different iron content to elute sequentially from DEAE cellulose columns with a gradient of increasing ionic strength (Lane, 1973). The structure of the protein molecule becomes more compact and as a result, the protein becomes less susceptible to proteolytic degradation (Brock *et al.*, 1976; Esparza and Brock, 1980).

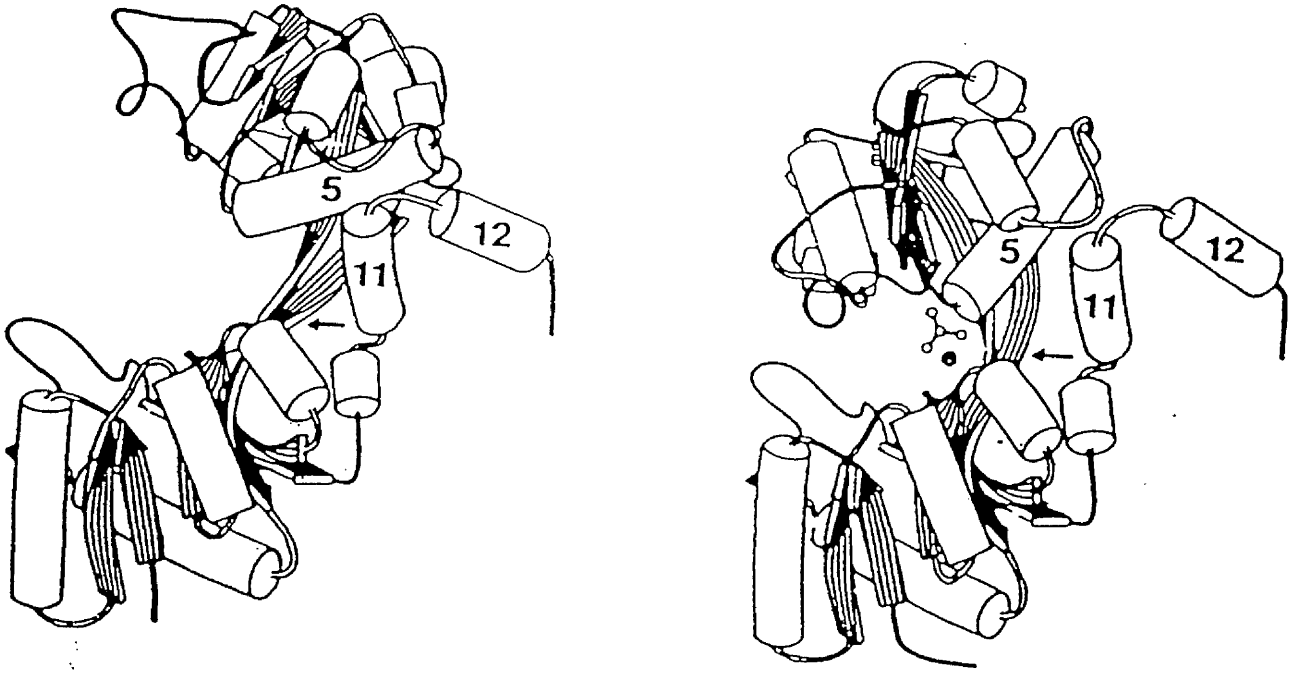

Fig. 7: Schematic representation of the N-lobe polypeptide chain fold showing the conformational change between open (left) and closed (right) forms. An arrow marks the approximate hinge point in the two anti-parallel b-strands connecting the two domains (from Anderson *et al.*, 1990)

1.1.3.7 Metal and Anion Substitution

Transferrins are capable of binding a large number of other metal ions other than iron at the same binding site. Metal ions which have been found to bind to transferrin include divalent and trivalent metal ions such as copper, vanadium, chromium, aluminium, cobalt, gallium, manganese, plutonium, indium, zinc, terbium, europium, platinum, americium and curium (reviewed by Huebers and Finch, 1987). Some of these cations, like gallium, bind with an affinity close to that of iron. Lanthanide ions also bind to transferrin but much more weakly.

Some iron chelates, such as nitrilotriacetate, can also act as synergistic anions. Substitution of oxalate for carbonate in the C-lobe of lactoferrin has been shown to occur without any change to the overall protein structure (Baker and Lindley, 1992).

1.1.3.6 Comparison Between Lactoferrin and Transferrin Structures

The lactoferrin structure is consistent with known structural data on transferrin. There is a high level of amino acid sequence homology between lactoferrin and transferrin. The iron binding sites in both proteins are also similar.

A comparison of the diferric human lactoferrin and rabbit transferrin structures (Baker and Lindley, 1992), has revealed two interdomain interactions which differ between the two proteins. These interactions could account for the characteristic differences in binding properties of lactoferrin and transferrin. Firstly, a salt bridge across the N-lobe domain interface between Lys 301----Glu 216 exists in diferric

lactoferrin but not in transferrin. An equivalent interaction does not occur in the C-lobe of lactoferrin either. Secondly, instead of a salt bridge, the corresponding Lys in transferrin (Lys 296) is differently oriented and makes close contact with another Lys (Lys 206) from other domain. The salt bridge is replaced by a hydrogen bond between Ser 298 and Glu 212. These two changes are close to the iron site in the hinge region and could account for the lower acid stability towards iron binding of the N-lobe of transferrin as compared to lactoferrin.

Another difference between lactoferrin and transferrin lies in the distribution and composition of the glycan chains. Those of lactoferrin are being distributed one on each lobe, whereas for transferrin, both glycan chains are bound to the C-terminal lobe only. Also as mentioned earlier, the glycans of milk lactoferrin has terminal fucose residues but those of neutrophilic lactoferrin like transferrin are devoid of fucose. Studies on the interaction of lactoferrin with the small intestine receptor (Davidson and Lonnerdal, 1988), suggest that the fucosylated glycans on the carbohydrate chain of milk lactoferrin are necessary for receptor recognition. However, Anderson *et al.*, (1989) have focussed attention on the first 55 residues of transferrin and the first 40 residues of lactoferrin, which contain at least nine basic side chains as the likely receptor binding region.

1.1.4 FUNCTIONS OF LACTOFERRIN AND TRANSFERRIN

1.1.4.1 Introduction

A number of biological functions have been proposed for transferrin and lactoferrin. Many of these are still controversial particularly in the case of

lactoferrin. Functions of lactoferrin and transferrin are dependent on the ability of the proteins to bind iron very tightly and reversibly. They both share the common function of controlling the levels of free iron in the biological fluids. While a major biological role as an iron transport and delivery protein has been established for transferrin, there is no well defined role for lactoferrin (Sanchez *et al.,* 1992). However both proteins have been implicated as bacteriostatic agents, aiding in the body's defences against bacterial infection. A role in the suppression of iron catalysed free-radical formation (Baldwin *et al.*, 1984) has been proposed for lactoferrin. Although there are reports of receptors for lactoferrin on intestinal cells, it is not clear whether lactoferrin plays a role in iron absorption. Lactoferrin has also been reported to stimulate growth of some cell lines but little is known about the mechanisms of growth stimulation.

1.1.4.2 Iron Donation to Cells

(a) Transferrin receptor (TfR)

It is now well accepted that iron donation is a major function of transferrin. Cells which require iron express a receptor for transferrin which has been well characterised (Sutherland *et al.*, 1981). Transferrin receptors are present on virtually all proliferating, differentiating, and haemoglobin-synthesizing cells (Trowbridge and Omary, 1981; Iacopetta and Morgan, 1983) (**Fig. 8**).

The major structural features of the transferrin receptor of different species, based on studies in human, mouse, rat and chicken cells are very similar (reviewed by Dautry-Varsat, 1986). The receptor is a transmembrane, homodimeric glycoprotein with a

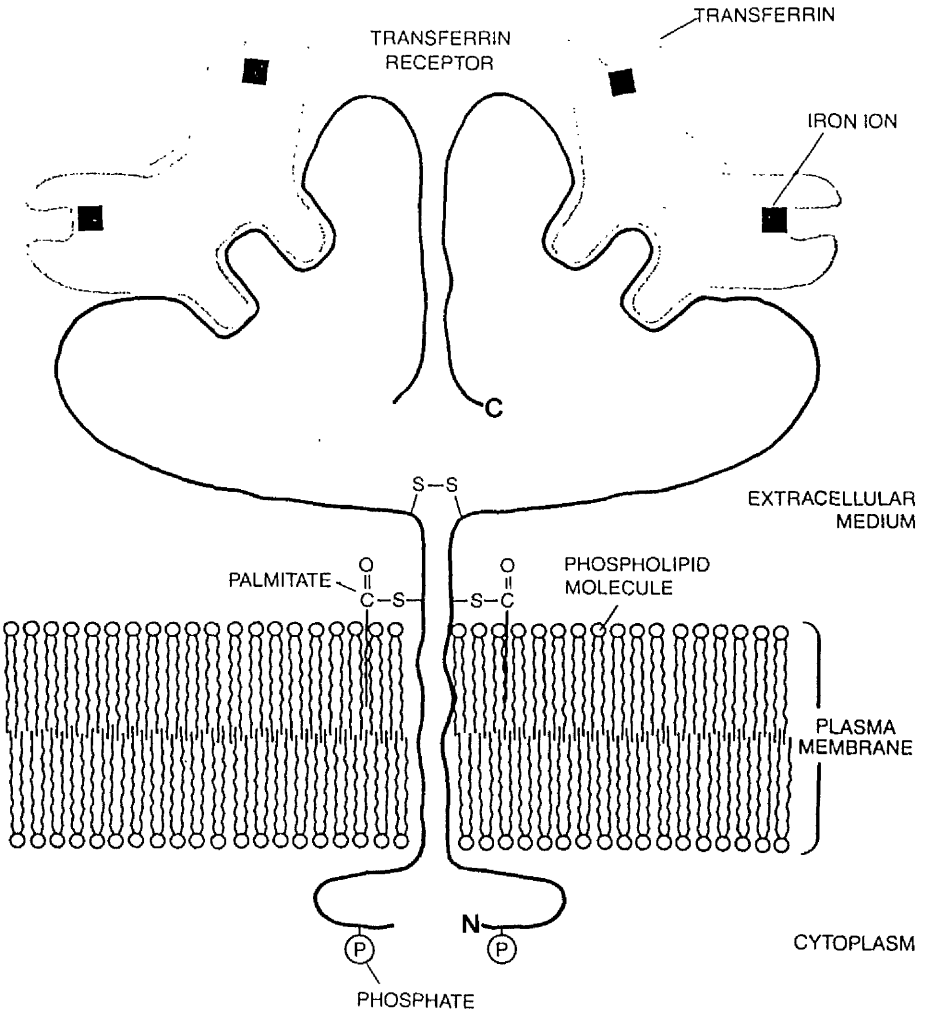

Fig. 8: Structure of the transferrin receptor (from Dautry-Varsat, 1986).

molecular weight of 180,000 dalton. The 90,000 dalton subunits, are covalently linked by a single disulphide bridge and can bind one transferrin molecule per subunit. Each subunit can be divided into three domains. The cytoplasmic N-terminal domain consists of 61 amino acids and is phosphorylated at residue Ser 24. The hydrophobic transmembrane domain which is inaccessible to proteolytic attack, consists of 28 residues; 14 hydrophobic and 14 uncharged residues. This domain presumably contains the signal for translocation of the receptor across the membrane.

Cys 62 at the internal face of the cytoplasmic membrane, is acylated by a palmitate. These fatty acids are thought to anchor the receptor hydrophobically to lipids in the plasma membrane of the cell. The rest of the amino acids (671 residues) make up the external domain. The disulphide bridges are formed between residues Cys 89 and Cys 98 with their homologues. The external domain is N-glycosylated at Asn 251, 317 and 727.

TfR preferentially binds iron-containing transferrin molecule rather than apotransferrin. Affinity constants for binding of diferric transferrin molecule have been found in the range of 5×10^6 - 2.3×10^9 M^{-1}, depending on temperature, pH and cell type (Young and Aisen, 1980). Using the interaction of different forms of ^{125}I-labelled transferrin and rabbit reticulocytes, Young *et al.* (1984) have found that the affinity constant for receptor binding is 4.6×10^6 M^{-1} for apotransferrin, 2.5×10^7 M^{-1} for monoferric transferrin (C-terminal). 2.8×10^7 M^{-1} for monoferric (N-terminal), and 1.1×10^8 M^{-1} for diferic transferrin. It therefore seems that the amount of iron taken up depends on the number of each molecular species present.

(b) Transferrin-to-Cell Cycle

In most mammalian cell types, the uptake of transferrin iron occurs by receptor mediated endocytosis (Theil and Aisen, 1987) (**Fig. 9**). Once the transferrin has bound to its receptor, the transferrin-transferrin receptor (Tf-TfR) complex accumulates in coated pits. Coated pits are specialised regions of the cell surface that mediate endocytosis of a variety of receptors and their extracellular ligands (Goldstein *et al.*, 1985). These pits are covered on the outer side with lattices of a fibrous protein, clathrin. Studies by Iacopetta *et al.* (1988) and Miller *et al.* (1991) have shown that recycling receptors such as those for transferrin can influence the formation of coated pits and promote the growth of clathrin lattices.

The molecules present in the coated pits on the other hand can interact with the cytoplasmic domains of the receptor proteins that become concentrated above them (Pearse, 1987; Morris *et al.*, 1989). An interaction between the transferrin receptor and these proteins has not been demonstrated but it has been shown that the ability of this receptor to internalise with high efficiency depends upon this interaction (Collawn *et al.*, 1990). The cytoplasmic domain of the transferrin receptor has been suggested to contain a specific signal sequence located within amino acid residues 19-28 that determines high efficiency endocytosis (Iacopetta *et al.*, 1988). Within 1-2 min, the coated pits are pinched off from the outer surface of the cell to be internalised as clathrin-coated vesicles. As the clathrin coated vesicles move deeper into the cytoplasm they rapidly lose their clathrin coats in an energy requiring process to form smooth surfaced vesicles.

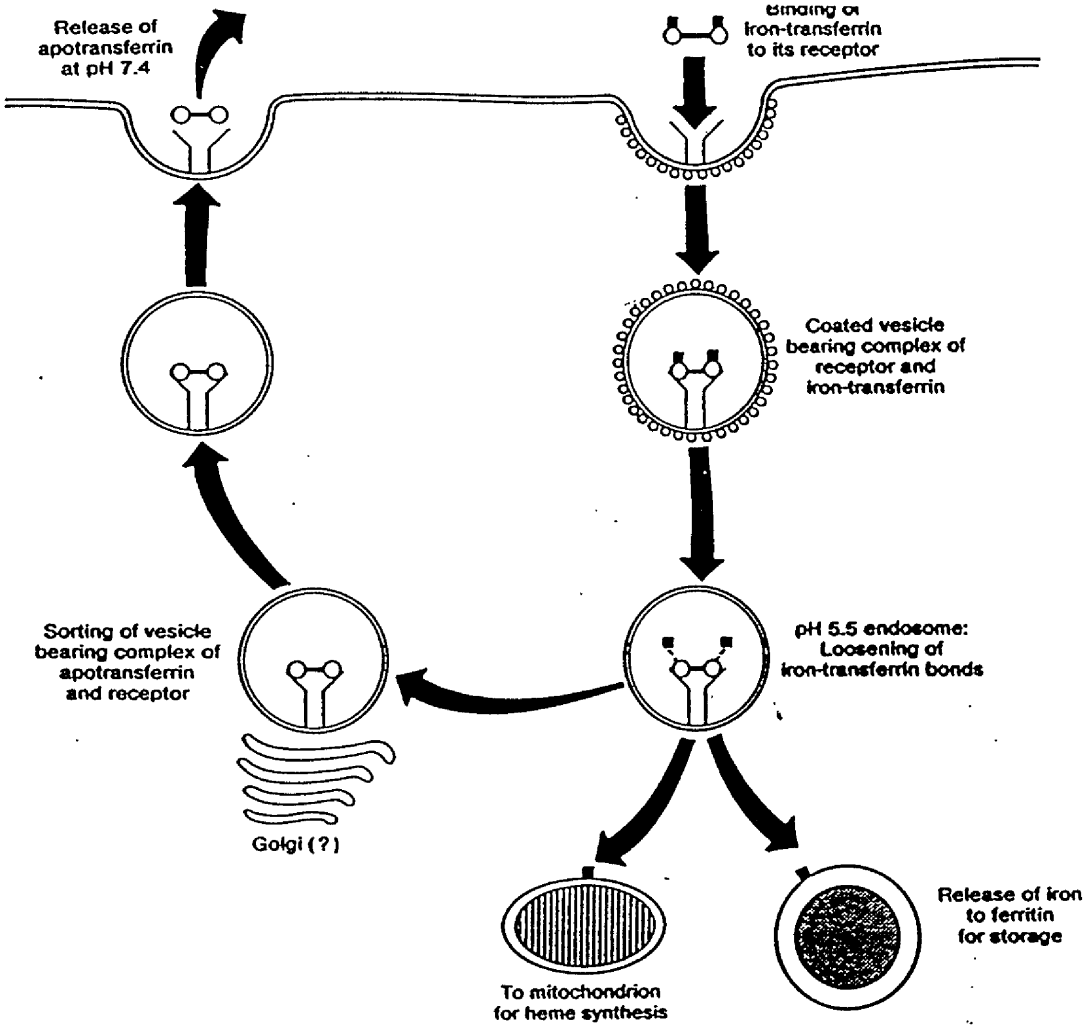

Fig. 9: Schematic events in delivery of iron from transferrin to the iron requiring cell via receptor mediated endocytosis (from Theil and Aisen, 1987).

The vesicles associate and diffuse to form an endosome, which has an internal pH of around 5-6.5 (van Renswoude *et al.*, 1982). This results in protonation of the anion (CO_3^{2-} or HCO_3^-) in the binding site adjacent to the iron and provokes its release . It has been shown that methylamine, ammonium chloride and chloroquine, which increase vesicular pH, decrease the release of iron from the protein to the cell (Morgan, 1981), without inhibiting internalisation of transferrin. Other studies employing mutants of CHO cells which are defective in endosomal acidification, have demonstrated that such cells cannot remove iron from internalised transferrin (Klausner *et al.*, 1983).

Earlier report by Fletcher and Huehns (1968) had suggested a nonhomogeneous transfer of iron from the two iron binding sites of transferrin with one binding site preferentially delivers iron to the erythron while the other supplies nonerythroid tissues. Further work has shown that iron release to the erythroid cells could occur from either mono- or diferric transferrin following an "all or none" phenomenon, leaving only apotransferrin (de Jong, 1991). Iron is subsequently transported into the cytoplasm, while apotransferrin remains bound to the TfR at the vesicular pH. As mentioned earlier, apotransferrin binds more strongly to the receptor at pH <6.5, so that when the recycling vesicles fuse with the plasma membrane at neutral or slightly alkaline pH of the extracellular fluid or of the growth medium, apotransferrin starts to dissociate from its receptor and is now available for another endocytic cycle. This cycle may repeat as many as 300 times before TfR is degraded (Omary and Trowbridge, 1981).

The return of apotransferrin to the cell surface after having released its iron at 37 °C takes 7-15 min (Hopkins, 1985; Bleil and Bretscher, 1982,

Hopkins and Trowbridge, 1983; Harding *et al.*, 1983; Iacopetta and Morgan, 1983; Watts, 1990). However *in vitro* studies showed that the rate of spontaneous dissociation of iron from transferrin at pH 5.0-5.5 is much too slow. When transferrin is bound to its receptor, the rate is substantially increased even when the pH is slightly increased (5.6-6.0) (Bali *et al.*, 1991; Sipe and Murphy, 1991). Studies by Schwartz *et al.*, (1984), Stoorvogel *et al.*, (1987) and Watts (1990) found that lysomotropic amines, chloroquine and primaquine inhibit exocytosis of internalised ligands including transferrin.

(c) Other Mechanisms of Iron Uptake from Transferrin

Apart from the well established receptor mediated endocytosis, other mechanisms have been proposed for the uptake of iron from transferrin, particularly in hepatocytes. These mechanisms involve the release of iron from transferrin to a plasma membrane component (Nunez *et al.*, 1982). Two models have been proposed for the release of iron at the plasma membrane.

In the first model, diferric transferrin can be reduced once bound to its receptor by transmembrane NADH: ferricyanide oxidoreductase (Crane *et al.,* 1985) which has been located in several cell types including hepatocytes (Clark *et al.*, 1982; Sun *et al.*, 1985). The reduction of iron to the divalent state destabilises the transferrin-iron bond and favours its transport through the plasma membrane (Grohlich *et al.*, 1977). Ferrous ion is trapped by appropriate plasma membrane iron ligands and subsequently internalised and taken up by the various biosynthetic pathways. Iron depleted transferrin is displaced from the receptor by diferric transferrin (Nunez *et al.*, 1983; Thorstensen and Romslo, 1988) (**Fig. 10**).

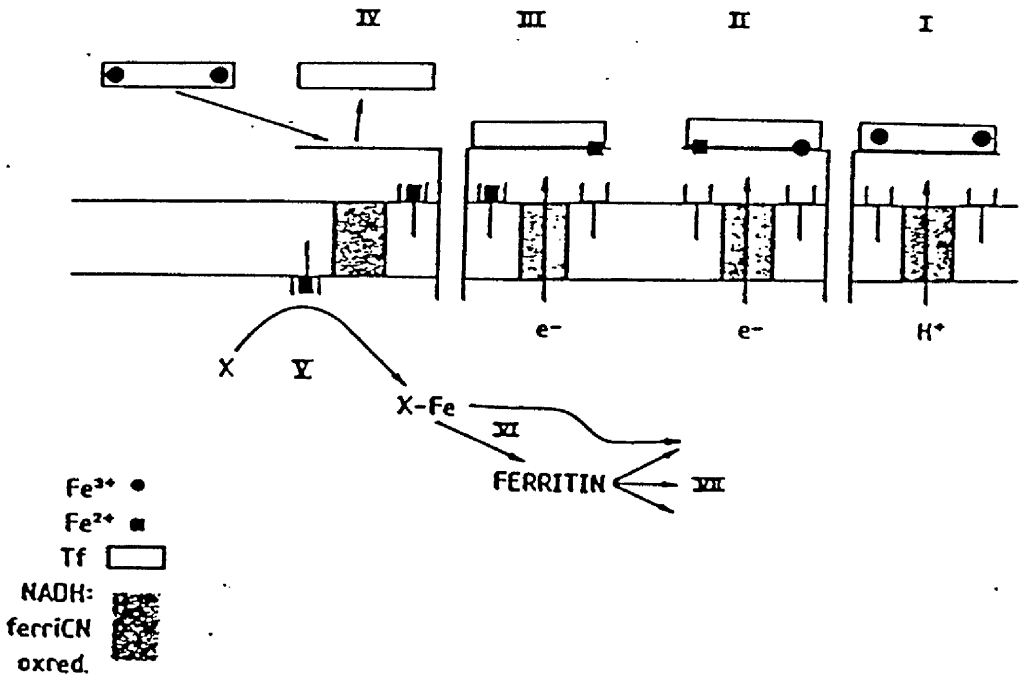

Fig. 10: Model for the uptake of iron from transferrin by rat hepatocytes. Diferric transferrin binds to the transferrin receptor which is located in close proximity to the proton- and electron- pumping NADH:transferrin (ferricyanide) oxidoreductase (I). The iron-transferrin bond is destabilised by proton fluxes, making ferric iron susceptible to reduction (II). Ferrous iron is trapped by a plasma membrane ferrous iron binder and translocator (III) and apotransferrin is displaced by another diferric transferrin (IV). This series of events (I-IV) is repeated until the transferrin/transferrin-receptor complex is hidden from the extracellular space by internalisation into an endosome. Iron translocated through the plasma membrane or the endosomal membrane is picked up by intracellular iron ligand (s) (V) which pass iron to the various intracellular iron requiring compounds (VI) and processes (VII) (from Thorstensen and Romslo, 1988).

In the second model, the binding of transferrin to its receptor promotes a comformational change in transferrin resulting in a decreased affinity of transferrin for ferric ion. Ferric ion that has been released is bound by a membrane ligand which is subsequently reduced (Nunez *et al.*, 1983) and available for chelation by intracellular iron carriers.

(d) Role of Lactoferrin in Iron Donation

Even though lactoferrin is structurally similar to transferrin in reversibly binding two ferric ions per molecule of the protein, the ability of lactoferrin to fuction as an iron donor like transferrin remains doubtful. Lactoferrin has been shown to bind to many cells especially monocytes and macrophages (Van Snick and Masson., 1976; Birgens *et al.*, 1983). In some of these studies, uptake of iron was observed, whereas in others, the amount taken up was considered insignificant. More detailed discussion on the possible role of lactoferrin in iron donation is included in Part Two of this chapter.

1.1.4.3 Role of Lactoferrin in Inflammation

(a) Role of Lactoferrin in Hypoferraemia

Van Snick *et al.* (1974) proposed that lactoferrin released from specific granules of neutrophils was involved in the hypoferraemia associated with inflammation. It was suggested that lactoferrin competes with bacterial siderophores to remove iron from plasma transferrin and unloading it to macrophages of the mononuclear phagocyte system where it is incorporated into ferritin. In so doing, the plasma iron decreases (**Fig. 11**).

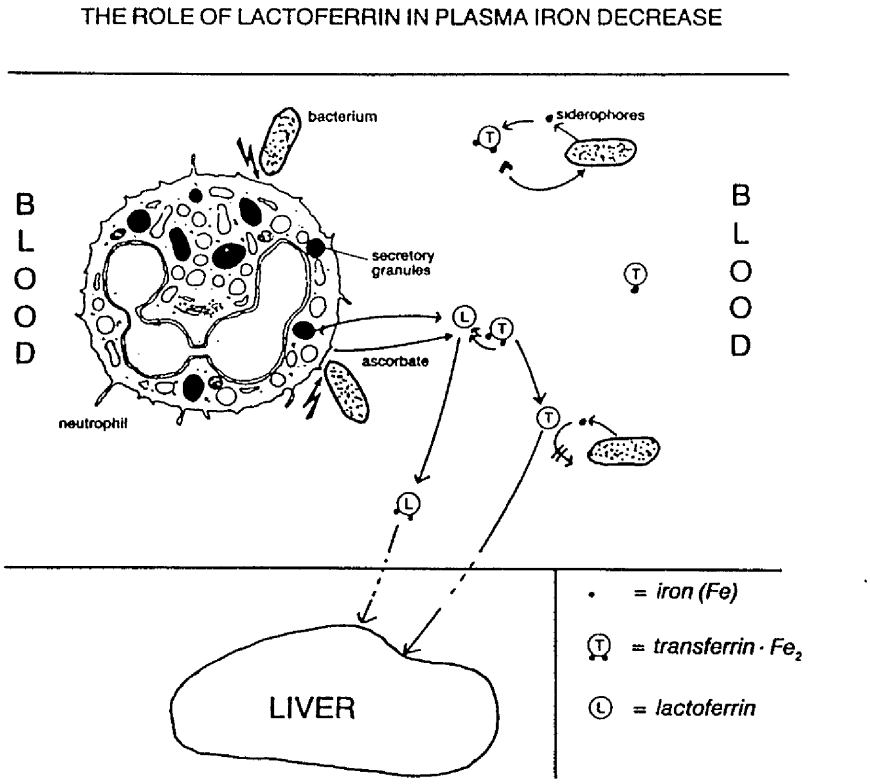

Fig. 11: Model for the cooperation of the different iron-binding proteins (transferrin and lactoferrin) in the iron-limiting mechanism conducted in blood, resulting in hypoferraemia and thus in iron deprivation of pathogenic microorganisms, which try to obtain the iron by their siderophores (From Sawatzki, 1987).

(b) The effect of Lactoferrin on Hydroxyl Radical Formation

During phagocytosis, neutrophils consume oxygen and convert it to superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), which react together to generate the highly reactive hydroxyl radical (OH·) (Babior, 1978). The direct reaction of superoxide anion and hydrogen peroxide known as Haber-Weiss reaction, proceeds at a significant rate only in the presence of a transition metal ion. Iron is the most likely candidate to be active *in vivo* (Flitter *et al.*, 1983).

$$Fe^{3+} + O_2^- \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH\cdot + OH^-$$

$$\text{Net:} \quad O_2^- + H_2O_2 \longrightarrow OH\cdot + OH^- + O_2$$

Hydroxyl radical, due to its high reactivity, can attack almost all biological molecules usually setting off free radical chain reactions (Halliwell *et al.*,1986). It is implicated in phagocytic bactericidal activity (Babior, 1978) as well as promoting lipid peroxidation with consequent cell damage (Lai and Piette, 1978 ; Gutteridge *et al.*, 1979). Generation of hydroxyl radical *in vivo* could be site specific depending on the location of metal ion complexes used in the Haber Weiss reaction. Hence the extent of the effect of hydroxyl radical varies according to the location of its generation.

The involvement of lactoferrin in the generation of hydroxyl radical is controversial (Andrews *et al.*, 1989). Ambruso and Johnston (1981) reported that iron-loaded lactoferrin from both milk and neutrophil is an efficient catalyst for hydroxyl radical formation and therefore an important contributor to the microbicidal activity of neutrophils. It was supported by the work of Bannister *et al.* (1982). However, other workers have reported

no evidence for the enhancement of hydroxyl production by lactoferrin (Winterbourn, 1983; Baldwin *et al.*, 1984; Gutteridge *et al.*, 1981). This is in line with the finding that in normal subjects lactoferrin has a very low iron content, well below saturation either in neutrophils (Bullen and Armstrong, 1979) or in milk (Fransson and Lonnerdal, 1980). Also as mentioned earlier in section 1.3.7, iron starts to mobilise from transferrin at pH values $\leq$ 6.5, which is actually the pH of the microenvironment of adherent phagocytic cells. Hence, lactoferrin that is present is expected to sequester any "free" iron that transferrin is unable to bind and thus offer a protective mechanism against hydroxyl radical formation. Further evidence that lactoferrin does not enhance hydroxyl radical generation is provided by the observation that activated human neutrophils did not produce hydroxyl radical unless iron ions were added to the reaction mixture, even though the activated neutrophils were found to release superoxide anions, hydrogen peroxide and lactoferrin extracellularly (Britigan *et al.*, 1986). Lactoferrin can also withstand oxidative stress and therefore makes a good candidate as an antioxidant at the sites of inflammation (Winterbourn and Molley, 1988; Halliwell *et al.*, 1988).

Thus, evidence from various studies seem to indicate that lactoferrin released from neutrophils inhibits rather than promotes the generation of hydroxyl radical (Britigan *et al.*, 1986; Flitter *et al.*, 1983). However conditions within the phagocytic vacuole with iron being made available from phagocytosed particles may cause lactoferrin to promote its production.

(c) Inhibition of Lipid Peroxidation by Lactoferrin

Exposure of membrane lipid to systems generating oxygen radicals in the presence of iron accelerates the process of lipid peroxidation which produces a mixture of organic peroxides. Lactoferrin as well as transferrin at physiological degrees of saturation are inhibitors of iron dependent lipid peroxidation (Gutteridge *et al.*, 1981). This is again due to their iron binding capacity, since saturation of the proteins abolishes their inhibitory effect. Therefore, secretion of lactoferrin from neutrophils in large amount during phagocytosis might be a protective mechanism by which surrounding tissues are protected against membrane damage especially in inflammatory joint diseases.

1.1.4.4 Role of Lactoferrin in Microbial Infections

(a) Introduction

Just as iron is essential to human and other animals, a source of iron is also required for the growth of almost all microorganisms, including those pathogenic to humans. To sustain growth and multiplication of microorganisms, iron must be present at a minimum concentration of 0.3 mM (Finkelstein *et al.*, 1983). The total concentration of iron in various biological fluids far exceeds the minimum requirements for microbial growth. However iron is relatively unavailable extracellularly as it is bound to transferrin and lactoferrin.

Microorganisms have to compete with their hosts for their supply of iron. Many of them have well-developed biochemical mechanisms for extracting and trapping the iron. These include the production of siderophores and

their receptors (Neilands,1981), and receptors for iron saturated transferrin and lactoferrin (Schryvers and Morris, 1988) that allow them to remove and utilise iron from these proteins.

(b) Growth Inhibition of Microorganisms by Transferrin and Lactoferrin

Many *in vitro* experiments have demonstrated the bacteriostatic effects of transferrin and lactoferrin. In some cases bactericidal effects of lactoferrin have also been reported (reviewed by Weinberg, 1984). Growth inhibition was observed with a wide range of microorganisms including gram-positive and gram-negative bacteria, aerobes, anaerobes and yeasts. Lactoferrin was found to be bactericidal on *Streptococcus mutans* and *Vibrio cholerae* but bacteriostatic against *E.coli* (Arnold *et al.*, 1982).

The bacteriostatic effect of these proteins was markedly reduced or abolished by the addition of iron to the growth media. This formed the basis for the argument that the important factor for the antibacterial effect is the percentage of iron saturation of these proteins. It was then hypothesised that these proteins act by depriving the microorganisms of their iron supply (reviewed by Weinberg, 1984).

Evidence from a number of studies, however indicates the anti-microbial action of lactoferrin is more complex than simple nutritional iron deprivation (Arnold *et al.*, 1977; Ellison *et al.*, 1988). Visca *et al.* (1990) point out the existence of additional anti-microbial mechanisms based on the interference between lactoferrin and microbial structures (both apo- and iron-saturated forms). Other mechanisms of action include blocking of microbial carbohydrate metabolism (Arnold *et al.*, 1982; 1977) and

destabilising the integrity of the outer membrane of gram-negative bacteria causing the release of lipopolysaccharides (Ellison *et al.*, 1988; 1990). A more recent study by Bellamy *et al.*, (1992) identified the bactericidal domain of the lactoferrin molecule which may be responsible for the membrane disruptive effects.

(c) Anti-microbial Activity of Lactoferrin in the Gut of the Newborn Infant

It has long been known that breast fed infants are better protected against gastrointestinal infections than infants fed with cow milk formula (Brock, 1980; Bullen *et al.*, 1972). *Escherichia coli*, a member of the normal intestinal flora is also a frequent aetiological agent of intestinal infections, in particular, infantile gastroenteritis. It has been estimated to affect more than 500 million children below 5 years of age with an annual mortality rate of 1 to 4 % worldwide (Snyder and Merson, 1982).

The anti-microbial activity of lactoferrin against *E.coli in vitro* has been well documented (Bullen *et al.*, 1972; Rogers and Synge, 1978; Spik *et al.*, 1978; Reiter *et al.*, 1975; Griffith and Humphreys, 1977). *In vitro* inhibition of *E.coli* requires the addition of bicarbonate to the milk or colostrum as to simulate the condition in the intestine as well as to enhance iron-binding by lactoferrin and prevent iron being made available to bacteria. Inhibition also requires specific antibodies, although good bacteriostatic activity in the absence of specific antibodies has also been reported (Arnold *et al.*, 1977; Law and Reiter, 1977). The bacteriostatic activity of lactoferrin was also enhanced by the presence of lysozyme (Montreuil *et al.*, 1985; Ellison and Giehl, 1991). The study by Ellison and Giehl (1991) found that while each protein alone is bacteriostatic, together

they can be bactericidal in a manner that was dose dependent and blocked by iron saturation of lactoferrin. Despite the wealth of *in vitro* observations on the antimicrobial role of milk lactoferrin, *in vivo* data is less convincing (Sanchez, 1992). Bullen *et al.* (1972) suggested based on the results of experiments with guinea pigs, that the protective effect of human milk in the small intestine toward *E.coli* could be due to lactoferrin and possibly transferrin as well, acting in concert with specific *E.coli* antibody. However the outcome of the experiment could have other interpretations as discussed by Brock (1980).

Another *in vivo* study by Antonini *et al.* (1977), showed that the number of coliforms in the intestine of newborn guinea pigs dosed with *E.coli* and fed cow's milk was lower if the milk was supplemented with ovotransferrin which is structurally related to lactoferrin. More recent clinical studies analysed the faecal flora of infants fed a diet supplemented with bovine lactoferrin (Balmer *et al.*, 1989; Roberts *et al.*, 1992). The addition of lactoferrin had little or no effect on the faecal flora of infants fed lactoferrin supplemented formula and controls with unsupplemented formula.

1.1.4.5 Role of Lactoferrin as a Growth Factor

Iron plays an important role in cell growth. Transferrin, being an iron transport protein has been shown to transport iron to growing cells (Aisen and Listowsky, 1980). It was first demonstrated by Hayashi and Sato (1976) to be an essential growth factor for a rat pituitary cell line, GH3 in serum free medium. Transferrin is now used for the culture of various cell lines in synthetic medium.

More recent studies have suggested that lactoferrin may also promote cell growth. Hashizume *et al.* (1983) reported that lactoferrin was an essential growth factor for lymphoma cell lines in serum free medium. Subsequently it was reported by Amouric *et al.* (1984) that lactoferrin stimulated the growth of the human adrenocarcinoma cell line HT 29 in the presence of a low concentration of iron, either of which alone was unable to promote cell growth. Further work by Nichols *et al.* (1990) showed that lactoferrin enhanced proliferation of rat mucosal crypt cells suggesting that it might play a part in maturation of the intestine of the newborn. The effect of lactoferrin on the proliferation of lymphocytes is not clear. Mazurier *et al.* (1989) have reported enhancement, whilst others (Richie *et al.*, 1987; Slater and Fletcher, 1987) have reported inhibition. However recent work by Djeha and Brock (1992a) has shown that apolactoferrin can overcome the inhibitory effect of "free" iron on lymphocyte proliferation, but itself inhibits proliferation when iron is bound to transferrin.

The mechanism by which lactoferrin is able to stimulate cell growth is unclear but appears somewhat different from that of transferrin (Hashizume *et al.*, 1987 ; Roiron-Lagroux and Figarella, 1990). Unlike transferrin which donates iron to the cell via the route described in section 1.4.2(a), lactoferrin does not seem to enhance growth in analagous manner. Lactoferrin is not internalised and little or no iron is taken up from the protein (Brock and Esparza, 1979; Oria *et al.*, 1988). In some cases, growth enhancement required iron (Azume *et al.*, 1989) whereas in others iron was unimportant (Nichols *et al.*, 1990; Oria *et al.*, 1988).

According to Roiron-Lagroux and Figarella (1990), lactoferrin may induce cell proliferation by releasing iron at the plasma membranes without itself being internalised. This process has been proposed for the uptake of iron

from transferrin (section 1.4.2(b)) in reticulocytes (Nunez *et al.*, 1983) and isolated rat hepatocytes (Morley *et al.*, 1985; Thorstensen and Romslo, 1988). The release of iron from lactoferrin at the plasma membrane could thus involve activation of membrane oxidoreductase activity (Sun *et al.*, 1991).

1.1.4.6 Role of Lactoferrin in Iron Absorption

The role of lactoferrin in iron absorption will be discussed in Part Three of this chapter.

PART TWO: REVIEW OF LITERATURE ON CELLULAR INTERACTIONS OF LACTOFERRIN

1.2.1 BINDING STUDIES OF LACTOFERRIN

1.2.1.1 Binding of Lactoferrin to Cells

A crucial step in understanding the biological role of lactoferrin is to study its interaction with cells. Lactoferrin binding has been reported on many cell types especially cells from the monocyte/macrophage system (see section 2.1.2), but also to lymphocytes (Van Snick and Masson, 1976), intestinal brush border membranes (Hu *et al.*, 1988; Davidson and Lonnerdal, 1985); a variety of cultured leukemic cell lines (Yamada *et al*, 1987) and human colon adenocarcinoma cell line (Roiron-Lagroux *et al.* 1989). Hepatocytes, Kupffer and liver endothelial cells have been observed

to bind lactoferrin as well (Courtoy *et al.,* 1984; Regoeczi *et al.*, 1985; McAbee and Esebensen, 1991; Ziere *et al.* 1992).

The existence of lactoferrin receptors has been reported on some of these cells. Lactoferrin receptors have been demonstrated in B and T-lymphocytes (reviewed by Birgens, 1991). Mazurier *et al.* (1989) have isolated the receptor from a triton X-100 soluble extract of phytohaemagglutinin-stimulated human lymphocytes. Purification using anti-ligand affinity chromatography yielded two major proteins molecular weights of 107 kDa and 115 kDa. A lactoferrin receptor has also been isolated from solubilised human fetal intestinal brush-border membranes (Kawakami and Lonnerdal, 1991). The molecular weight of the receptor was found to be 110 kDa by SDS-Page.

Quantitative binding data, dissociation constants and molecular weights of the receptors in studies dealing with lactoferrin binding to various cell types are summarised in **Table 1.**

1.2.1.2 Binding of Lactoferrin to Monocytes and Macrophages

(a) Mononuclear-Phagocyte System

The macrophage and its precursors in the same cell lineage make up the mononuclear phagocyte system. The cells include promonocytes and their precursors in the bone marrow, monocytes in the circulation and macrophages in tissues (reviewed by Johnston, 1988). The transit time in the bone marrow from the first monocytic precursor to the mature monocyte is about six days. The monocytes randomly leave the bone marrow within 24 hours and remain in the circulation with a $t_{1/2}$ of approximatelythree

days (Whitelaw, 1972). Migration of monocytes into the different tissues appears to be a random phenomenon in the absence of localised inflammation. Once in the tissues, monocytes probably do not reenter the circulation but instead undergo transformation into tissue macrophages with morphological and sometimes functional properties that are characteristic for the tissus in which they reside. The life span of individual macrophages in human tissues is believed to be months, but precise data are not available (Johnston, 1988).

The most important step in the maturation of macrophages from the standpoint of function is the conversion of the normal resting or resident cell to the activated macrophages. Macrophage activation is accomplished during infection through the release of macrophage-activating lymphokines from T-lymphocytes specifically sensitised to antigens for the infecting organisms (North, 1978). Activated cells have enhanced capacity to kill intracellular microorganisms and tumor cells.

There are many functional activities carried out by mononuclear phagocytes. Perhaps the best known of all the functions is its ability to phagocytose and kill microorganisms. In response to invading microorganisms, the phagocytes undergo metabolic stimulation, i.e. the respiratory burst. It results in marked increase in oxygen consumption, oxidation of glucose via the hexose monophosphate shunt and generation of reactive oxygen intermediates such as hydrogen peroxide and superoxide anion (Babior *et al.*, 1978). Superoxide anion and hydrogen peroxide interact with each other in a cycle of reactions to form the potent oxidant, hydoxyl radical, which is probably the most important microbicidal oxygen species. The production of the hydroxyl radical is catalysed by iron, the overall reaction is known as the Haber-Weiss reaction (see section 1.4.3(b)).

TABLE I

Dissociation constants (Kd), and molecular weights of receptors for lactoferrin bound to various cell types

Cells	Molecular weight of receptor (kDa)	Kd (nM)	Ref
Enterocytes			
Rabbit	100	830	Mazurier *et al.*(1985)
Mouse	130	380	Hu *et al.*(1988,)
Monkey	-	-	Davidson & Lonnerdal (1988)
HT - 29 Human	-	3.9	Derisbourg (1990)
HT 29 - D4 Human	-	408	Doiron-Lagroux *et al.* (1989)
Lymphocytes			
Human lymphocytes	-	43	Bennett & Davis (1981) Birgens *et al.* (1984)
Activated lymphocytes	110	83	Mazurier *et al.*(1989)

Superoxide and hydrogen peroxide are produced within most cells in microsomes, mitochondria, peroxisomes or elsewhere by the action of certain cytochromes, amino acid oxidases and other enzymes.

Another major function of the mononuclear macrophage system is in regulation of iron reutilisation especially those of the liver and spleen cells, which in normal conditions is highly efficient. They remove damaged and senescent red blood cells, convert the haem moiety to bilirubin and eventually return the iron into circulation, making it available to the haemopoietic system and the rest of the cells or it can be stored as ferritin. In inflammatory lesions, macrophages may also phagocytose other iron-containing cellular debris.

The activity of mononuclear phagocytes against cancers in human is less well understood, but these are thought to suppress the growth of spontaneously arising tumors. This control activity may not involve the phagocytic process. Macrophages may kill tumor cells by means of secreted products including lysosomal enzymes, oxygen metabolites and tumor necrosis factor-cachectin (Johnston, 1988).

One of the essential factors involved in the monocyte's protective function is its capacity to undergo diapedesis across the endothelial wall of blood vessels and to migrate to sites of microbial invasions in tissues. These cells would be expected to enter sites of inflammation.

Other equally important function of macrophages is processing antigen and presenting it to lymphocytes (Unanue and Allen, 1987). Monocytes and macrophages are also primary sources of the polypeptide hormones termed

interleukin-1 (IL-1) which have a particularly potent effect on the inflammatory response.

(b) Binding of Lactoferrin

The ability of lactoferrin to bind to cells of the mononuclear macrophage system was first reported by Van Snick *et al.* (1976), who showed that lactoferrin bound to mouse peritoneal macrophages. Since then many studies have confirmed the binding of lactoferrin to monocytes and macrophages (Bennett and Davis, 1981; Steinmann *et al.*, 1982; Birgens *et al.*, 1983; Bartal *et al.*, 1987 ; Oria *et al.*, 1988 ; 1993).

Binding of lactoferrin to these cells has been suggested to occur through specific receptor (Van Snick *et al.*, 1976 ; Birgens *et al.*, 1983), but so far no receptor molecule has been isolated. Others have suggested that the binding is relatively non-specific (Goavec *et al.*, 1985). Quantitave binding data, dissociation constant and number of binding sites per cell have been determined in various studies as summarised in **Table II**.

The specificity of lactoferrin binding to monocytes and macrophages has been demonstrated in competitive binding studies with various proteins. Binding of bovine lactoferrin to a monocytic cell line was inhibited by excess bovine lactoferrin but not by human lactoferrin, suggesting that the recognition mechanisms for human and bovine lactoferrin are not identical (Oria *et al.*, 1993). Transferrin, despite its structural homology to lactoferrin, did not inhibit the binding of lactoferrin (Van Snick and Masson, 1976; Bennett and Davis, 1981; Birgens *et al.* 1983).

TABLE II

The parameters of lactoferrin binding to macrophages and monocytes

Cells	Kd (nM)	Sites / cell (x 10^{-6})	Ref
Alveolar macrophages	-	2	Markowetz *et al.*(1979)
Peritoneal macrophages (murine)	1500	22	van Snick & Masson (1976)
Peritoneal macrophages (murine)	28	-	Imber & Pizzo (1983)
Alveolar macrophages (human)	1700	54	Campbell (1982)
Alveolar macrophages (human)	500	12	Goavec *et al.* (1985)
Blood monocytes (human)	1800	33	Bennett & Davis (1981)
Blood monocytes (human)	4.5	1.6	Birgens *et al.* (1983)
Blood monocytes (human)	430	10	Moguilevsky *et al.*(1985)
Blood monocytes (human)	3560	34	Britigan *et al.* (1991)

Other proteins such as IgG (Van Snick and Masson, 1976; Bennett and Davis, 1981; Birgens *et al.*, 1983), bovine albumin (Campbell, 1982), cytochrome c (Van Snick and Masson, 1976) and various milk proteins (Oria *et al.*, 1993) have no effect on binding of lactoferrin.

1.2.2.3 The Nature of Lactoferrin Binding Sites

The nature of the recognition site on the cells responsible for binding of lactoferrin is not known for certain. The protein moiety of lactoferrin molecule has been shown to be of pivotal importance in binding. According to Rochard *et al.* (1989), the cell binding site may be located in the N-terminal lobe of the molecule (residues 4-90 and/or 258-281). Further study by Ziere *et al.* (1992) using liver cells had reported that N-terminal arginine residues which resemble the arginine-rich receptor binding sequence in apoE, may be responsible for the interaction of lactoferrin with its receptor.

Others have suggested that electrostatic interactions, specific and non-specific, exist between lactoferrin and the negatively charged cell surface. This conclusion is based on competition binding studies between lactoferrin and other cationic proteins mentioned above. However the results of these studies are often not consistent. Cationic proteins such as lactoperoxidase (pI 9.6; McAbee and Esbensen, 1991 ; Oria *et al.*, 1993), protamine sulphate (pI 12.1; Yamada *et al.*, 1987 and McAbee and Esbensen, 1991) and other neutrophil granule glycoproteins, elastase and cathepsin G (Campbell, 1982) decreased the binding of lactoferrin. Another basic protein, lysozyme (pI 11.4) was found to be inhibitory by Moguilevsky (1985) but not inhibiting by other workers (Yamada *et al.*, 1987, McAbee and Esebensen, 1991).

Removal of sialic acid, one of the major sources of negative charge by neuramidase decreased binding of lactoferrin (Yamada *et al.*, 1987). On the other hand, Moguilevsky *et al.* (1985) found that sialic acid as well as glycosaminoglycans and phospholipids did not contribute to the binding. Taken together these findings, it seems that the binding of lactoferrin to the cells may not be due solely to electrostatic interactions between the protein and acidic groups on the cell surface.

Recognition of lactoferrin by the cell could also be mediated by the glycan moiety of lactoferrin, with fucose being one of the sugars playing an important role in the association (Prieels *et al.*, 1978; Goavec *et al.*, 1985). However, subsequent studies showed that binding of lactoferrin did not require recognition of the sugar components. Glycoconjugates bearing fucose, galactose, N-acetylglucosamine or mannose did not inhibit binding of lactoferrin to receptors on murine peritoneal macrophages (Imber and Pizzo, 1983).

1.2.3 UPTAKE OF IRON FROM LACTOFERRIN

1.2.3.1 Iron Uptake by Monocytes and Macrophages

Just as binding studies of lactoferrin to cells of the monocytes/macrophage system was first reported by Van Snick *et al.*,(1976), so also was the first report of uptake of iron from lactoferrin. They reported that iron-loaded lactoferrin when bound to murine peritoneal macrophages delivered its iron into intracellular ferritin. Similar findings were later obtained by Moguilevsky *et al.* (1985) and Birgens *et al.* (1988) using human monocytes. However iron uptake in these studies was found to be extremely slow with detectable net accumulation of iron and incorporation

into ferritin being observed only after about 24 h of incubation. Indeed a study by Oria *et al.*(1988) clearly indicated that within 6 h of incubation there was no net accumulation of iron from lactoferrin by a murine macrophage cell line, P388 D1, whereas there was a substantial uptake of iron from transferrin.

The earlier workers who reported iron uptake from lactoferrin suggested that lactoferrin was internalised into the cell after binding to its plasma membrane receptor, in order to release its iron. According to Moguilevsky (1985) once inside the cell, lactoferrin unlike transferrin was degraded concomitantly with the binding sites, probably in the lysosomes, and in the process iron was released. Further studies by Birgen and Kristensen (1990) reported that the interaction of lactoferrin with monocytes caused a change in the molecular conformation of lactoferrin, resulting in inability to rebind to its receptor, suggesting lactoferrin cannot operate in a cyclic manner to deposit iron like transferrin.

1.2.3.2 Iron uptake by other cells

Besides monocytes and macrophages, studies dealing with possible lactoferrin-mediated iron uptake has been carried out with reticulocytes which showed no evidence for such a process (Zapolski and Princiotto, 1976 ; Brock and Esparza, 1979). Translocation of iron from lactoferrin was also not observed in human lymphocytes (Birgens, 1991). Roiron *et al.* (1990) found that unlike transferrin, lactoferrin was not internalised by a human adenocarcinoma cell line, HT 29-D4 even though lactoferrin might have relesed its iron to the cell (Roiron *et al*, 1989).

Studies with hepatocytes revealed that these cells bound and endocytosed lactoferrin regardless of its iron content (McAbee and Esbensen, 1991). This is different from the action of transferrin which interacts with cells only in the diferric form. Hence, a possible role for hepatocytes could be to clear apo- and diferric lactoferrin that have been released from granulocytes, especially during inflammation. In this way, lactoferrin was taken up not to supplement nutritional iron requirements of the cells but rather to regulate it's blood levels.

Clearance of lactoferrin by liver hepatocytes could also occur by adsorptive pinocytosis (Regoeczi *et al.* 1985).

1.2.3 INTERACTION OF MILK PROTEINS WITH LACTOFERRIN

1.2.3.1 Milk Proteins

(a) Protein Content of Human Milk

Human milk has many unique properties that benefit the breast-fed infant. Some of these properties reside in the protein fraction of the milk. These include host defence factors (immunoglobulins, lysozyme and lactoferrin), digestive enzymes (lipases, proteases and amylase) as well as specific binding proteins and growth factors.

Human milk, compared to the milks of other animals, is relatively poor in protein. The amount of whey protein is also higher than casein. Colostrum, (milk obtained 1 to 5 days following parturition) and transitional milk (6 to 10 days following parturition) have higher amount of protein than mature milk. The increase is largely due to a higher amount of whey protein rather

than casein. The amount of protein in colostrum is about 23 mg/ml and in transitional milk about 16 mg/ml (Bezkorovainy, 1977).

(b) Casein

Casein in human milk is present exclusively in micellar form. The casein micelles in human milk are considerably smaller than those in cow's milk. Each micelle consists of a group of protein subunits associated and linked together with PO_4^{2-} and Ca^{2+}. The major casein subunit is β-casein, representing 50% of the total casein fraction. Cow's milk, however contains a large proportion of of α-casein which is apparently absent in human milk (Lonnerdal, 1985a). Casein can form complexes with some of the whey proteins. It was shown that casein prepared by acid precipitation contained lactoferrin, serum albumin and α-lactalbumin (Lonnerdal, 1985b).

(c) Whey proteins

Whey proteins have received much more attention than casein. This may be due to the physiological activities of some of the whey components. This review will only focus on some of these whey proteins.

α-lactalbumin

α-lactalbumin has a very high nutritional value (Forsum, 1973) and an amino acid composition well adapted to the requirements of the newborn infant. Besides the nutritional role of α-lactalbumin, this protein is part of the enzyme lactose synthase, which is responsible for lactose synthesis in the mammary gland. α-lactalbumin also has a metal-ion binding site which binds calcium in a 1:1 molar ratio (Segawa and Sugai, 1983).

Immunoglobulins

Human colostrum is rich in immunoglobulins especially secretory IgA(sIgA), with monomeric IgA, IgG and IgM being minor components. The sIgA molecule is resistant against proteolysis and therefore able to exert its function in the gut of the newborn.

Lactoperoxidase

Lactoperoxidase is an enzyme of human and cow's milk. It joins with two other chemical factors, hydrogen peroxide and thiocyanate to produce a defense against a variety of human pathogens. Lactoperoxidase system works most effectively at pH levels below that of milk, hence stomach acids (pH 2.0) help serve this function. As the enzyme is active at relatively low pH, it is most likely to survive the acid condition (reviewed by Lonnerdal (1985b).

Lysozyme

Lysozyme is another important human whey protein with a concentration 3000x higher than in cow's milk. This enzyme catalyses cleavage of the chemical bond between N-acetylglucosamine and N-acetylmuramic acid which are part of the cell wall structure bacteria. Therefore lysozyme from milk can function as a bacteriostatic agent in the intestine of a breast-fed infant. Lysozyme has a very high isoelectric point (pI 11) and consequently is positively charged at physiological pH. It can therefore form aggregates with other proteins which are anionic at this pH.

1.2.3.2 Association of Lactoferrin with Milk Proteins

Both human and bovine lactoferrin have been shown to interact with milk proteins and other macromolecules. Human lactoferrin interacts with casein, α-lactalbumin, albumin (Hekman,1971), sIgA (Watanabe *et al.*,1984) and lysozyme (Jorieux *et al.*,1985). It has also been shown to undergo polymerisation in the presence of calcium to form a tetramer (Bennett *et al.*,1981).

In general the interaction between lactoferrin and milk proteins has been considered to be non-specific. However there are reports suggesting some degree of specificity in the interaction. Lampreave *et al.*(1990) reported a stoichiometric relationship for the interaction of lactoferrin with β-lactoglobulin and with albumin; 2:1 in the first case and 1:1 in the second.

The nature of the interaction between lactoferrin and these proteins is not known for certain. The dissociation of the lactoferrin-milk protein complex by increasing the ionic strength points to electrostatic interaction. This is in agreement with the pronounced tendency of lactoferrin to become associated with more acidic proteins such as albumin or casein. Watanabe *et al.*(1984) also found that the binding of lactoferrin to IgA increased with the molarity of salt. Their findings also suggested that binding of lactoferrin to IgA could occur through the formation of disulphide bonds. However not all interactions between lactoferrin and milk proteins can be attributed to electrostatic forces because human lactoferrin has been found to interact with basic proteins like lysozyme and lactoperoxidase.
The importance of these associative properties of lactoferrin is not known. It is also not known for certain whether these properties have any effects on the binding of lactoferrin to cells.

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PART THREE: REVIEW OF LITERATURE ON THE ROLE OF LACTOFERRIN IN IRON ABSORPTION

1.3.1 FACTORS AFFECTING IRON ABSORPTION

There are two fundamental factors which influence the amount of iron being absorbed from the gastrointestinal tract. The first is the physiological and nutritional status of the individual, and the second is the nature of the diet.

1.3.1.1 Physiological factors

The absorption of iron is linked to the individual's need. Growth is most rapid in infancy and around puberty, so the requirement for iron is greatest around these times. Absorption of iron is also dependent on the iron status of the individual. In general, absorption is increased in iron deficiency and depressed in iron overload.

Both iron deficiency and iron deficiency anaemia occur with greater frequency during the first two years of life than at any other period in the life span (Oski, 1983). However in the early period up to 6 months, anaemia is not a problem among healthy full term infants (Wharton, 1989), irrespective of whether they are breast fed or fed with iron supplemented or unsupplemented formulas. The reason for this is that these infants have

sufficient endogenous iron to meet their requirements (Aggett *et al.*1989). As a result, iron absorption in the early postnatal period is lower than at any later time in childhood. The amount and bioavailability of dietary iron are therefore less important before 4 months of age (Cook and Bothwell, 1984). Nonetheless as infants grow their endogenous iron reserves become increasingly marginal and become appreciably depleted after 6 months of age. Without adequate intake, iron deficiency frequently occurs in these term infants between the ages of 9 to 24 months.

Data on the iron status of infants who have been exclusively breast fed beyond 6 months are limited. McMillan *et at.*(1976) found that human milk, fed to the exclusion of other foods, was adequate to preserve a positive iron balance in four infants. These data were supported by the work of Saarinen *et al.*(1977) and Siimes *et al.*(1984) who studied 56 and 36 infants respectively that were exclusively breast-fed for 9 months. They both found no indication of iron deficiency among these infants. Instead infants who were breast fed had higher iron status than infants fed iron-supplemented formula and solid foods (**Fig. 12**). Similar finding was obtained from the work of Pastel *et al.*(1981) on seven healthy infants in the Peruvian Andes who had been exclusively breast-fed for 7 to 12 months. These studies suggested that exclusive breast feeding was sufficient to maintain adequate iron nutrition for the first year of life, even without iron supplementation. However iron sufficiency may be reduced in infants given solid foods (Saarinen *et al.*, 1977) and hence iron supplementation might be required.

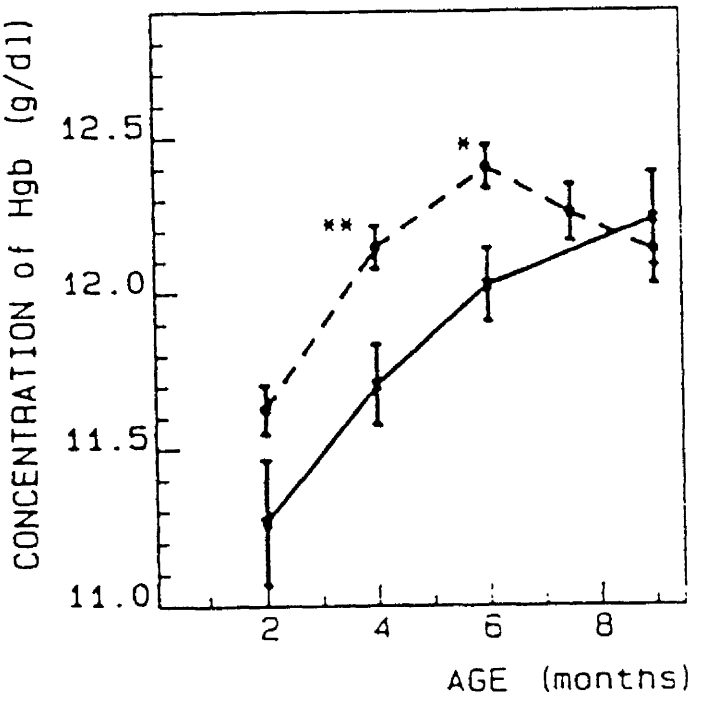


Fig. 12: Changes in haemoglobin concentrations of exclusively breast-fed infants (dashed line) and infants fed-supplemented formula (solid line) (from Siimes *et al.*, 1984).

1.3.1.2 Dietary factors

The reason for the delayed onset of iron depletion in breast fed infants is attributed to the greater bioavailability of iron from breast milk (Saarinen *et al.*, 1977), which is not paralleled by artificial feed based on cow's milk or soy protein isolate (Saarinen and Siimes; 1979). The finding that plasma ferritin levels at 6 months in breast fed babies were higher than those of infants receiving a formula without added iron further suggests a better absorption of iron from breast milk (Wharton, 1989). The reason why the iron in breast milk is so well absorbed is not known. However, protein mediated uptake of iron by the intestinal mucosal cell has received much attention. Lactoferrin, due to its high level in milk has been postulated to promote iron absorption but the mechanism of action is not known (Cox *et al.*, 1979; Fransson *et al.*, 1983; Kawakami *et al.*, 1988).

As the diet becomes more complex, absorption of iron may be affected by some components of the diet. The presence of chelating agents which bind iron in the diet can either enhance or inhibit its absorption. The enhancing effect of organic acids, sugars and amino acids is primarily due to their ability to form stable complexes with iron. Meat enhances the absorption of non-haem iron by a factor of 2-3 (Cook and Monsen, 1976), but the factor responsible for the facilitating effect is still unknown. The enhancing effect of ascorbic acid on non-haem iron absorption is profound whether it is contained naturally in the food or is added as synthetic vitamin (Derman *et al,*1980; Lynch and Cook, 1980; Sayers *et al.*, 1974). The facilitating effect of ascorbic acid is due to its ability to form a soluble complex with iron at a low gastric pH, thus preventing formation of insoluble hydroxides.

The presence of tannin in tea and to a lesser extent in coffee results in the formation of insoluble iron tannates. Tannins are also widely distributed in vegetable foods and may be partly responsible for the overall low bioavailability of iron in many such foods (Gillooly *et al.*, 1983). Another important inhibitor of iron absorption is bran, which impairs iron absorption due to the presence of phytate (Simpson *et al.,* 1981)

1.3.2 PATHWAYS OF IRON ABSORPTION

1.3.2.1 Overall Pathway

Iron absorption occurs predominantly in the proximal end of the duodenum. The precise path as well as the actual proteins and ligands involved in the absorption of iron are not fully understood (Flanagan, 1989). However the process may involve the following steps:

(1) iron binding to the brush border of enterocytes
(2) iron uptake by enterocytes
(3) iron transport within enterocytes
(4) transfer of part of the iron from enterocytes to the blood
(5) return of the remaining iron to the lumen when enterocytes are shed from the villous tips

1.3.2.2 Iron Uptake by Enterocytes

The mechanism for the uptake of iron from the intestinal lumen by the enterocyte is illustrated in **Fig. 13** (Flanagan, 1989).

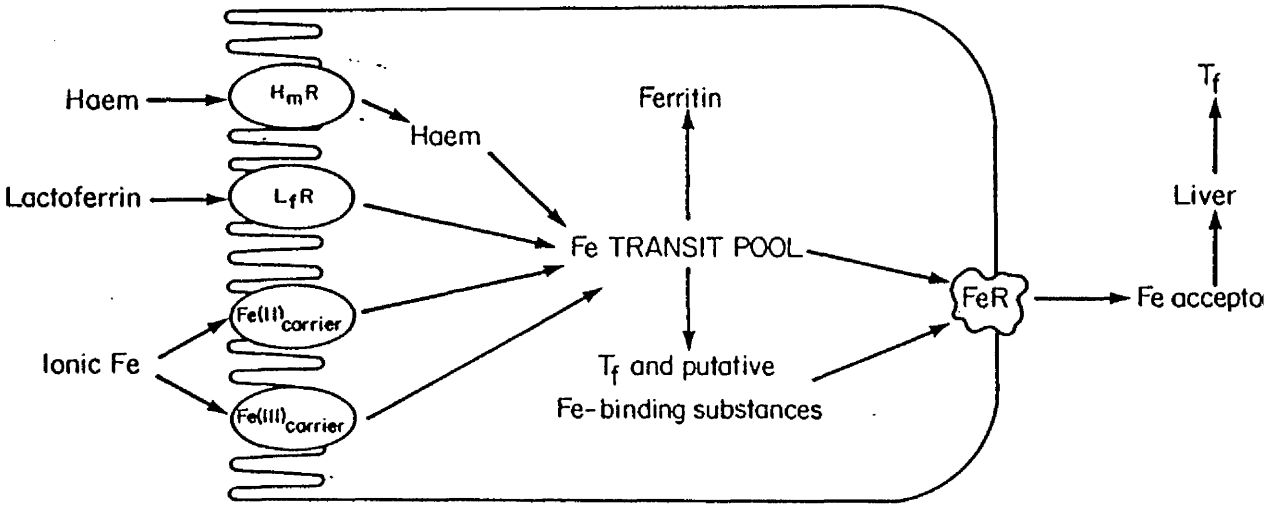


FIG. 13: Possible mechanisms for the absorption of iron by enterocytes. HfR, haem receptor; LfR, lactoferrin receptor; Fe(II) carrier, microvillus membrane carrier for chelated Fe(II); Fe(III) carrier, microvillus membrane carrier for chelated Fe(III); FeR, putative basolateral membrane carrier (from Flanagan, 1989).

Iron that is ingested may enter one or two common pools before being absorbed. The smaller pool comprises heme iron compounds derived from meat sources. Iron in this form is highly available. It is absorbed by a pathway distinct from that of ionic iron since intraluminal factors such as acidity and chelating agents do not affect the process (Turnbull *et al.*,1962). Furthermore, the intestinal haem receptor has been partially characterised from both pig and human brush border membranes (Grasbeck *et al.*, 1979). It is likely that haem is released from haemoglobin during intraluminal digestion and taken up by the intestinal mucosa. Once in the cell, iron is then released from porphyrin by haem oxygenase.

The second pool of iron is composed of all other forms of food iron as well as any organic iron present. This iron is much less bioavailable than haem iron and its absorption is dependent on other dietary factors in the meal as mentioned above. Much evidence indicates that absorption of ferrous iron is preferred over ferric iron but the precise biochemical explanation is unclear. However the iron must be present in a soluble form to be taken by the mucosal cell. Ferrous iron being more soluble is more effectively absorbed than ferric iron. In the acid condition of the stomach, most of the dietary iron is solubilised but upon passing into the alkaline medium of the duodenum, any free iron will be precipitated as insoluble ferric hydroxide which cannot be absorbed.

Non-haem iron can also be taken up by the mucosal cell via specific protein. Transferrin is unlikely to be involved in the luminal uptake because repeated studies have failed to identify transferrin receptors in the intestinal brush border membranes (Cox *et al.*, 1979; Parmley *et al.*,1985 ; Banerjee *et al.*,1986). On the other hand, lactoferrin receptors have been identified in the brush border membranes of rabbit (Mazurier *et al.*,1985), mouse (Hu *et*

al.,1990), rhesus monkey (Davidson and Lonnerdal, 1988) and human fetal and infant brush border membranes (Lonnerdal, 1992).

The receptor from infant rhesus monkey intestine exhibited saturation kinetics and the binding was found to be specific for both human and monkey lactoferrin but not for bovine lactoferrin or human transferrin. The receptor had a higher affinity for diferric lactoferrin than apolactoferrin (Davidson and Lonnerdal, 1988).

Partial proteolysis of lactoferrin did not seem to inhibit its binding to the intestinal receptor. The human lactoferrin receptor was found to have a molecular weight of about 110 kDa (see **Table 2**). Binding of lactoferrin to its receptor was significantly reduced by removal of terminal fucose residues, implying that fucosylated glycans of lactoferrin could be important for receptor recognition.

Despite having identified lactoferrin receptors in the brush border membrane, the role of lactoferrin in iron absorption is not known for certain. By using human mucosal biopsies, Cox *et al.*(1979) found that human lactoferrin has the ability to deliver iron to mucosal cells of the small intestine of human adults. In another study, a significant increase in red blood cells, haematocrit and haemoglobin values was obtained from iron-deficient rats fed with iron-saturated lactoferrin as compared to ferrous iron (Kawakami *et al.*,1988), indirectly demonstrating that iron from lactoferrin was more readily absorbed, as also suggested earlier by Fransson *et al.* (1983).

Contrary to those findings, McMillan *et al.* (1976) showed that infant formula fortified with bovine lactoferrin administered to adult humans did

not increase iron absorption. This was supported by an *in vivo* study in rats (Fairweather-Tait *et al.*,1986) and in newborn infants (Fairweather-Tait *et al.*,1987) in which the effect of lactoferrin on iron absorption was determined by comparing iron retention from ^{58}Fe-labelled bovine lactoferrin with that from ^{58}Fe chloride added as a supplement to the infant formula. Both studies reported that bovine lactoferrin had no effect on iron absorption in rats or newborn infants. Infact, earlier work by De Vet and Van Gool (1974) reported a negative correlation between iron absorption and the duodenal concentration of lactoferrin in adult humans. De Laey *et al.* (1968), studied the effects of human lactoferrin on iron absorption using everted duodenal sacs from rats and guinea pigs. They concluded that exogenous apolactoferrin significantly decreased the mucosal iron uptake, whereas no significant uptake was observed with diferric-lactoferrin.

Considering both sides of the argument plus the lack of evidence that the receptor proteins on the mucosal membrane actually mediate uptake or transport of lactoferrin-bound iron, the role of lactoferrin in iron absorption remains controversial. However reviews by Brock (1980) and more recently by Sanchez *et al.* (1992), suggest that lactoferrin may act to regulate iron absorption, and perhaps even to facilitate excretion of iron in the immediate postnatal period rather than promote iron absorption, thereby protecting against too large of an iron accumulation at a period of low need for iron. Later in infancy, the proteolytic activity of the infant's gut is increased and lactoferrin can be degraded and the bound iron released and can then be absorbed.

1.3.3 ABSORPTION OF LACTOFERRIN BY NEWBORN INFANTS

1.3.3.1 Survival of Lactoferrin in the Gastrointestinal Tract

The possible involvement of lactoferrin in iron absorption and protection against gastrointestinal infection, depends upon whether it can remain in the intestinal tract without being destroyed by gastric acidity or proteolytic activity (reviewed by Brock, 1980). In newborn infants, the proteolytic activity in the duodenum is better developed than the gastric activity. Both acid and pepsin production are low in relation to body weight, hence there is little gastric proteolytic activity. However these infants can still digest milk proteins, presumably due to pancreatic protease production, which appears to be comparable with that of adults (Hadorn *et al.*, 1968; Lindberg, 1974).

In vitro studies by Brines and Brock (1983) have shown that apolactoferrin was more sensitive than iron saturated lactoferrin to the action of trypsin and human lactoferrin was found to be less sensitive than bovine lactoferrin. Bovine apolactoferrin was rapidly degraded by trypsin to small peptides, whereas the iron-saturated form was relatively resistant to prolonged tryptic digestion and gave rise only to large fragments which still retained their iron-binding capacity (Brock *et al.*, 1976). Pepsin, however can partly digest human iron-saturated lactoferrin, leaving a half-molecule containing one iron-atom.

Nevertheless, in *in vivo* studies, several groups have reported that intact lactoferrin had been recovered in the feces of term infants (Davidson and Lonnerdal, 1985; Prentice *et al.*, 1989; Spik *et al.*, 1982) as well as in premature infants (Schanler *et al.*, 1986; Goldman *et al.*, 1990). The fecal excretion of lactoferrin was greater in breast-fed infants than those fed with

cow's milk based formula. IgA was also detected in the feces of the newborn infants but not lysozyme, suggesting that the infants was capable of digesting proteins but somehow lactoferrin and IgA escaped digestion.

The digestive ability increases with age, presumably as a result of an increase in gastric proteolytic activity (Brock, 1980). This is in line with the finding that the amount of lactoferrin excreted was found to decrease with age, so that by 6 and 12 weeks of age, about 99% of lactoferrin was degraded in exclusively breast-fed infants (Prentice *et al.*, 1987).

1.3.3.2 Plasma and Urine Levels of Lactoferrin

Intact lactoferrin and two large fragments have been detected in the urine of premature infants receiving human milk (Hutchens *et al.*, 1990) and have been shown to be of maternal origin (Hutchens *et al.*, 1991). This suggests that lactoferrin was partially degraded in the gut and that significant absorption of this protein occurred in the premature infant. However, some lactoferrin fragments could have been present in the milk before ingestion, as reported by Calvo *et al.*(1991).

Even though lactoferrin had been detected in the urine of premature infants receiving human milk, it seems less likely that lactoferrin absorption occurs in term infants, as no difference in plasma lactoferrin concentrations was found between breast and bottle fed infants (Scott, 1989).

AIMS OF THESIS

Many biological roles have been proposed for lactoferrin but so far none has been fully established. Several of these proposed functions imply an interaction with cells of the mononuclear phagocyte system, but the nature of this interaction is unclear. The first part of this thesis reports a study of lactoferrin interaction with the human promonocytic cell line, U937 and compares its behaviour with that of transferrin, the serum iron transport protein. The binding pattern of lactoferrin to these cells and the possible involvement of a receptor molecule are examined. In addition the effect of heat treatment and other milk proteins on the binding of human and bovine lactoferrin to these cells have also been investigated.

Due to its close structural similarity to transferrin, one of the main functions that has been ascribed to lactoferrin is in iron transport and uptake. Work along this line has been conducted specifically to study the ability of lactoferrin to donate its iron to these cells and the possible mechanism of iron donation involved.

Apart from iron donation, binding of lactoferrin to the cells may also exert some other biological effects. Two of these potential effects have been studied. The first of these is to determine if lactoferrin can stimulate the growth of U937 cells as has been reported by others using different cell types. Secondly, since cells may be exposed to "free" iron especially in conditions of iron overload or at the sites of inflammation and may acquire it in due process, it is important to study whether binding of lactoferrin to these cells can inhibit the uptake of this iron.

Another area of controversy involves the role of lactoferrin in iron absorption. The second part of this thesis focusses on this aspect of lactoferrin function. Caco-2, a human colon carcinoma cell line capable of differentiating and forming a mucosal cell monolayer in culture is used as an *in vitro* model to study the involvement of lactoferrin in the iron transport mechanism. Since lactoferrin from maternal origin has been detected in the urine of preterm newborn infants, it has been postulated that lactoferrin and its iron may be transported across the mucosa. It is therefore worthwhile to find out if similar transport occurs in newborn term infants and further to investigate if there is a difference between the plasma lactoferrin level in breast-fed and formula-fed infants.

CHAPTER TWO

INTERACTIONS OF LACTOFERRIN WITH A PROMONOCYTIC CELL LINE, U937; ITS ROLE IN IRON UPTAKE

2.1 INTRODUCTION

A considerable amount of work has been done on lactoferrin since it was first isolated in 1960. This work has led to a better understanding of its chemical structure and its physicochemical properties. Consequently much attention has been focussed on the similarities between lactoferrin and transferrin (Aisen and Leibman, 1972; Metz-Boutigue *et al.*, 1978). They have nearly identical molecular weight, with similar amino acid composition and high identity of sequence. They both bind two ferric ions per molecule of the protein with high affinity, concomitantly with the binding of two bicarbonate or carbonate ions. However the binding affinity of lactoferrin for iron far exceeds that of transferrin by a factor of 260 and lactoferrin requires exposure to pH 2 to be free of its bound iron while transferrin releases its iron at pH 4.5 (Brock, 1985).

Work of similar magnitude has also been carried out to study the functional activities of lactoferrin. While transferrin has a well defined function in iron transport to cells, the biological significance of lactoferrin remains obscure. Although lactoferrin has many structural and biochemical similarities to transferrin, its role in iron transport is not clear. The first suggestion that lactoferrin might play such a role came from the work of Van Snick *et al.* (1974) who demonstrated that lactoferrin could bind to peritoneal macrophages and transfer its iron to these cells. They also reported that during acute inflammation induced by the administration of endotoxin to rats, injection of lactoferrin caused a decreased level of plasma iron. These observations led them to propose that lactoferrin might contribute to the hypoferraemia associated with inflammation by capturing iron released from transferrin as a result of a reduced pH in the microenvironment and diverting this iron to the macrophages. No similar

studies have been published nor have their findings been supported by later studies. In addition direct evidence that lactoferrin acts as an iron donor is scarce. Work with transferrin has shown that in most cells, the uptake of iron requires the expression of a receptor that allows endocytosis of transferrin and intracellular iron release to occur (Dautry-Varsat, 1986). Although there are reports on the existence of specific lactoferrin receptors on various types of cells (reviewed by Birgens *et al.*, 1991), no such mechanism of iron transport has been described for lactoferrin. A putative receptor molecule has been isolated from activated lymphocytes (Mazurier *et al.*, 1989), but the nature of the molecule and its interaction with lactoferrin remains largely unknown. It is also not clear whether these membrane proteins can mediate uptake of iron from lactoferrin in an analagous way to that of transferrin.

In order to understand better the possible involvement of lactoferrin in cellular iron uptake, it is important to examine its interaction with cells in particular cells of the reticuloendothelial system. To accomplish this purpose, a human promonocytic cell line, U937 was chosen for the study.

U397 originated from the pleural fluid of a patient with diffuse histiocytic lymphoma (Sundstrom and Nilsson, 1976). Morphologically the cells are monoblastic and the histochemical profile reveals monocytic lineage. The cells grow as a single-cell suspension culture with a doubling time originally reported to be 95 h (Sundstrom and Nilsson, 1976). However recent passages of the line grow more quickly, 20-48 h per population doubling (Harris and Ralph, 1985). The cells are round with small cytoplasmic projections present on some cells. The mean diameter is 12.5 μm with a range of 8.1-16.9. Maturation of the cell line increases the size of the cells and acquires a lobated nucleus. The surface marker study

revealed the presence of C3 and Fc receptors on the cells (Sunstrom and Nilsson, 1976). The Fc receptors bind IgG1 with an affinity comparable to that of Fc receptors on normal mononuclear phagocytes (Anderson and Abraham, 1980).

Therefore, considering the monocytic characteristics of U937, this cell line was used in this research to study the cellular interaction of lactoferrin and to determine the effects of this interaction on the cells. A comparison is made between the interactions of lactoferrin and that of transferrin. The specific objectives of the study include the following:

1. to study the binding and dissociation pattern of lactoferrin and transferrin

2. to determine iron uptake from lactoferrin and transferrin

3. to determine whether lactoferrin and transferrin are internalised by the cells

4. to determine the effect of lactoferrin on the uptake of non-transferrin bound iron

5. to investigate the effect of lactoferrin and transferrin on cell proliferation

2.2 MATERIALS

2.2.1 Cell Line

U937 was kindly provided by the Department of Biochemistry, Royal Infirmary, Glasgow.

2.2.2 Human Milk

Human milk was supplied by the Department of Paediatrics, Queen Mother's Hospital, Glasgow.

2.2.3 Proteins

2.2.3.1 Lactoferrin

Human lactoferrin was purified from milk according to the procedure reported by Johansson (1969). Bovine lactoferrin was obtained from Fina Research (Seneffe, Belgium). Heat-treated bovine lactoferrin was prepared at the Veterinary Faculty, University of Zaragoza, Spain, according to the method described by Sanchez *et al.* (1992).

2.2.3.2 Transferrin

Apo-transferrin (Behringwerke, Marburg, Germany) solution was prepared in PBS-1 % bicarbonate at the required concentration and filter sterilised. When necessary, it was saturated with iron at appropriate percentages with FeNTA. The purity of human transferrin was ckecked by SDS-PAGE.

2.2.3.3 Other proteins

Bovine α-lactalbumin and ovine β-lactoglobulin were obtained from Veterinary Faculty, University of Zaragoza, Spain. Human α-lactalbumin, IgA, lactoperoxidase, lysozyme and heparinase III were obtained from Sigma (Poole, U.K.)

2.2.4 Radioisotopes

^{125}I, carrier free (500 mCi/mg), ^{59}Fe-citrate (10 mCi/mg) and ^{3}H-thymidine (52 Ci/mmol) were purchased from Amersham International, Amersham, U.K.

2.2.5 Monoclonal Antibodies

Monoclonal antibodies to the monocyte cell-surface markers, CD3, CD4, CD11b, CD14, CD25, CD45 and HLA-DR were purchased from Becton Dickinson. Monoclonal antibodies specific for CD3, CD11b, CD14, CD25 and CD45 were labelled with fluorescein isothiocyanate (FITC), while the remaining antibodies were labelled with R-phycoerythrin (PE).

2.2.6 Reagents

Reagents used in all the experiments were of analytical grade. Most of them were prepared in iron-free apparatus i.e. plastic, or glassware rendered iron-free by soaking in 10 % HCl overnight and rinsing three times in deionised, distilled water.

2.2.6.1 Standard Culture Medium

The complete culture medium consisted of RPMI 1640 (w/o Hepes; Flow Laboratories, Irvine, U.K.), supplemented with penicillin (100 units/ml; Flow), streptomycin (100 mg/ml; Flow), sodium pyruvate (0.1 mg/ml; Flow) and 10 % final concentration of fetal calf serum (Flow). When required, serum free medium was prepared using human serum albumin (Behringwerke) at 1 mg/ml final concentration in place of fetal calf serum.

2.2.6.2 Human Serum Albumin Solution

A stock of 1 % (w/v) human serum albumin (Behringwerke) was prepared in RPMI-1640 medium (w/o Hepes; Flow), filter sterilised and stored at 4 °C. The solution was tested by radial immunodiffusion assay (kindly done by Dr. S. McGregor) and found not to contain any detectable transferrin.

2.2.6.3 Phosphate Buffered Saline

This buffer contained NaCl (8 g/l), KCl (0.2 g/l), Na_2HPO_4 (1.15 g/l) and KH_2PO_4 (0.2 g/l) (BDH Chemicals Ltd., Poole, England) in deionised, distilled water and filter sterilised.

2.2.6.4 Ferric Nitrilotriacetate

FeNTA was prepared at a molar ratio of 4:1 of NTA to iron, to ensure the formation of low molecular weight rather than hydrated polymeric complexes (Spiro *et. al.,* 1967). One volume of a freshly prepared solution of $FeCl_3$ (40 mM; BDH) was slowly added dropwise with constant stirring, to an equal volume of NTA (160 mM, pH 7.0; Sigma) and the pH finally

adjusted to 5.0 with 1 M sodium hydroxide to prevent the NTA-free acid precipitating. The final concentration of iron was 10 mM.

2.2.6.5 Acetic acid/sodium acetate buffer (pH 4.0)

This contained 410 ml acetic acid (0.2 M; BDH) and 90 ml sodium acetate trihydrate (0.2 M; BDH), made up to 1 litre.

2.2.6.6 Tris-HCl buffer (0.05 M, pH 8.0)

This buffer contained 250 ml Tris (0.2 M, BDH) and 26.8 ml HCl (1 M), made up to 1 litre.

2.2.6.7 Coupling buffer

This buffer contained 8.4 g sodium bicarbonate and 29.2 g sodium chloride in 1 litre of deionised and distilled water, with pH adjusted to 8.3.

2.2.7 Reagents for SDS-PAGE

2.2.7.1 Stock acrylamide solution (40 %)

38.96 g acrylamide (BDH)
1.04 g bisacrylamide (BDH)
Made up to 100 ml with distilled water, filter sterilised and kept in the dark at 4 °C.

2.2.7.2 Running gel (10 %)

The running gel was made up as follows:

8.75 ml stock acrylamide (40%)

8.75 ml 1.5 M tris-HCl, pH 8.8

16.3 ml distilled water

0.7 ml SDS (10 % w/v)

2.2.7.3 Stacking gel

Stacking gel was prepared by mixing the following in a universal:

1.25 ml 0.5 M tris-HCl, pH 6.8

1.0 ml stock acrylamide (40 %)

7.5 ml distilled water

100 µl SDS (10 % w/v)

2.2.7.4 Tank buffer

24 g tris (Boehringer, Mannheim)

115.2 g glycine (Sigma)

4.0 g SDS (Sigma)

Made up to 4 litre with distilled water.

2.2.7.5 Sample Buffer

50 % (v/v) glycerol (BDH)

0.25 M tris-HCl, pH 6.8 (Boehringer)

5.0 % (w/v) SDS (Sigma)

5.0 % (v/v) β-mercaptoethanol (Sigma)

0.0125 % (w/v) bromophenol blue (BDH)

2.2.7.6 Fixer

10 % (v/v) Trichloroacetic acid

10 % (v/v) acetic caid

30 % (v/v) methanol

2.2.7.7 Staining Solution

0.25 % (w/v) Coomassie brilliant blue

50 % (v/v) methanol

10 % (v/v) acetic acid

The solution was stirred overnight and filtered before use.

2.2.7.8 Destaining Solution

20 % (v/v) methanol

10 % (v/v) acetic acid

made up with distilled water

2.3 METHODS

2.3.1 Isolation of Human Lactoferrin

2.3.1.1 Ion-Exchange Chromatography

Lactoferrin was isolated from milk by the method of Johansson (1960) with slight modification. One litre of frozen human milk was diluted with two volumes of deionised and distilled water containing 30 mg ferrous ammonium sulphate (BDH) and was stirred gently with a magnetic stirrer for 30 min. To this 3 g of CM-Sephadex C-50, Cl^- form (Pharmacia, Uppsala, Sweden) was added and stirred for 1 h after which the gel was allowed to settle. The gel was washed by suspending it in 1 litre of deionised, distilled water for 10 min and then decanted. This washing was repeated twice until the water looked clear indicating that most of the unbound proteins had been washed away. The gel was then packed into a column made from a 60 ml syringe, and washed with 400 ml tris-HCl (0.05 M) at a flow rate of 10 ml/h which eluted small amounts of uncoloured proteins. About 2.5 ml fraction was collected for every 15 min. The column was eluted until the A_{280} nm reading was negligible. The washing step was repeated with 200 ml each of tris-HCl (0.05 M) buffer containing increasing molarity of NaCl, from 0.5 M to 2 M when lactoferrin was eluted from the column. The A_{280} nm readings of all fractions were taken and plotted in the elution profile as shown in **Fig. 14**.

Two peaks were observed, the first appeared when the gel was eluted with 0.05 M Tris/0.5 M NaCl, but the major lactoferrin peak appeared when the column was eluted with 0.05 M Tris/2 M NaCl, the protein eluting as a clear red solution.

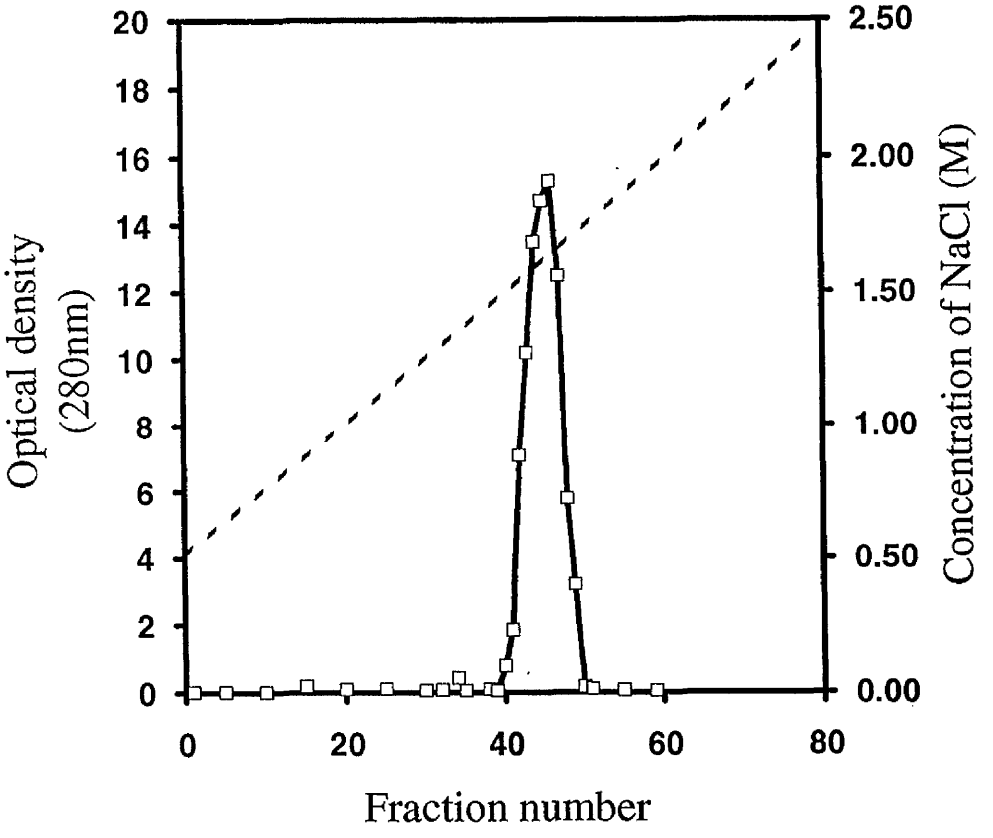


Fig. 14 : Elution profile of lactoferrin prepared from human milk according to the method of Johansson (1960), which had been passed through CM-Sephadex C-50, using 0.05 M Tris, pH 8.0, followed by increasing concentration of NaCl.

About 35 ml of the eluate was pooled from fractions 40-50 and the A_{280} nm and A_{465} nm were measured, the ratio of A_{465}:A_{280} was calculated. The pooled fraction was concentrated using an Amicon filter until the volume was reduced to 4 ml and this was further concentrated to about 1 ml using Amicon ultrafiltration cell equipped with a PM 10 membrane (Amicon Co., Lexington, Massachusettes, U.S.A.). The Amicon cells were centrifuged in a fixed angle rotor (Super minor, Mk 2, MSE). The purity of the pooled fraction was checked by SDS-PAGE as shown in **Fig. 15**. Two bands were observed, one corresponding to lactoferrin and the other being weak could be an impurity.

2.3.1.2 Gel Filtration

A second chromatographic step was necessary in order to remove the impurity, consisting of gel filtration through Ultrogel ACA 34 (LKB, Uppsala, Sweden), which has a separation range of molecular weights between 20,000-350,000 daltons. The column, 34 cm x 3.0 cm was equilibrated overnight with tris-HCl (0.05 M, pH 8.0) containing 0.5 M NaCl. The concentrated lactoferrin prepared from section 2.3.1.1 was applied and the column was eluted with the same buffer at a flow rate of 16.8 ml/h at 2.1 ml/tube. The A_{280} nm of each fraction was measured and an elution profile was plotted (**Fig. 16**). All fractions contributing to the major peak (15 ml; fractions 20-28) were pooled, concentrated and the A_{465}/A_{280} was found to be 0.041, suggesting that the preparation was quite pure. Indeed it was found on SDS-PAGE to give a single band, having the same molecular weight as transferrin (**Fig. 17**).

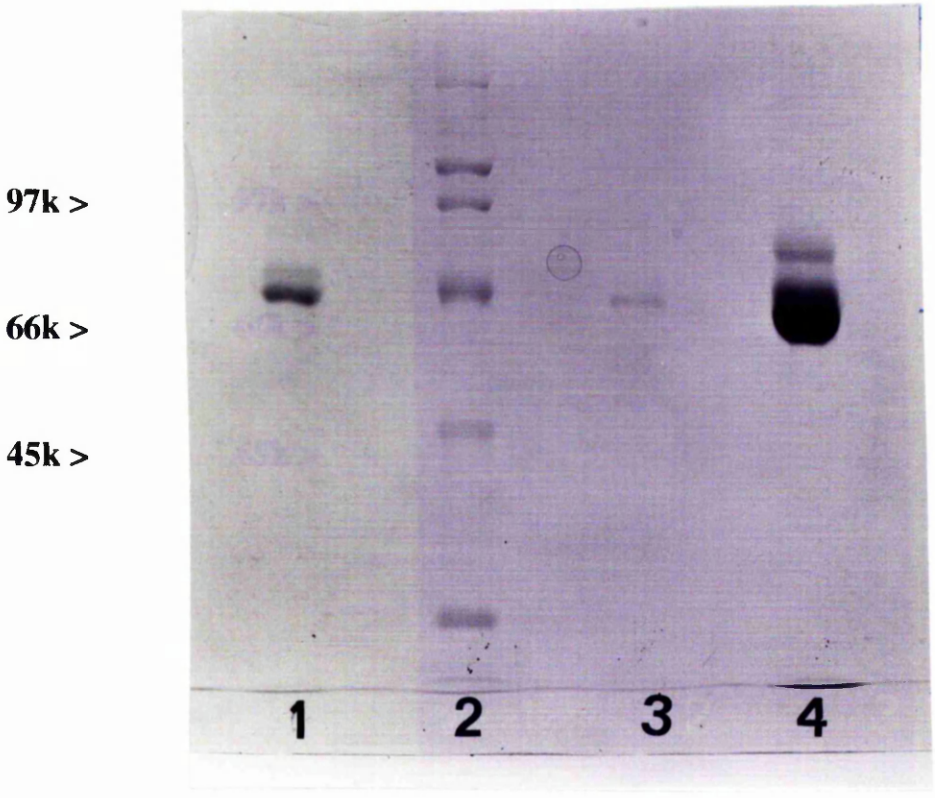


Fig. 15: Electrophoretic patterns of human lactoferrin preparations from milk by passing through CM-Sephadex C-50, according to the method of Johansson (1960). The lanes are referred to as: (1) 10 µl of lactoferrin preparation, (2) standard molecular weight markers, (3) 1 µl lactoferrin preparation and (4) 100 µl lactoferrin preparation applied to the column.

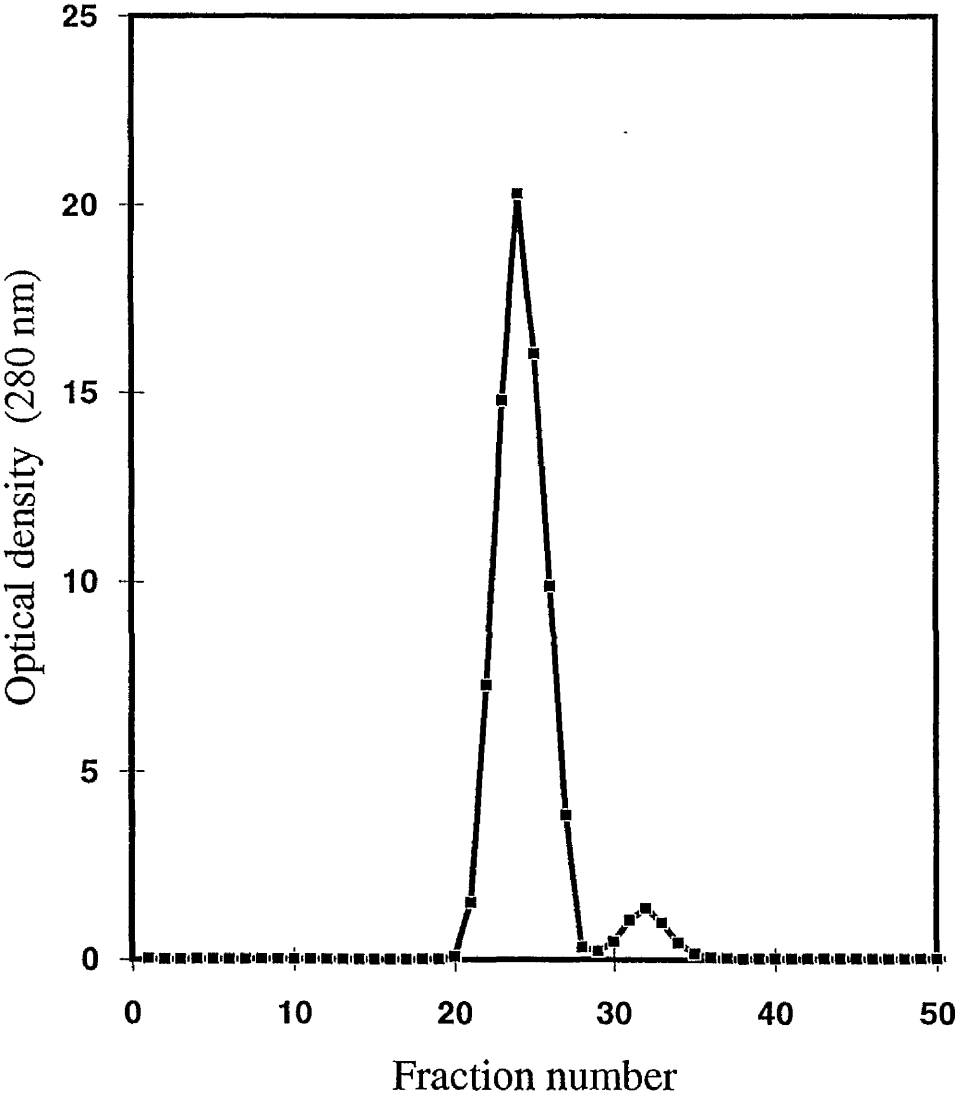


Fig. 16: Gel filtration on Ultrogel ACA 34 of human milk prepared from passage through CM-Sephadex C-50.

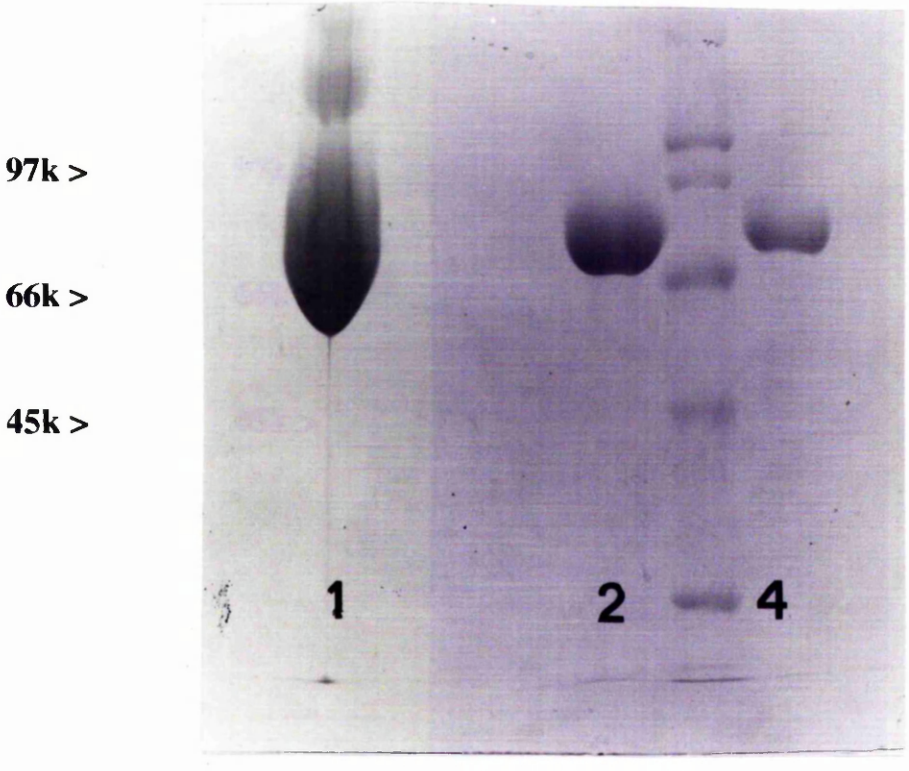


Fig. 17: Electrophoretic patterns in SDS-PAGE of human lactoferrin which has been passed through CM-Sephadex C-50 and Ultrogel ACA 34. The lanes are referred to as: (1) 100 µl of lactoferrin preparation, (2) 10 µl of lactoferrin preparation (3) standard molecular markers and (4) 1 µl lactoferrin preparation.

2.3.2 SDS-PAGE

The LKB 2001 Vertical Electrophoresis System was used with 1.5 mm spacers.

2.3.2 1 Preparation of the Running Gel

About 20 ml of running gel was prepared per slab in a 100 ml Buchner flask. The gel solution was degassed for about 30 seconds, after which 450 µl of 10 % (w/v) ammonium persulphate (Sigma) and 25 µl TEMED (Sigma) were added. The gel solution was pipetted into the gel former to a mark made previously at about 1 cm from the bottom. A few ml of water-saturated butanol was added to remove any air bubble and to ensure a straight surface. The gel was allowed to solidify for about 30 minutes after which butanol was tipped out and the remaining space was washed twice with distilled water.

2.3.2.2 Preparation of Stacking Gel

The stacking gel was mixed with 100 µl ammonium persulphate (10 % w/v) and 10 µl TEMED. A small volume of stacking gel was used to wash the top of the running gel before pouring the stacking gel. A comb was carefully inserted in the stacking gel and was allowed to set for about 15 min after which the comb was carefully extracted.

2.3.2 3 Running the Gel

Three volumes of lactoferrin preparation i.e. 1, 10 and 100 µl as well as 10 µl of SDS molecular weight markers (Sigma) were mixed with 20 µl

sample buffer and heated at 100 °C for 5 min before loading the samples in appropriate spaces in the stacking gel. A constant voltage giving a current of 25 mA per slab was applied until the dye entered the running gel when the current was increased to 45 mA. Current was switched off when the frontline of the dye was about 1 cm from the bottom edge of the gel. The gel was placed in a fixing solution for about 20 min until the bluish frontline turned yellowish. It was then stained for 1.5 h and later destained overnight. The gel was rinsed twice with water, carefully transferred to a piece of blotting paper, placed on a gel dryer plate, and covered with a cling film. The plate was connected to a vacuum freeze-dryer for 4-6 h to dry ; overdrying the gel would cause it to crack. The gel was later photographed.

2.3.3 Desaturation of Lactoferrin

Lactoferrin as prepared in section 2.3 was iron-saturated. It was rendered iron free according to the protocol described by Mazurier and Spik (1980) by dialysis overnight, against acetic acid/sodium acetate buffer (0.1 M) containing EDTA (40 mM) and sodium phosphate (0.2 M) at pH 4.0, with two changes of buffer. It was finally dialysed against PBS. The ratio of $A_{465} : A_{280}$ was determined to estimate the percentage of iron saturation of lactoferrin.

2.3.4 Radiolabelling of Lactoferrin and Transferrin

Lactoferrin and transferrin were labelled singly either with ^{59}Fe or ^{125}I or whenever required they were labelled with both isotopes.

2.3.4.1 Radiolabelling with ^{59}Fe

Lactoferrin and transferrin were dissolved in PBS-1 % bicarbonate. To obtain iron saturated ^{59}Fe-labelled proteins, sufficient ^{59}Fe citrate (Amersham) to give 30 % iron saturation was added and the proteins were left overnight at 4 °C to allow specific binding of ^{59}Fe. FeNTA (20 mM) was then added to fully saturate the proteins. This sequential addition of ^{59}Fe followed by unlabelled FeNTA ensured that all radioiron was specifically bound to the proteins.

2.3.4.2 Radiolabelling with ^{125}I

Labelling with ^{125}I (Amersham) was carried out by the chloramine-T method (Greenwood *et al.*, 1963). Lactoferrin and transferrin were dissolved in PBS at the high concentration of 20 mg/ml because the effectiveness of iodination is proportional to the protein concentration. To 1 mg of protein was added 5 µl (0.5 mCi) ^{125}I (Amersham) and were mixed gently by sucking a few times into the micropipette. Five µl of chloramine T (1 mg/ml; Sigma) was next added and was mixed for only 20 seconds after which 5 µl sodium metabisulphite (1 mg/ml; Sigma) was immediately added. Lastly 5 µl potassium iodide (5 mg/ml; Sigma) was mixed with the solution. The protein solution was then passed through a prepacked Sephadex G25 column (Pharmacia LKB Technology AB, Uppsala, Sweden) to remove any unbound ^{125}I. Fractions of about 1 ml were collected until the radioactivity in the gel as measured by a hand γ-detector diminished. Five µl aliquots were taken from each fraction and counted for ^{125}I in a Compugamma 1232 gamma counter (LKB-Pharmacia, Croydon, U.K.). Fractions giving rise to the first ^{125}I-peak were pooled and the A_{280}

nm was measured to determine the protein concentration. The protein solution was filter sterilised and kept at 4 °C.

2.3.4.3 Double labelling with ^{59}Fe and ^{125}I

To label the proteins with both ^{59}Fe and ^{125}I, they were first labelled with ^{59}Fe and then saturated with FeNTA according to the procedure in section 2.3.4.1 except that the proteins were dissolved in PBS instead of PBS-1 % bicarbonate. The proteins were next labelled with ^{125}I as in section 2.3.4.2.

2.3.5 Affinity Chromatography

Four affinity columns were prepared which were: Sepharose-sheep anti-human transferrin (S-Sa-HTf), Sepharose-normal sheep immunoglobulin (S-NSIg), Sepharose-rabbit anti-human lactoferrin (S-Ra-HLf) and Sepharose-normal rabbit immunoglobulin (S-NRIg).

2.3.5.1 Production of Antibodies Against Lactoferrin

The inoculum was prepared by emulsifying 1 ml of complete Freund's adjuvant (Difco) with an equal volume of lactoferrin (1 mg/ml) in a plastic bijou, which was taped to a vortex and shaken vigorously for 20 min. The stability of the emulsion was tested by dropping a small volume of the emulsion into a beaker of water. The emulsion was ready to be injected if the drop retained its globular form and did not diffuse in the water. The emulsified inoculum was injected into two rabbits (1 ml per rabbit) by multiple subcutaneous inoculations at various sites followed by 3 weeks rest. After 3 weeks, a booster was given (1 mg/ml of lactoferrin in PBS) to each rabbit as before, followed by a week's rest and then a trial bleed.

Approximately 20 ml blood was collected from the ear vein of each rabbit into a glass universal and allowed to clot at room temperature. The serum was then pooled, transferred to plastic conical-bottomed test tubes (Sterilin) and centrifuged twice at 500 g for 5 min to remove any erythrocytes. The antibody titre was checked by the ring precipitin test as follows. A small volume of lactoferrin solution (10 μg/ml; 100 μg/ml and 1 mg/ml) was gently layered on top of a similar volume of antiserum in a Durham tube. The formation of strong precipitin bands with all three concentration of the protein indicated a very good titre.

2.3.5.2 Preparation of IgG from Anti-Serum

IgG was prepared from rabbit anti-serum by ammonium sulphate precipitation. Ten ml of ice-cold serum was pipetted into a small beaker and saturated ammonium sulphate solution was gradually added with constant stirring, and then left to stir for 1 h. The precipitate was recovered by centrifugation at 3000 rpm for 10 min at 4 °C, resuspended in 20 ml of 50 % saturated ammonium sulphate solution and left to stir for another 1 h. The precipitate was spun down as before and resuspended in 5 ml PBS. It was then dialysed against 2 x 2 litre coupling buffer to remove traces of ammonium sulphate. The purity of IgG preparation was checked by measuring its A_{280} nm and A_{250} nm. The ratio of A_{280}:A_{250} was found to be 2.7, indicating that the preparation was pure. Finally the concentration of the IgG preparation was adjusted to 5-10 mg/ml.

2.3.5.3 Coupling of Lactoferrin and Transferrin to CNBr-Activated Sepharose 4B

The required amount of freeze-dried CNBr-activated Sepharose 4B (Pharmacia Ltd., Milton Keynes, U.K.) was swollen in 1 mM HCl for 15 min, then washed with the same solution on a sintered glass filter (200 ml/g gel). The gel was finally washed and resuspended in the coupling buffer (see section 2.2.6.7). The proteins to be conjugated were previously dialysed against the coupling buffer for 2 days, the concentration then adjusted to 5-10 mg/ml as judged by spectroscopic absorption at 280 nm. Two volumes of the swollen gel were immediately mixed with one volume of the protein (5 mg protein per ml swollen gel). The mixture was left rotating in an end-over-end mixer for 2 h at room temperature after which it was centrifuged and the supernatant saved to calculate by the difference the amount of the protein coupled. Excess ligand was washed away with the coupling buffer on the glass filter and any remaining active groups was blocked by treatment with 2 volumes of 1 M glycine for 2 h at room temperature. The final product was washed with 3 cycles of 0.1 M sodium acetate buffer (pH 4) containing 0.5 M NaCl followed by the coupling buffer, to remove traces of non-covalently adsorbed materials.

2.3.5.4 Preparation of Insolubilised Sepharose-Antibody Conjugates

The gel prepared as in section 2.3.5.3 was placed in a column made from a 10 ml syringe. Ten ml of anti-serum was passed through the column twice. The column was washed with PBS to elute any unbound protein. It was then washed with 3 M KSCN (BDH) in 0.1 M $NaHCO_3$ (pH 8.5) to elute bound antibody. The washes containing the antibody were next passed through prepacked Sephadex G-25 columns (Pharmacia) to remove any

salts. About 10 ml fraction was collected and its A_{280} nm was measured. It was then coupled with 0.5 g CNBr-activated Sepharose 4B gel following similar procedure as described in section 2.3.5.3. The conjugates were stored at 4 °C in PBS containing 0.02 % (w/v) sodium azide.

2.3.5.5 Specific Activity of Sepharose-Antibody Conjugates

The binding capacity and cross reactivity of the immunoabsorbents were checked by passing 5 μg of ^{59}Fe-lactoferrin and ^{59}Fe-transferrin through 50 μl of each gel. Cross reactivity of Sepharose-rabbit anti-human lactoferrin with transferrin was negligible but for Sepharose-sheep anti-human transferrin, it was necessary to pass through 1 mg of unlabelled lactoferrin to reduce cross reactivity with lactoferrin to < 10 %. The binding capacities of the anti-transferrin and anti-lactoferrin immunoabsorbents were 82 and 92 μg of ligand per ml of settled gel respectively.

2.3.6 Cell Culture

U937 cells were routinely cultured in RPMI 1640 medium supplemented with 10 % FCS in a humidified atmosphere of 95 % air and 5 % CO_2. They were subcultured every 3 days at 3 x 10^5 cells/ml and were maintained for 8-10 weeks. Except where otherwise stated, serum-free medium containing HSA (1 mg/ml) in place of FCS was used in all experimental procedures in order to eliminate interference by transferrin and other proteins present in the serum. The iron concentration of RPMI 1640 was estimated at 6-12 ng/ml by carbon furnace atomic absorption spectroscopy. Log-phase cells obtained 18 h after subculture were used in all experiments. The cells were washed twice with warm Hanks buffered salts solution (HBSS; Flow),

resuspended in serum-free medium and incubated for 30 min at 37 °C to ensure exocytosis of serum proteins prior to use.

2.3.7 Binding of Lactoferrin and Transferrin to U937 Cells

Log-phase cells (1 x 10^6 cells/ml) were incubated with 10 μg/ml ^{125}I-labelled lactoferrin or transferrin and an equal amount of unlabelled transferrin or lactoferrin respectively, to ensure that the two cultures contained both iron-binding proteins. The tubes were incubated either at 4 °C or 37 °C for various time intervals from 30 to 360 min. At each time interval, an aliquot (0.5 ml) of cell suspension was removed and immediately placed in ice water, and the cells spun down at 1500 rpm for 5 min in a refrigerated centrifuge; the supernatant removed and the cells were washed with ice-cold HBSS. The washing was repeated three times and prior to the last wash the cell suspension was transferred to a fresh tube to minimise the problem of non-specific binding of radioisotope to the tube. Radioactivity in the supernatants, washes and cell pellets was measured in a Compugamma 1232 gamma counter.

2.3.8 Determination of Lactoferrin and Transferrin Receptors on U937 Cells

A polystyrene 96-well round bottom plate (Flow) was coated with 100 μl of gelatin (0.25 % in 1 % BSA-PBS; Sigma) per well at 37 °C for 1 h. Aliquots of 25 μl of log-phase cells (2 x 10^7 cells/ml) were placed in each well, giving 5 x 10^5 cells/well. Cells were incubated either with varying amounts of ^{125}I-labelled proteins alone (2 - 24 μg/ml in 0.2 % BSA-PBS), to determine the total binding sites, or with the same amounts of labelled proteins plus a 200 fold excess of competing unlabelled protein, to

determine the non-specific binding. The plate was incubated at 4 °C for 1 h after which the cells were harvested using a cell harvester (Ilacon, Tonbridge, U.K.) on to a glass fibre mat which had been prewashed with 0.2 % BSA-PBS. Each filter was cut out and counted in the gamma counter. The number and affinity of the receptors were calculated by the method of Scatchard (1949).

2.3.9 Determination of Specificity of Lactoferrin and Transferrin Binding to U937 Cells

To log-phase cells in plastic conical tubes were added 10 μg/ml ^{125}I-labelled lactoferrin or transferrin each with the addition of equal amount of opposite unlabelled proteins or PBS as a control. All tubes were incubated at 4 °C for 30 min after which the cells were treated as in section 2.3.7.

2.3.10 Effect of Milk Proteins on Binding of Lactoferrin to U937 Cells

To duplicate tubes of 1 ml cell suspensions (10^6/ml) were added ^{125}I-labelled human or bovine lactoferrin (10 μg/ml) and unlabelled competing proteins (α-lactalbumin, β-lactoglobulin, lactoperoxidase, IgA and lysozyme) at 10 μg/ml or 200 μg/ml and incubated for 30 min at 4 °C. The cells were then treated as in section 2.3.7.

2.3.11 Effect of Heparinase on Binding of Lactoferrin and Transferrin

Log-phase cells were prepared as in 2.3.6 but were resuspended in warm serum-free RPMI 1640 medium plus 0.2 % BSA (Sigma). To 200 μl aliquots of cell suspension (10^6/ml) were added 10 μl of PBS (control), 1 or 2 units of Heparinase III (Sigma) and incubated at 37 °C for 1 h after which

the cells were immediately placed in ice water. ^{125}I-labelled lactoferrin or transferrin (10 μg/ml) was added to appropriate tubes and were then incubated at 4 °C for 30 min. All tubes were treated as in section 2.3.7.

2.3.12 The Effect of Fucose on Binding of Lactoferrin and Transferrin

To U937 cells prepared as in section 2.3.11 were added 10 μl fucose (5 mg/ml; kindly provided by Dr. D.A.R Simmons, Department of Bacteriology, Western Infirmary, Glasgow) or glucose (5 mg/ml; Fisons, Loughborough, U.K.) and mixed well. ^{125}I-labelled lactoferrin or transferrin (10 μg/ml) was added and the cells were incubated at 4 °C for 1 h. All tubes were treated as in section 2.3.7.

2.3.13 Effect of lactoferrin on the binding of monoclonal antibodies to monocyte surface markers

To one ml aliquots of cell suspension (5 x 10^6/ml) were added lactoferrin (10 μg/ml) or PBS (control) and these were incubated at 4 °C for 30 min. Five μl of monoclonal antibodies to monocyte cell surface markers were next added and incubated for 30 min after which the fluorescence was measured in the Fluorescence Activated Cell Sorter (FACSCAN, Becton Dickinson). In another experiment, increasing concentrations of lactoferrin (0, 10, 50 and 250 μg/ml) were used to confirm the effect of monoclonal antibodies against CD4 and DR on the binding of lactoferrin to the cells.

2.3.14 Iron Uptake from Lactoferrin and Transferrin by U937 Cells

Iron uptake from lactoferrin and transferrin was determined by incubating 10 ml of cell suspension (2 x 10^5 cells/ml) at 37 °C with ^{59}Fe-labelled lactoferrin or transferrin (50 μg/ml) plus unlabelled transferrin or lactoferrin (50 μg/ml) respectively at various time intervals. The third tube contained equal amount of unlabelled lactoferrin and transferrin to study the ^{3}H-thymidine uptake of these cells, in parallel with the measurement of iron uptake. At each interval 2.5 ml cell suspension was removed and treated as in section 2.3.7.

2.3.15 Exchange of Iron Between Lactoferrin and Transferrin During Cell Culture

In order to examine the possibility that there might be a transfer of ^{59}Fe from one iron-binding protein to another during culture, 50 μl aliquots of each culture supernatant were removed and passed through 50 μl of anti-transferrin and anti-lactoferrin immunoabsorbents. The gels were washed with 1 ml PBS to remove all unbound protein and the washes and gels assayed for ^{59}Fe activity.

2.3.16 The Effect of Lactoferrin on Iron Uptake from Other Sources

To determine the effect of lactoferrin on iron uptake from other sources, cells were incubated with 50 μg/ml ^{59}Fe-transferrin or 8 mM Fe as ^{59}FeNTA in the presence or absence of 50 μg/ml of unlabelled apolactoferrin or 50 % Fe-saturated lactoferrin.

2.3.17 Internalisation of Lactoferrin and Transferrin (Acid Treatment)

Cells (2 x 10^6 in 200 µl) were incubated with 50 µg/ml ^{125}I-labelled lactoferrin or transferrin at 4 °C or 37 °C for 30 min, then spun down and washed three times with ice cold PBS. Surface and intracellular distribution of the labelled proteins was determined by treating the cells with 200 µl of 0.25 M acetic acid/0.5 M NaCl, pH 2.3 for 5 sec, followed by addition of 100 µl of 1 M sodium acetate which raised the pH to 6.3. Cells and supernatant were separated by rapid centrifugation through Versilube F50 oil (Alfa, Wokingham, U.K.).

2.3.18 The Effect of Primaquine on Internalisation of Lactoferrin and Transferrin

To determine whether endocytosed proteins were recycled back to the cell membrane, cells that had been initially incubated with ^{59}Fe,^{125}I-labelled lactoferrin or transferrin (50 µg/ml) were treated for 30 min at 37 °C with 3 mM primaquine (Sigma) following removal of unbound labelled proteins. The cells were then subjected to acid-washing as in 2.3.17.

2.3.19 Dissociation Studies

Cells were preincubated with ^{59}Fe,^{125}I-labelled lactoferrin or transferrin (10 µg/ml). Unbound proteins were removed by centrifugation as in 2.3.7 and the washed cells reincubated in the presence of 1 mM desferrioxamine (CIBA, Horsham, U.K.). In another experiment, desferrioxamine was replaced by apotransferrin or apolactoferrin (50 µg/ml). At various time intervals, aliquots were removed and centrifuged. Radioactivity in the cells and supernatants was assayed. In some experiments, the supernatant was

treated with 10 % (w/v, final concentration) trichloroacetic acid to check whether proteins released into the medium had been degraded.

2.3.20 Measurement of Cell Proliferation

Log-phase cells (3 x 10^5/ml) in serum-free medium were incubated with appropriate additions (apolactoferrin, lactoferrin, apotransferrin and transferrin) at 50 μg/ml for 28 h after which 5 x 100 μl aliquots were pulsed with 1 μCi ^{3}H-thymidine (Amersham) for 4 h. The cells were then harvested on glass fibre mats using a cell harvester (Skatron, Lierbyen, Norway), which were usually left overnight on the bench to dry. Individual discs, each with contents of a single well, were added to 2 ml of scintillation fluid (LKB, Croydon, Surrey, England) and counted for 5 min on a scintillation counter (Packard, Pangbourne, Berkshire, England).

2.3.21 The Effect of Milk Proteins on Cell Proliferation

Measurements of cell proliferation was as in 2.3.24 except that samples contained 50 μg/ml native apolactoferrin or Fe-transferrin plus 50 μg/ml test protein (milk proteins or heated lactoferrin) or 100 μg/ml native lactoferrin alone. Controls contained no added proteins.

2.4 RESULTS

2.4.1 Binding Studies of Lactoferrin and Transferrin to U937 Cells

2.4.1.1 Binding pattern of Lactoferrin and Transferrin

Binding of lactoferrin and transferrin to U937 cells as a function of time and temperature is shown in **Fig. 18** and **Fig.19** respectively. Binding of lactoferrin and transferrin was found to be temperature dependent; at 4 °C, binding of both lactoferrin and transferrin remained almost constant but at 37 °C, lactoferrin associated with the cells increased gradually throughout the 6 h incubation period. Binding of transferrin at 37 °C was more rapid than that of lactoferrin and reached a plateau after 2 h. At both temperatures, the cells bound about ten times more lactoferrin than transferrin.

To characterise the binding in more detail the specific and non-specific binding was determined (**Figs. 20** and **21**) and the results subjected to Scatchard analysis. Again, it was found that total binding of lactoferrin to the cells was about 10 times greater than total binding of transferrin. However, most of the lactoferrin binding was non-specific, although Scatchard analysis revealed that there was nevertheless a specific element (**Figs. 22** and **23**). The number of lactoferrin binding sites per cell (3.0×10^6) was similar to that for transferrin (1.9×10^6). The dissociation constant for the specific binding of lactoferrin to U937 cells was 4-fold higher (83 nM) than that for transferrin (21 nM).

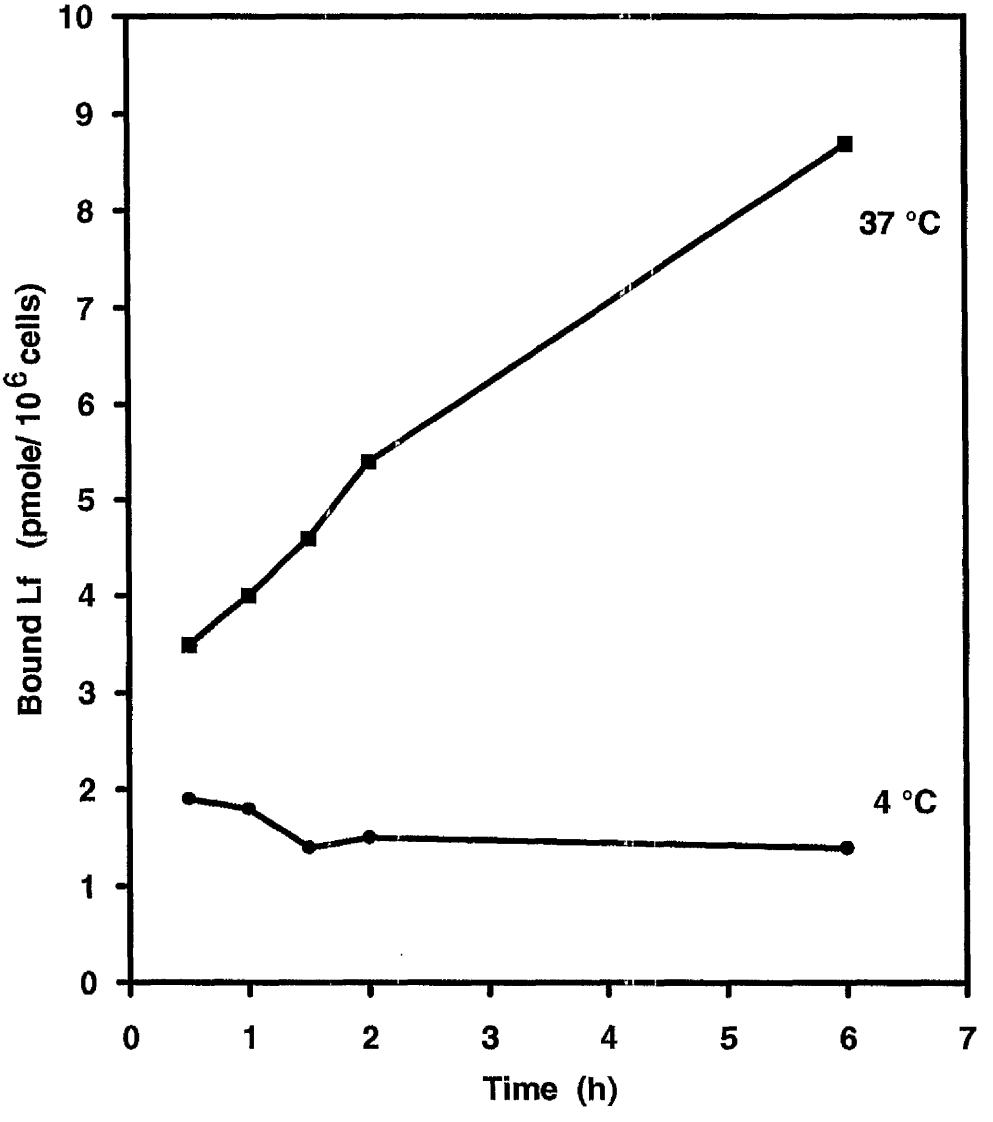


Fig. 18 : Effect of temperature and time on the binding of lactoferrin to U937 cells. Each point is a mean of duplicate experiments which did not vary by more than 10 %.

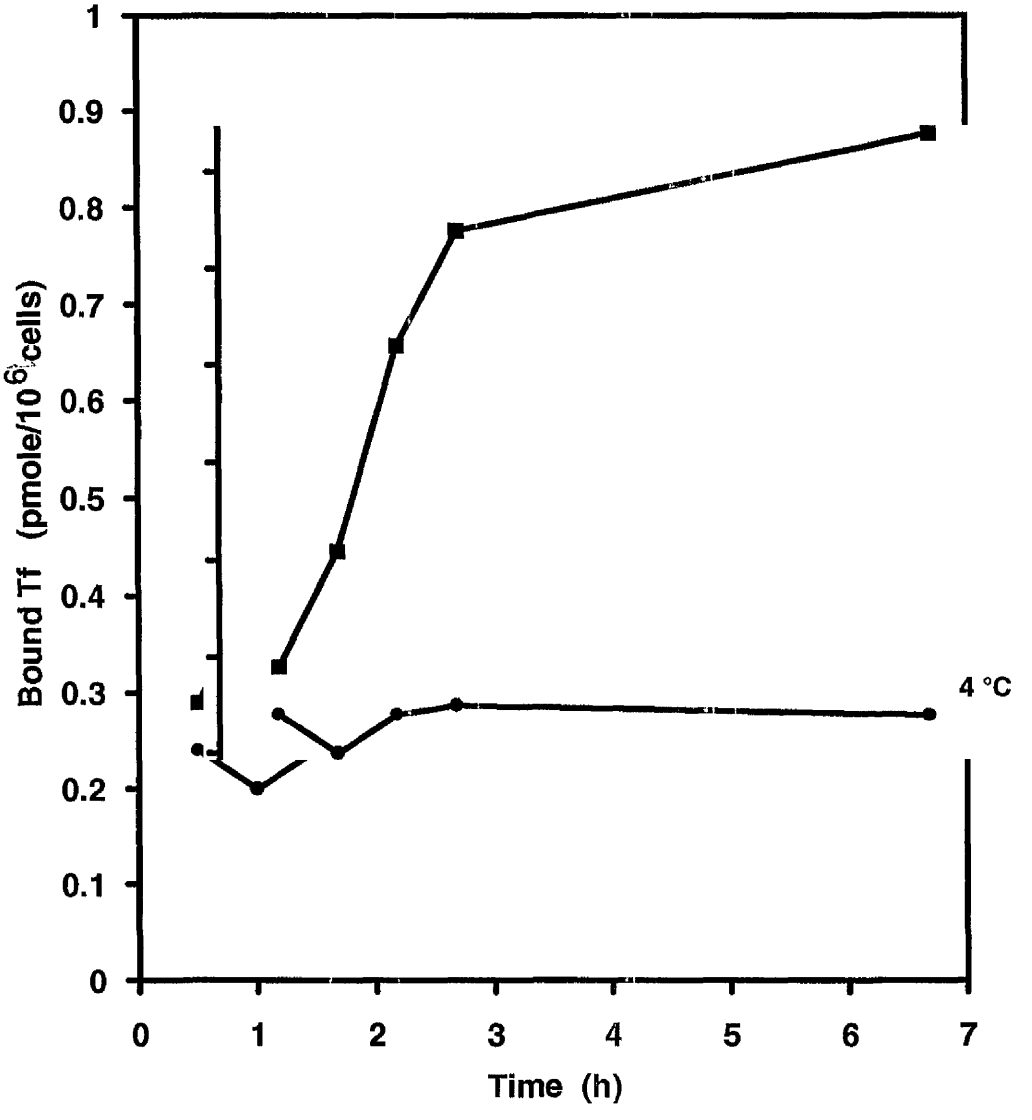


Fig. 19 : Effect of temperature and time on the binding of transferrin to U937 cells. Each point is a mean of duplicate experiments which did not vary by more than 10 %.

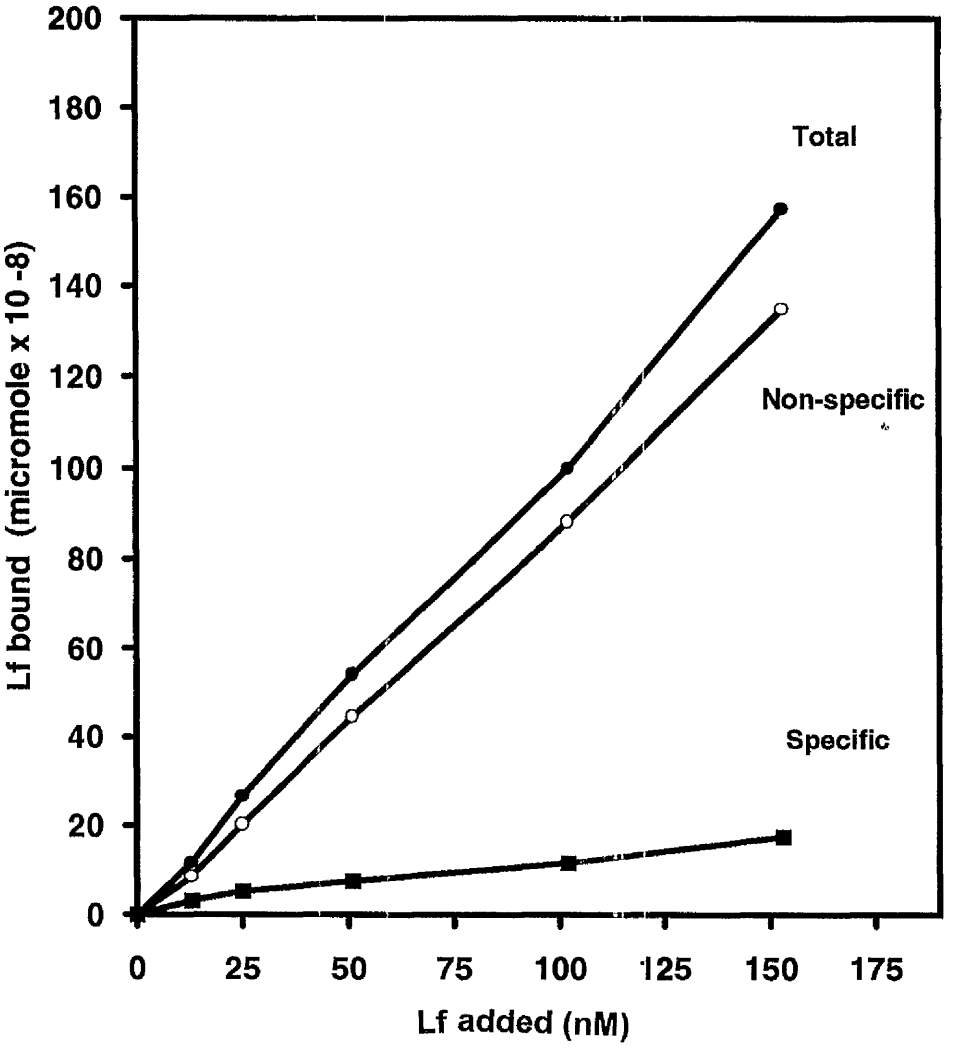


Fig. 20 : Binding of ^{125}I-labelled lactoferrin to U937 cells in the absence (total binding) or presence of 200-fold excess of unlabelled lactoferrin (non-specific binding). The difference between the two accounts for the specific binding. Values represent means of duplicate experiments.

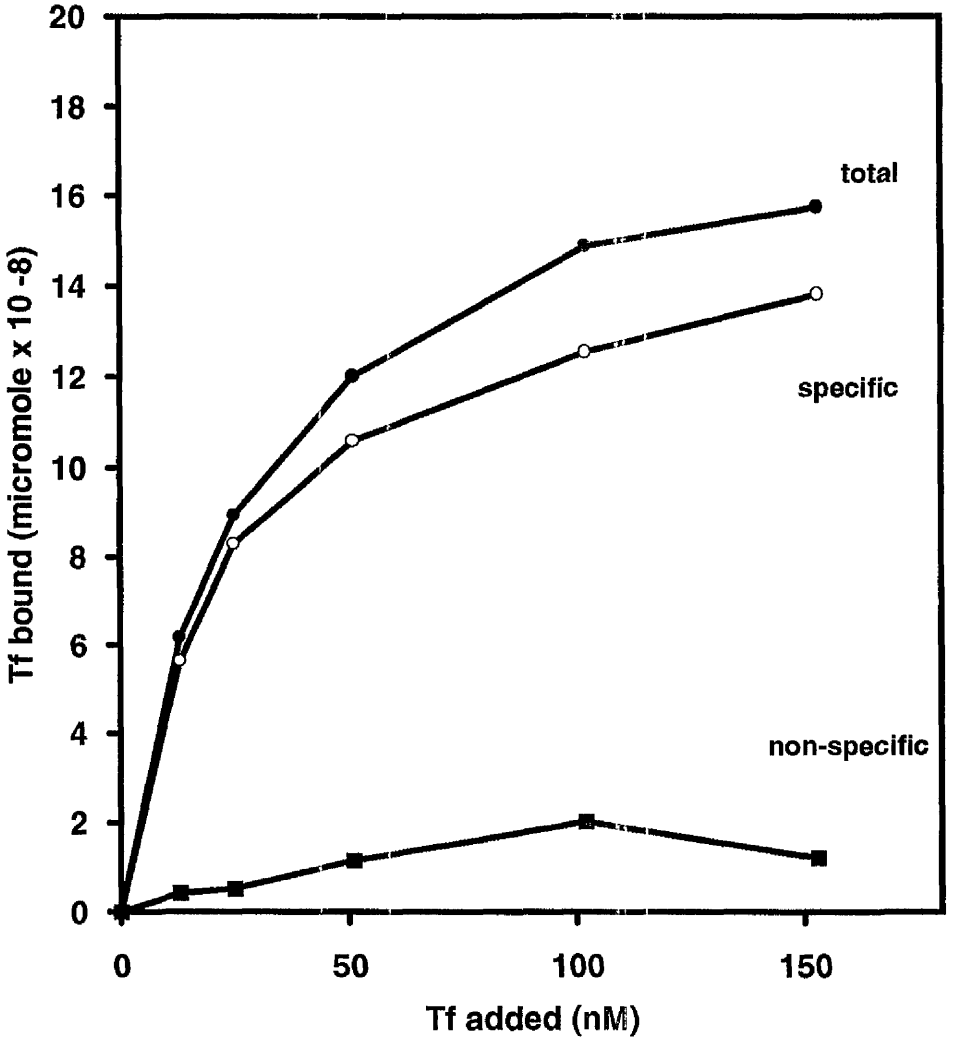


Fig. 21 : Binding of ^{125}I-labelled transferrin to U937 cells in the absence (total binding) or presence of 200-fold excess of unlabelled transferrin (non-specific binding). The difference between the two accounts for the specific binding. Values represent means of duplicate experiments.

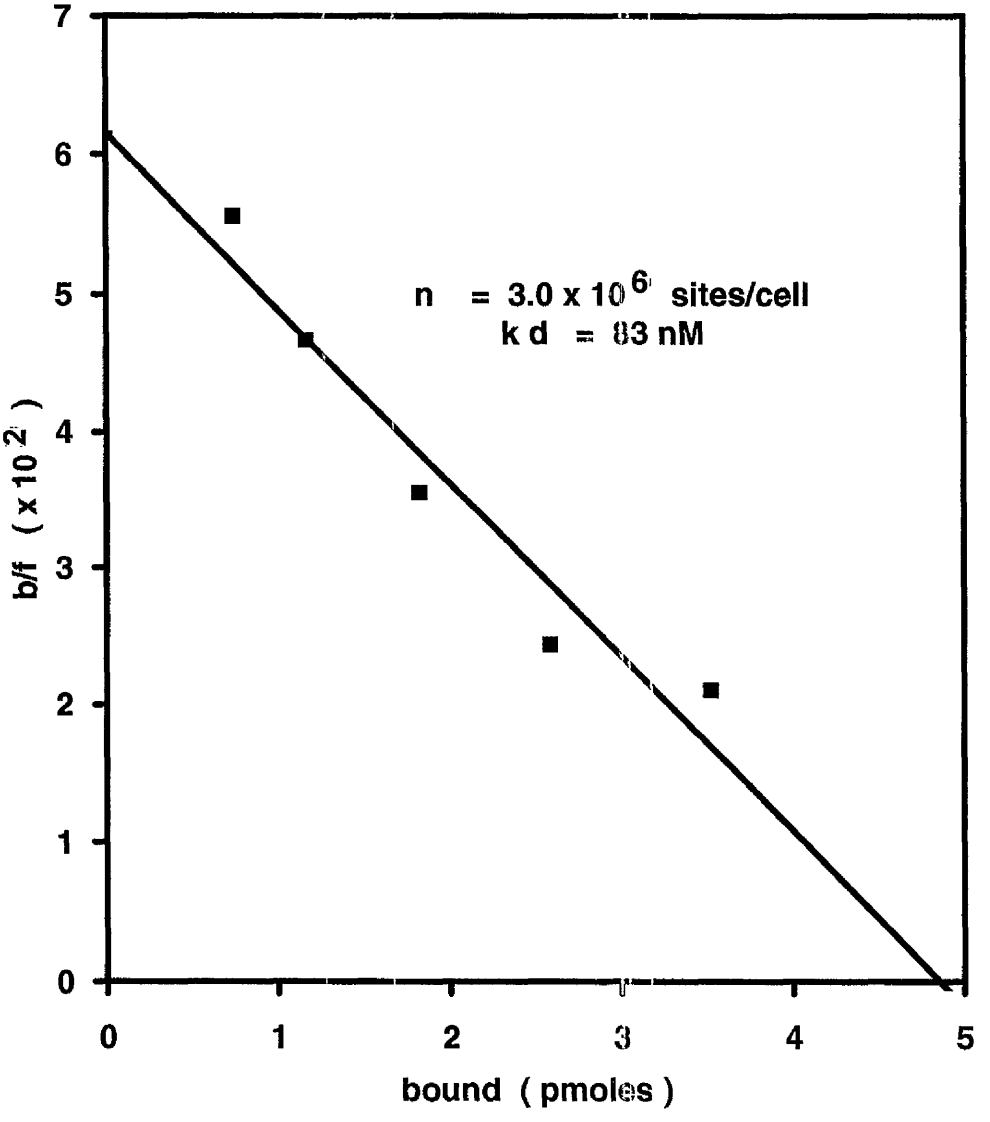


Fig. 22 : Scatchard plot for the specific binding of lactoferrin to U937 cells.

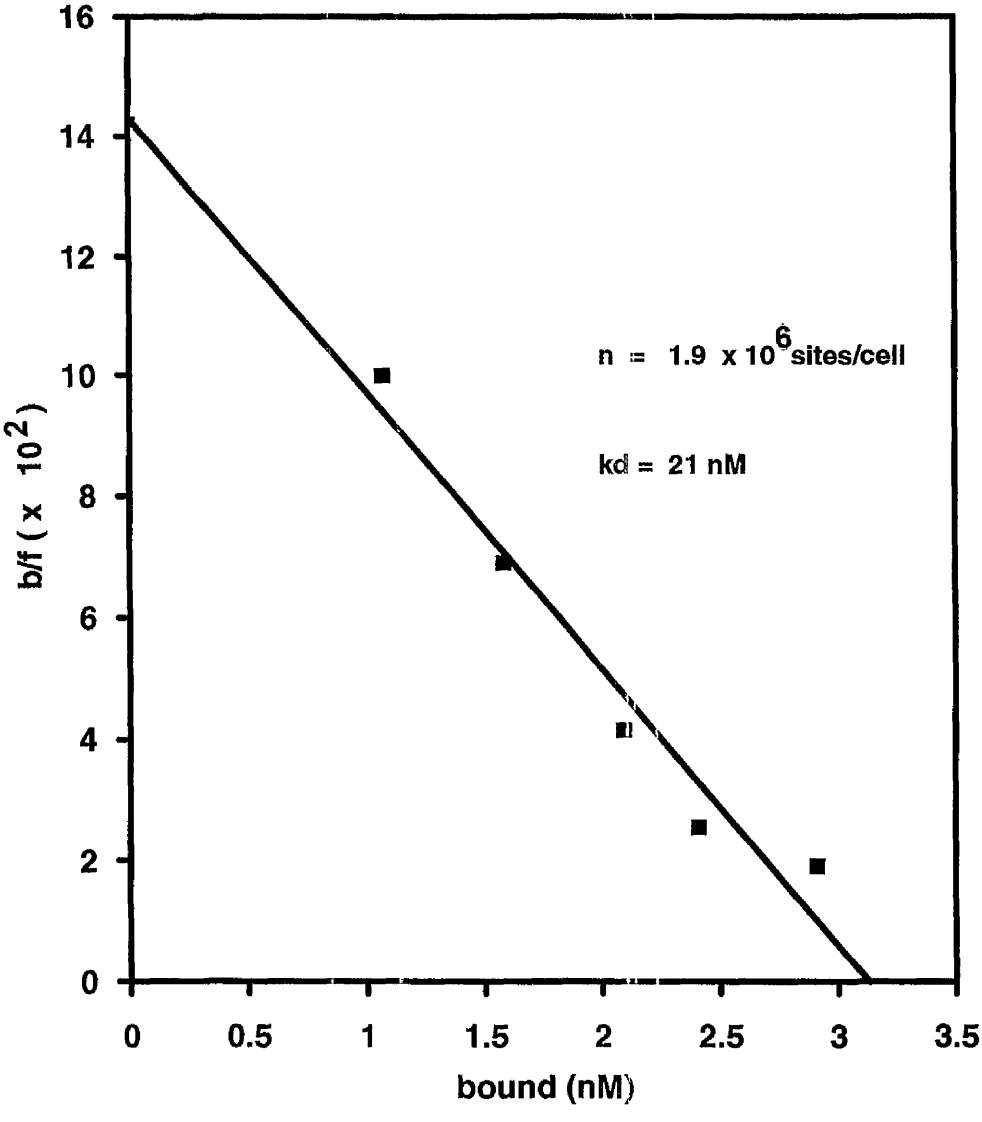


Fig. 23 : Scatchard plot for the specific binding of transferrin to U937 cells.

2.4.1.2 Competitive Binding of Lactoferrin and Transferrin to U937 Cells

In order to determine whether lactoferrin sharesthe same binding sites as transferrin on U937 cells, these cells were incubated with labelled lactoferrin or transferrin and an equal amount of unlabelled proteins. Binding of lactoferrin was not greatly affected by Fe-transferrin or apotransferrin (**Fig.24**), and neither form of lactoferrin affected transferrin binding (**Fig. 25**). These findings suggest that lactoferrin binding sites are different from those for transferrin.

In other competitive experiments to investigate whether fucose residues present in human milk lactoferrin were involved in its binding to the receptor, it was found that binding was not inhibited by 30 mM fucose, nor glucose of similar concentration (**Table III**). Furthermore treatment of the cells with heparinase (1-2 units/ml), which cleaves the acidic sugar residues did not result in reduced binding of lactoferrin as well (**Table III**).

When monoclonal antibodies were added with lactoferrin to the cells, it was found in preliminary experiments that in most cases lactoferrin did not inhibit the binding of monoclonal antibodies to CD3, CD11b, CD 14, CD 25 and CD 45 but somehow in these initial experiments anti-CD4 and anti HLA-DR showed a significant shift in the peak fluorescent channel as compared to the control. However further test to determine the effect of increasing concentration of lactoferrin did not alter the peak fluorescent channel values (**Fig. 26**). These results indicate that none of of the nine monoclonal antibodies tested was found to share the same binding site as lactoferrin on U937 cells.

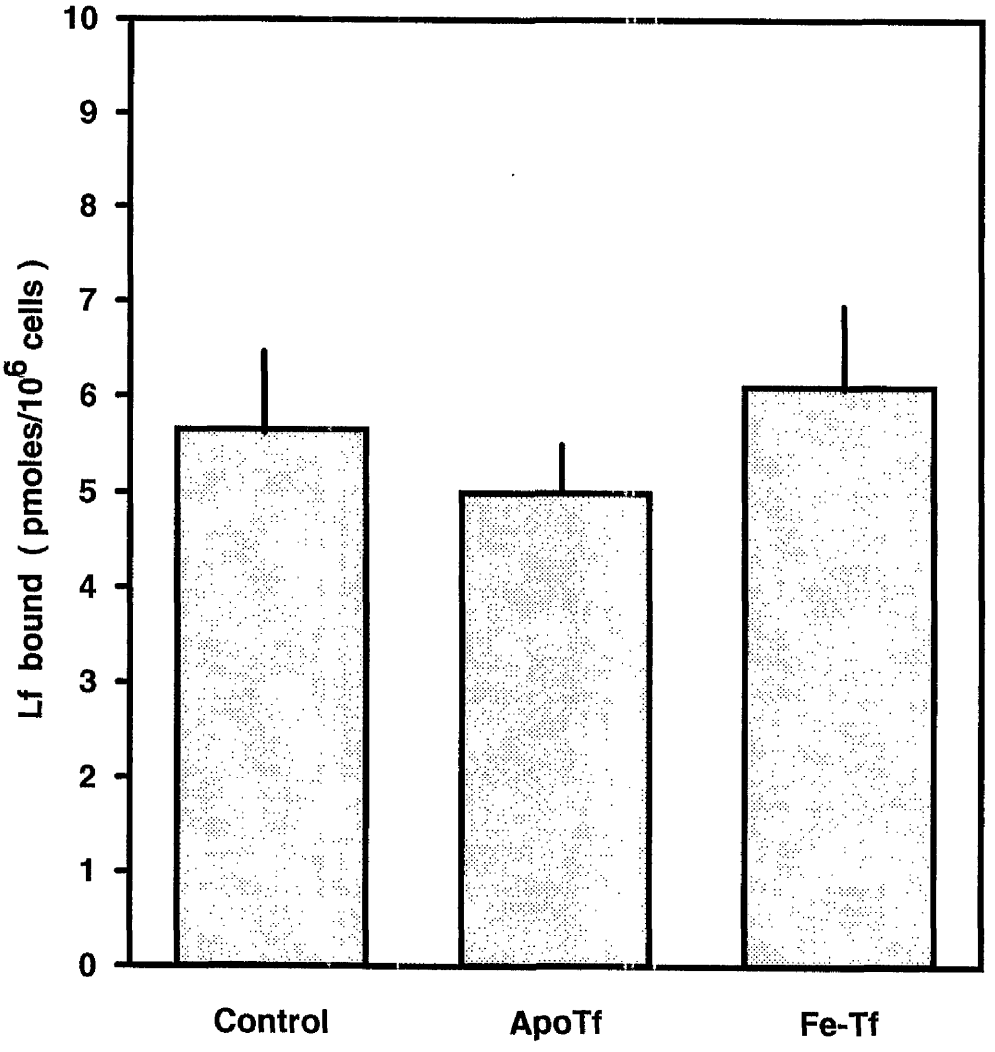


Fig. 24 : Effects of transferrin on the binding of lactoferrin to U937 cells. Values represent means ± SD (n = 4).

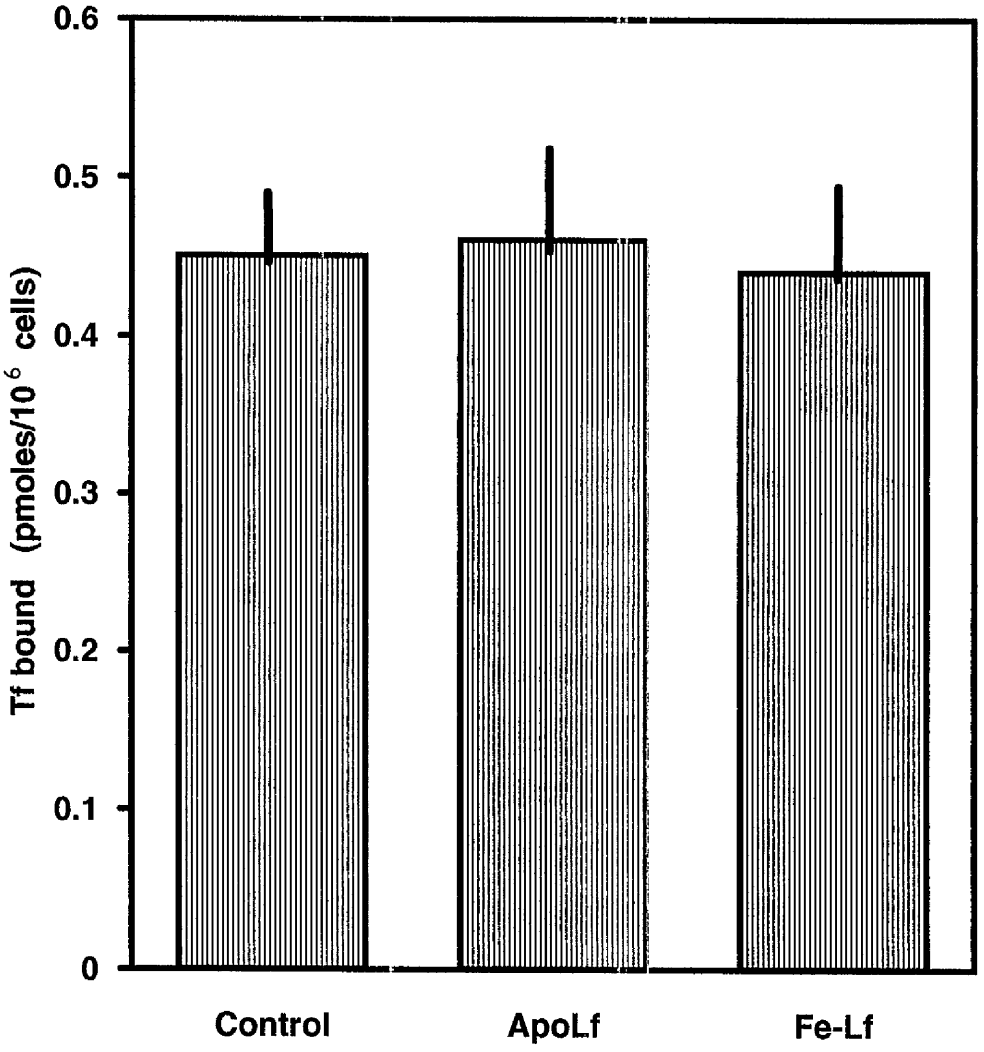


Fig. 25 : Effects of lactoferrin on the binding of transferrin to U937 cells. Values represent means ± SD (n = 4).

TABLE III

Effect of heparinase, fucose and glucose on the binding of lactoferrin and transferrin to U937 cells

U937 cells were incubated with heparinase III (1-2 units/ml) for 1 h at 37°C in serum-free RPMI medium plus 0.2 % BSA, after which the tubes were immediately placed in ice water before adding 10 μg/ml ^{125}I-lactoferrin or ^{125}I-transferrin and incubated at 4°C for 30min. In another experiment, the cells were incubated with 30mM L-fucose or D-glucose together with labelled lactoferrin or transferrin in serum-free RPMI medium containing 0.2% BSA for 30min at 4°C. At the end of incubation, cells were washed 3 x with HBSS· and assayed for cell-associated radioactivity. Values represent means ± S.D. for three determinations

	Cell-associated radioactivity sample/control x 100 (%)		
Addition	Heparinase	L-Fucose	D-Glucose
Lactoferrin	96.7 ± 19.1	100.7 ± 13.3	101.4 ± 0.05
Transferrin	78.7 ± 10.8	101.9 ± 4.3	103.8 ± 14.9

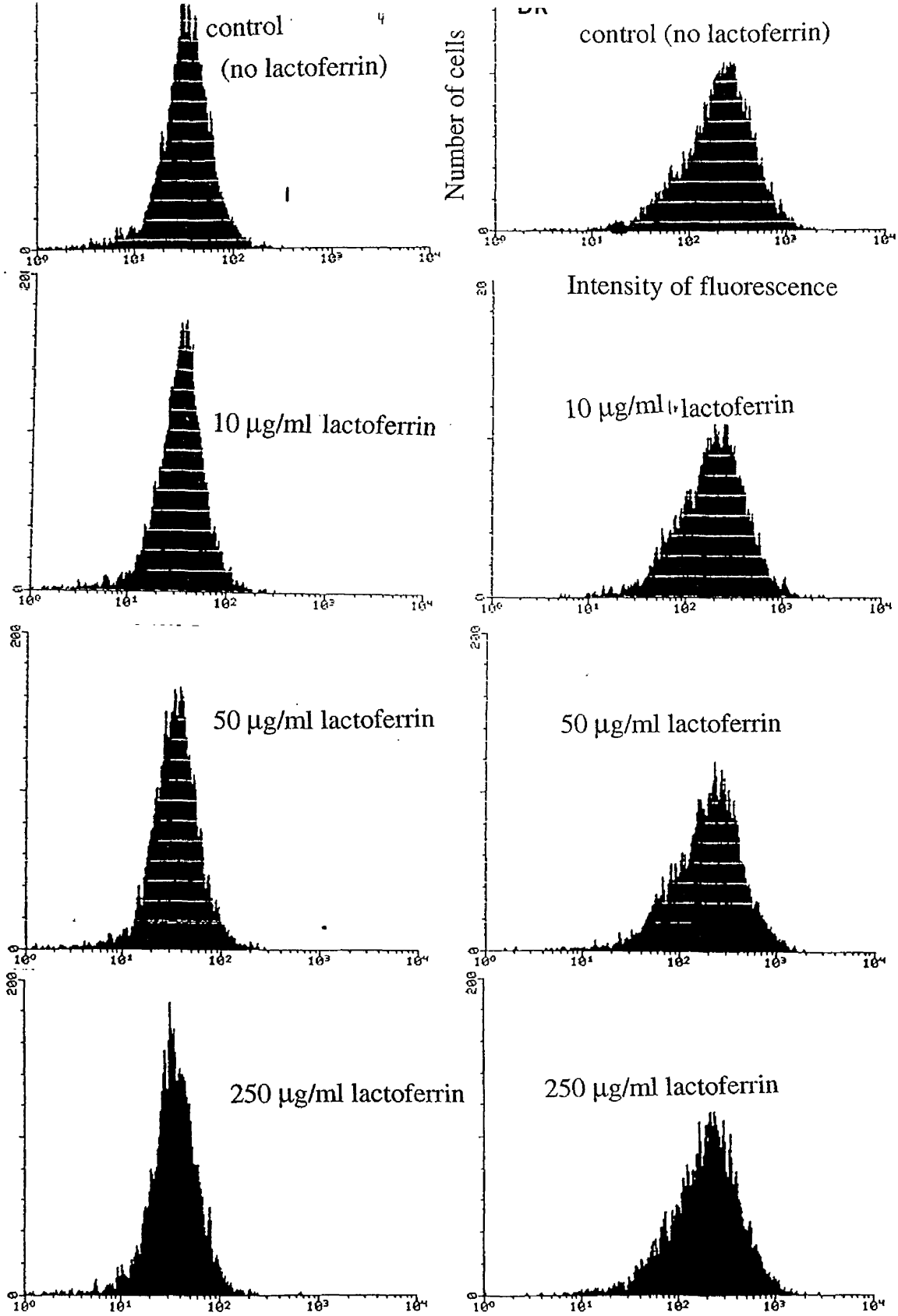


Fig. 26: The effect of different concentrations of lactoferrin on the binding of monoclonal antibodies to CD4 and DR as observed from immunofluorescence measurements.

2.4.1.3 Effect of Milk Proteins on Binding of Lactoferrin to U937 Cells

Just like human lactoferrin, there was also an appreciable binding of bovine lactoferrin to U937 cells; binding of bovine lactoferrin being 47 % higher than human lactoferrin. Binding of labelled bovine lactoferrin was reduced by the presence of unlabelled bovine lactoferrin, especially with excess of it which markedly reduced binding to about one third of the total binding (**Fig. 27**). Bovine lactoferrin on the other hand did not inhibit binding of labelled human lactoferrin, in the presence of even 20-fold excess of bovine lactoferrin; if anything binding was slightly increased (**Fig. 28**). This suggests that the recognition mechanisms for human and bovine lactoferrin are not identical. Most of the other milk proteins tested had no effect on the binding of either human or bovine lactoferrin to U937 cells. Human IgA at 200 μg/ml was somewhat inhibitory for bovine lactoferrin, but the effect was not observed when the IgA concentration was reduced to 10 μg/ml, and neither concentration had any effect on the binding of human lactoferrin. In contrast bovine lactoperoxidase, even at 10 μg/ml, inhibited binding of human lactoferrin, but had no effect on the binding of bovine lactoferrin. Lysozyme, like bovine lactoferrin showed a slight tendency to increase binding of human lactoferrin, but again there was no effect on the binding of bovine lactoferrin.

2.4.1.4 Effect of Heat Treatment on Binding of Bovine Lactoferrin to U937 Cells

Figure 29 shows that binding of labelled bovine Fe-lactoferrin to U937 cells was inhibited by an excess of native lactoferrin both in the apo and diferric forms.

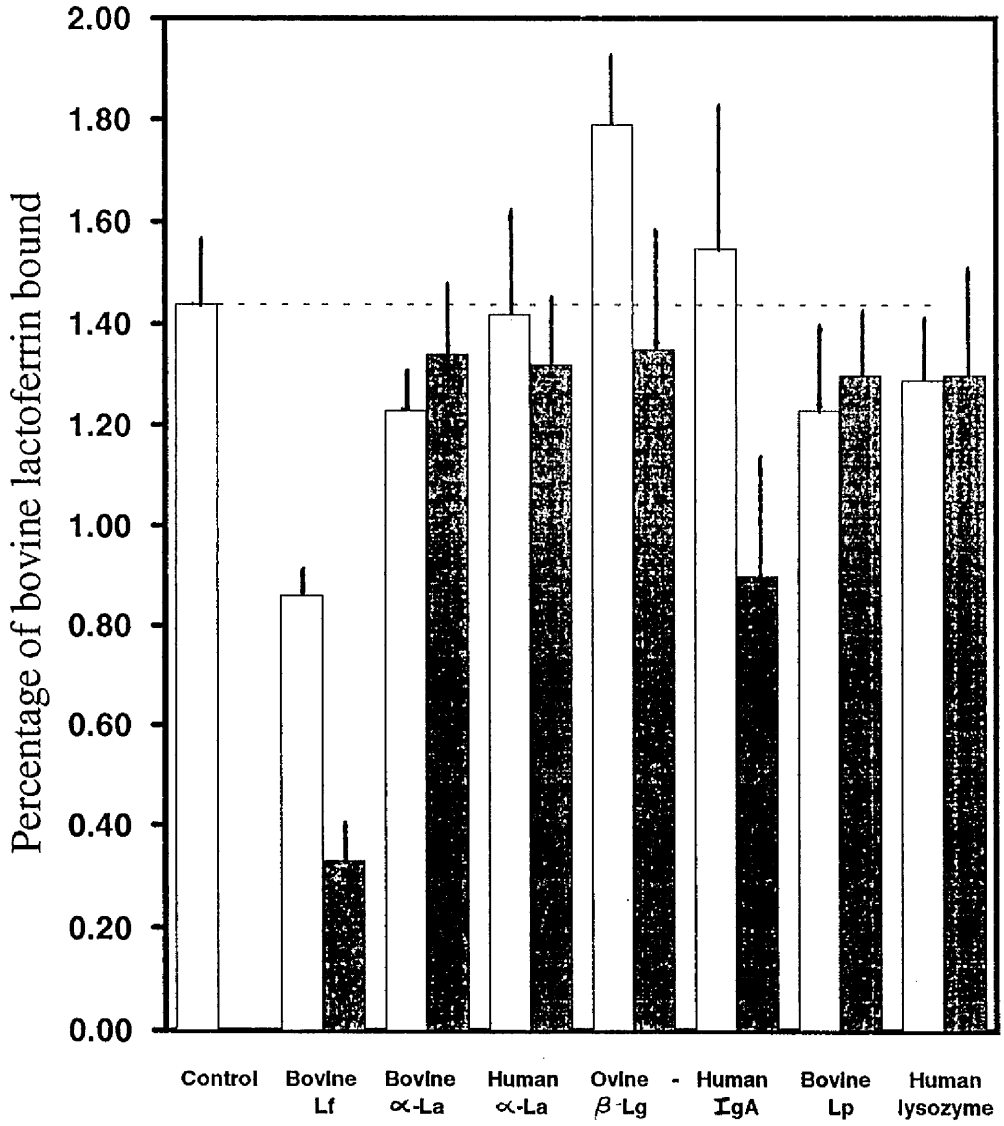


Fig. 27 : Effect of milk proteins on the binding of bovine lactoferrin (Lf) to U937 cells. Columns and vertical bars represent the mean values ± SD (n = 3).(Milk proteins: α-La, α-lactalbumin; β-Lg, β-lactoglobulin; Lp, lactoperoxidase.(□ 10 μg/ml; ▩200 μg/ml milk proteins)

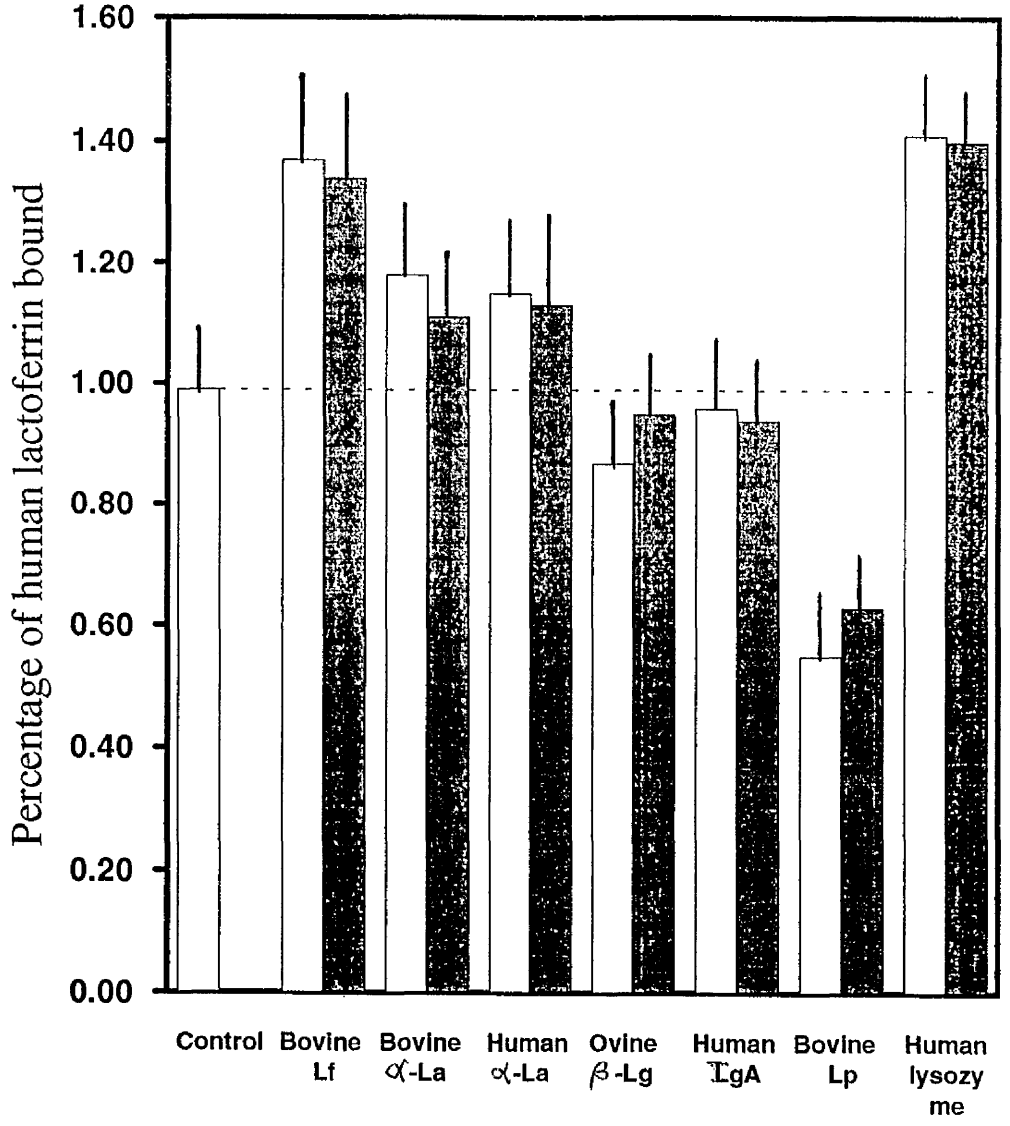


Fig. 28 : Effect of milk proteins on the binding of human lactoferrin (Lf) to U937 cells. Columns and vertical bars represent the mean values ± SD (n = 3). (Milk proteins: α-La, α-lactalbumin; β-Lg, β-lactoglobulin; Lp, lactoperoxidase. (□ 10 µg/ml; ■ 200 µg/ml milk proteins)

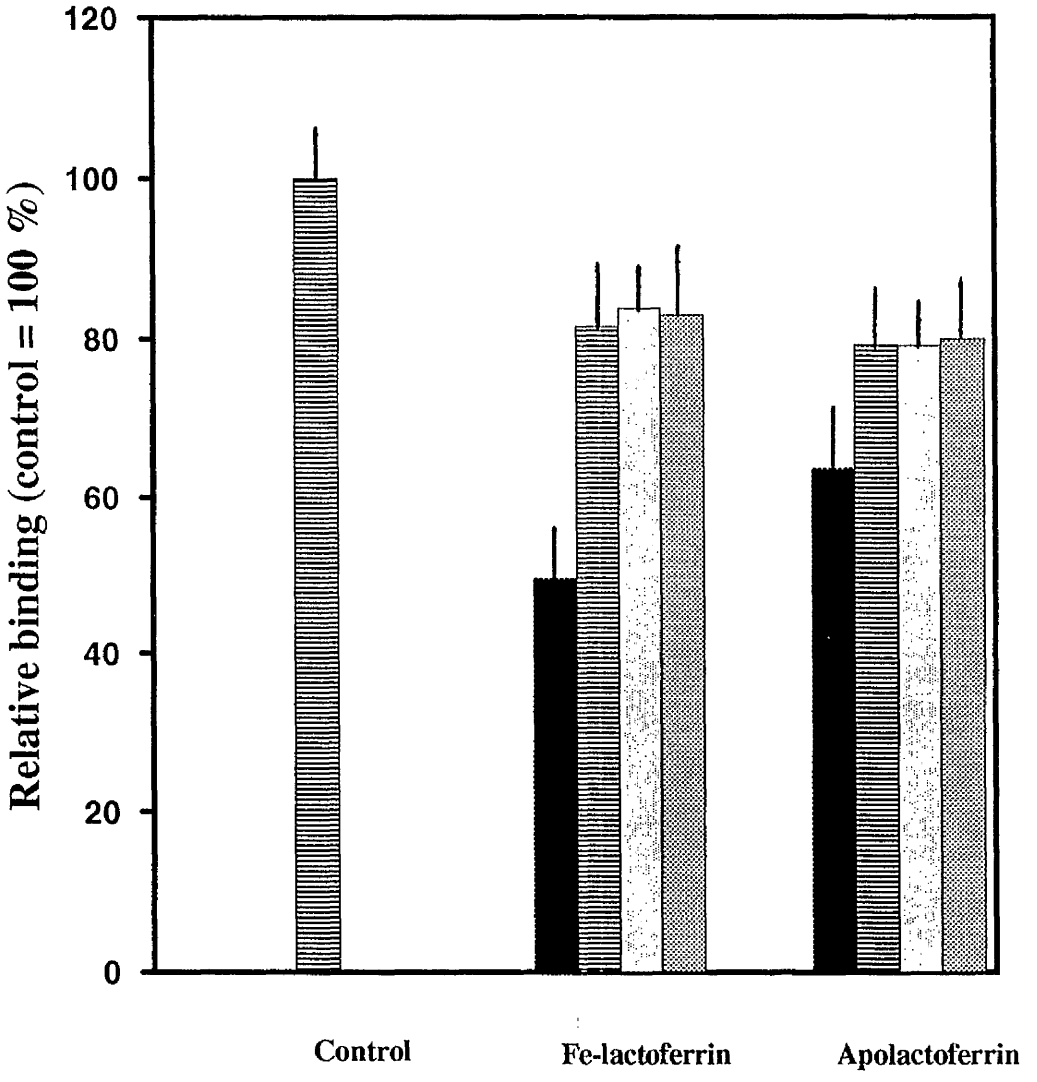


Fig. 29 : Effect of heat treated Fe-lactoferrin or apolactoferrin on the binding of native bovine lactoferrin. The cells were incubated with ^{125}I-labelled native bovine Fe-lactoferrin (10 µg/ml) and 50 µg/ml of either untreated lactoferrin ■ , or lactoferrin heat -treated at 72 °C for 20 sec ▥,85 °C for 20 min ▤. or 137 °C for 8 sec ▩ Columns and vertical lines represent the mean values ± SD (n = 3).

This result further confirms the earlier finding in section 2.4.1.2. The three heat treatments of lactoferrin (72 °C for 20 sec, 85 °C for 20 min and 137 °C for 8 sec) caused it to become less inhibitory with only 20 % inhibition instead of 34 % for the native lactoferrin. There was no difference in the inhibitory effects of lactoferrin subjected to the different heat treatments, or between apo- and Fe-lactoferrin.

2.4.2 Iron Uptake Studies

Iron uptake by U937 cells from ^{59}Fe-labelled lactoferrin or transferrin over a 56 h period at 37 °C is shown in **Fig. 30**. Initially, there seemed to be more iron uptake from lactoferrin than from transferrin, but after this initial delay, there was a progressive and linear increase in iron uptake from transferrin. With lactoferrin, there was no evidence of any sustained iron uptake prior to 26 h, and thereafter uptake was much slower than from transferrin, so that by the end of 56 h incubation, there was a 9-fold increase for transferrin as compared to only less than double for lactoferrin.

However, when a similar experiment was performed over a shorter time period, using doubly labelled (^{59}Fe, ^{125}I) proteins, it was evident that much of the apparent iron uptake from lactoferrin was due to the much greater binding of this protein to the cells than to net iron uptake (**Fig. 31**). Initially at 2 h incubation the amount of cell-associated protein and iron in pmol/10^6 cells for transferrin were 0.61 and 7.74 respectively and for lactoferrin were 2.95 and 7.06.

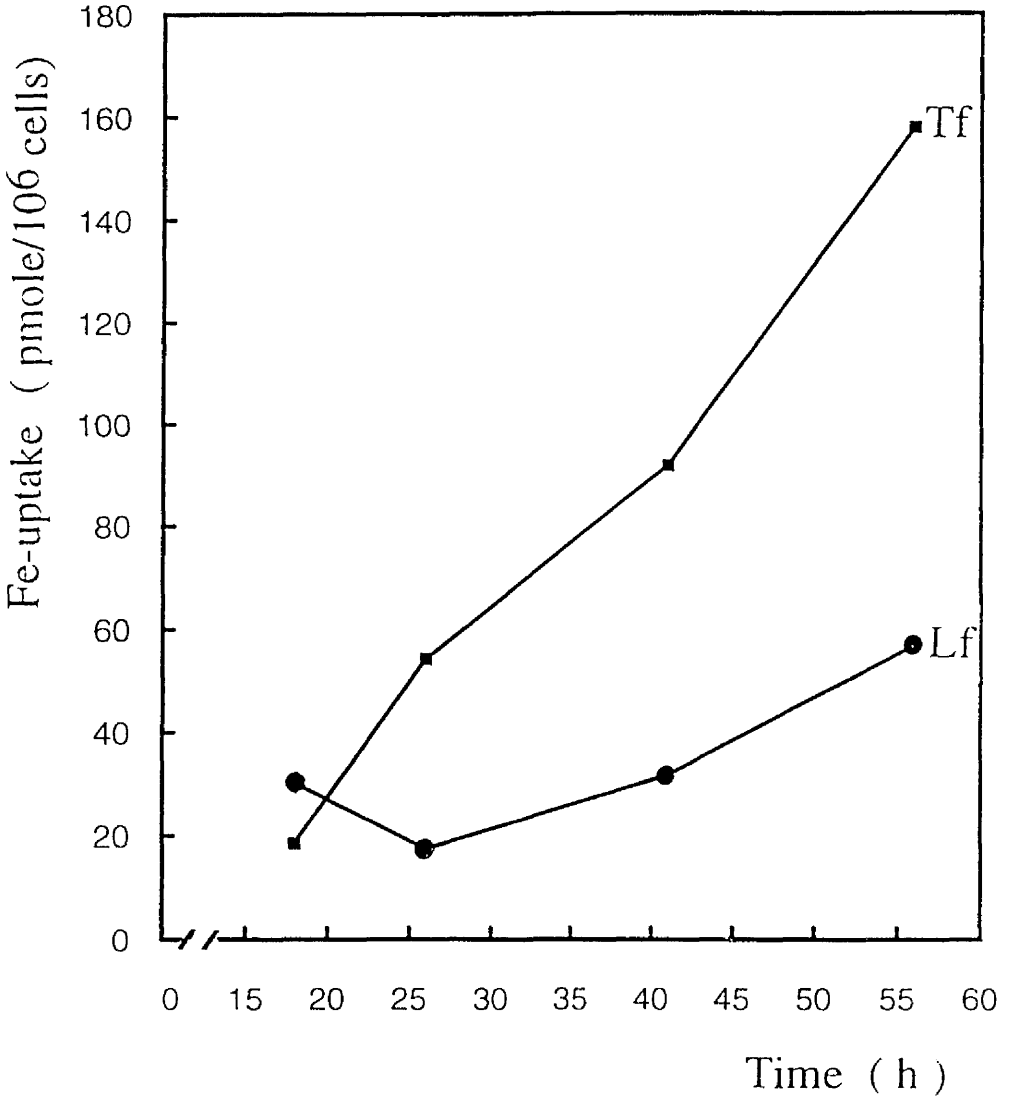


Fig. 30 : Iron uptake from transferrin (Tf) and lactoferrin (Lf) by U937 cells. Each point is a mean of duplicate determinations which did not vary by more than 10 %.

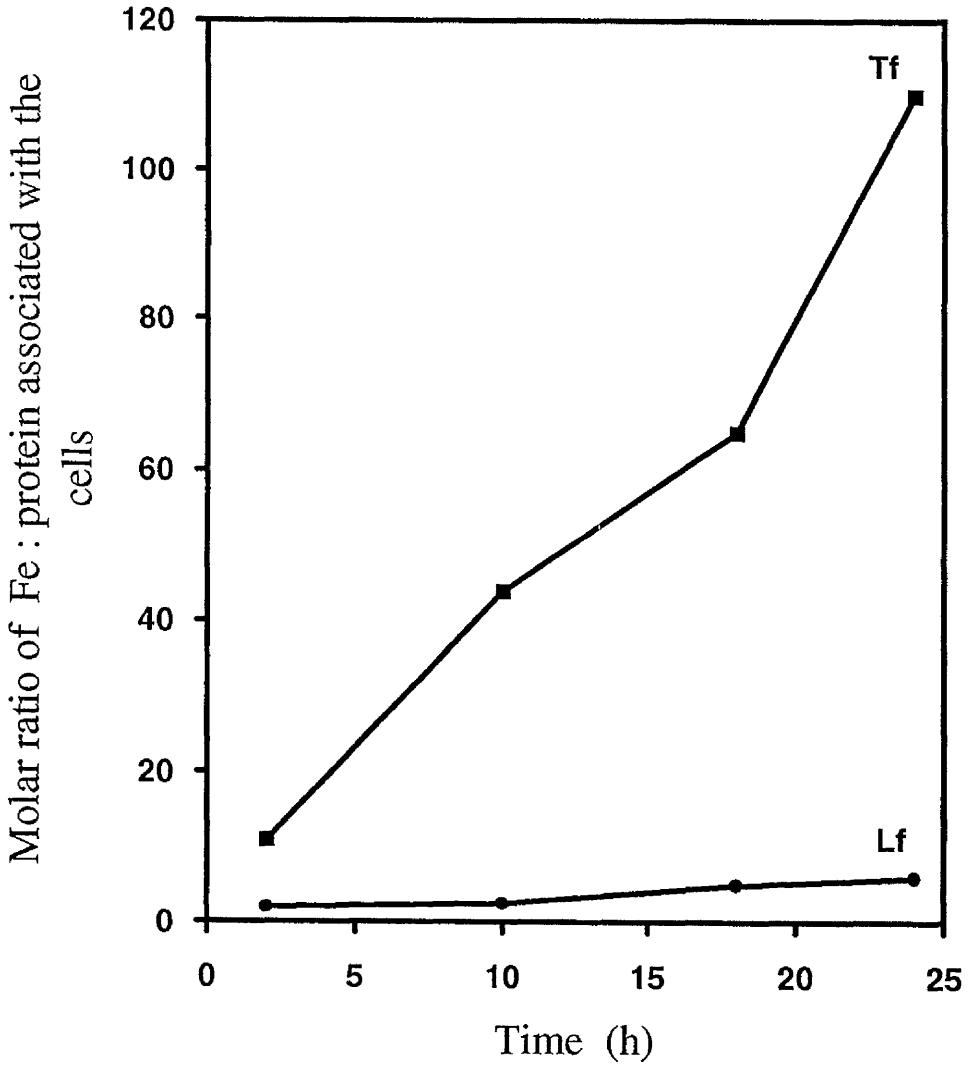


Fig. 31 : Molar ratio of iron : protein bound to U937 cells incubated with labelled lactoferrin (Lf) or transferrin (Tf).

After 18 h the amount of transferrin bound remained fairly constant (0.76 pmol/10^6 cells) but the amount of ^{59}Fe associated with the cells had increased 7 x (51.50 pmol/10^6 cells), whereas with lactoferrin both protein and iron had increased (5.9 and 35.50 pmol/10^6 cells). Thus as shown in the graph, the molar ratio of cell-associated iron:lactoferrin was 2.4 at 2h and increased only slightly to 5.9 after 18 h, but for transferrin the graph clearly illustrates that there was a steady accumulation of iron relative to transferrin, the ratio of iron to protein being 12.7 at 2 h and increased to 66 at 18 h.

2.4.3 Effect of Lactoferrin on Uptake of Iron from Other Sources

Since lactoferrin itself had little or no ability to donate iron to U937 cells, it was of interest to determine whether it could affect uptake of iron from other sources. Both apolactoferrin and Fe-lactoferrin significantly reduced uptake of non-transferrin bound iron in the form of FeNTA, but neither had any effect on the uptake of iron from transferrin (**Fig. 32**).

2.4.4 Internalisation of lactoferrin and Transferrin

In order to determine whether bound lactoferrin and transferrin were internalised, the cells were incubated with doubly labelled proteins for 30 min at 4 °C or 37 °C and the cell-surface bound proteins were then removed by rapid acid wash.

Table IV shows that this procedure released 80 % of the cell-associated lactoferrin regardless of whether incubation was carried out at 4 °C or at 37 °C. In contrast 65 % of bound transferrin was removed from the cell surface after incubation at 4 °C but only 41 % after incubation at 37 °C.

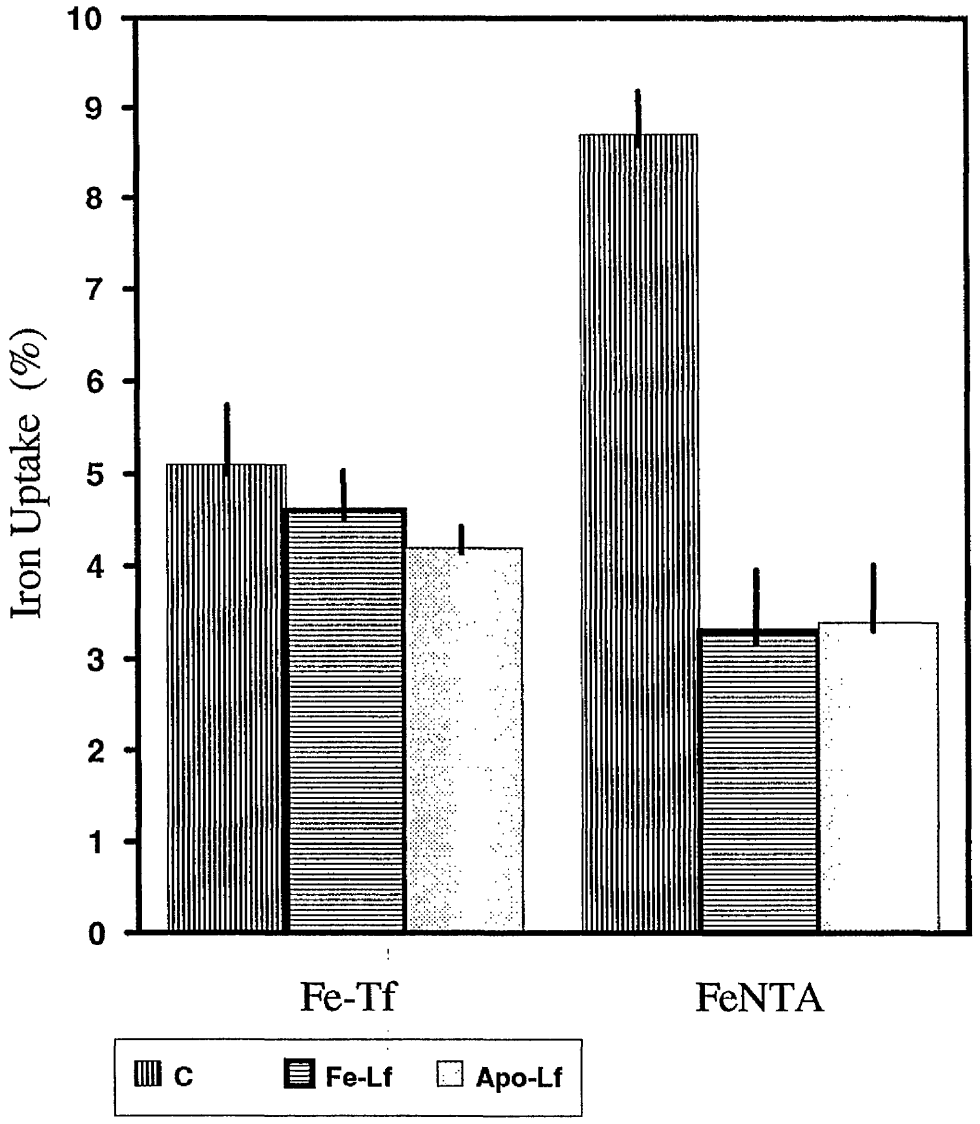


Fig. 32 : Iron uptake by U937 cells from Fe-transferrin (Fe-Tf) and ferric nitrilotriacetate (FeNTA). Values represent means ± SD (n = 4).

TABLE IV

Internalisation of lactoferrin and transferrin by U937 cells

U937 cells were incubated with 50μg/ml of ^{125}I-labelled lactoferrin or transferrin for 30 min. Unbound protein was removed and the proportion of surface-bound protein determined by acid washing. Values represent means ± S.D. of three experiments

	% of bound protein retained after acid washing	
Incubation temperature	4°C	37°C
Lactoferrin	16±1	19±1
Transferrin	35±1	56±6

Thus at 37 °C more than half of bound transferrin but < 20 % of lactoferrin had been internalised.

To determine whether the small proportion of cell-bound lactoferrin resistant to acid washing had really been endocytosed, the effect of primaquine on the interaction of doubly labelled lactoferrin with the cells was investigated and the results compared with that of transferrin. The amount of transferrin internalised doubled in the presence of primaquine (**Table V**), indicative of slower recycling of internalised transferrin to the cell surface. The molar ratio of iron:transferrin retained within the cells decreased in the presence of primaquine, indicating a reduced rate of iron deposition, which is consistent with slower cycling of transferrin. In contrast, the amount of lactoferrin retained by the cells after acid washing was similar in the presence or absence of primaquine and the molar ratio of iron:lactoferrin associated with the cells was close to 2 in both cases. This confirms that lactoferrin was not being internalised by the cells to any measurable extent.

2.4.5 Exchange of Iron Between Lactoferrin and Transferrin During Cell Culture

The previous results indicate that U937 cells do not internalise lactoferrin but despite this, the results shown in **Figs. 30** and **31** (see section 2.4.2.1) do indicate an apparent slow net accumulation of iron from lactoferrin. Since iron is taken up much more rapidly from transferrin, it seemed possible that these apparently contradictory results might be explained by a transfer of some ^{59}Fe from lactoferrin to transferrin during culture. To determine whether such a transfer occurred, supernatants from 18 h cultures containing ^{59}Fe-lactoferrin and unlabelled transferrin or vice versa were

TABLE V

Effect of primaquine on internalisation of lactoferrin and transferrin by U937 cells

Cells were labelled with ^{125}I, ^{59}Fe-labelled lactoferrin or transferrin for 30min at 4°C. Unbound protein was removed and the cells incubated with primaquine (3mM) at 37°C for a further 30min prior to acid washing. Values represent means ± S.D. of three experiments

	% of bound protein retained after acid-washing		Molar ratio Fe:protein	
Primaquine	+	-	+	-
Lactoferrin	11±2	13±2	1.8	2.4
Transferrin	83±5	40±4	4.1	7.1

passed through anti-transferrin and anti-lactoferrin immunosorbents. After correction for non-specific binding it was found that 16.6 % of ^{59}Fe originally bound to lactoferrin had transferred to transferrin, as against only 5.9 % of iron transferred from transferrin to lactoferrin (**Table VI**). Thus the apparent uptake of iron from lactoferrin could be due to a prior transfer of some of ^{59}Fe from lactoferrin to transferrin. No transfer of ^{59}Fe was detected in medium prior to incubation with the cell.

2.4.6 Release of Iron, Lactoferrin and Transferrin by U937 Cells

To determine whether transfer of ^{59}Fe results from cell-associated lactoferrin releasing its iron at the cell surface, the relative rates of dissociation of iron and transferrin/lactoferrin from U937 cells was investigated. When cells previously incubated with ^{125} I,^{59} Fe-lactoferrin or transferrin were washed and reincubated at 4 °C in fresh medium containing neither protein, but with 1 mM desferrioxamine (to prevent re-entry of released iron into the cells), about 80 % of ^{59} Fe dissociated from the cells over a 90 min period (**Fig. 33**) as compared to only about 60 % of the lactoferrin itself, indicating that some iron was dissociating from the membrane-bound lactoferrin. It seems unlikely that desferrioxamine itself is responsible for this release as exchange of iron between transferrins and desferrioxamine is extremely slow under physiological conditions (Pippard, 1989). Furthermore similar results were obtained when apotransferrin was used instead of desferrioxamine to capture iron released from lactoferrin; 28 % of iron remaining cell-associated after 90 min, as compared with 20 % in the presence of desferrioxamine, while the proportion of lactoferrin remaining bound to the cells at this time was 41 % in both cases. The small decrease in iron release resulting from substitution of apotransferrin for

TABLE VI

Transfer of iron from transferrin to lactoferrin and vice versa during culture of U937 cells

Route	Fe - transfer (%)
Transferrin → lactoferrin	5.9
Lactoferrin → transferrin	16.6

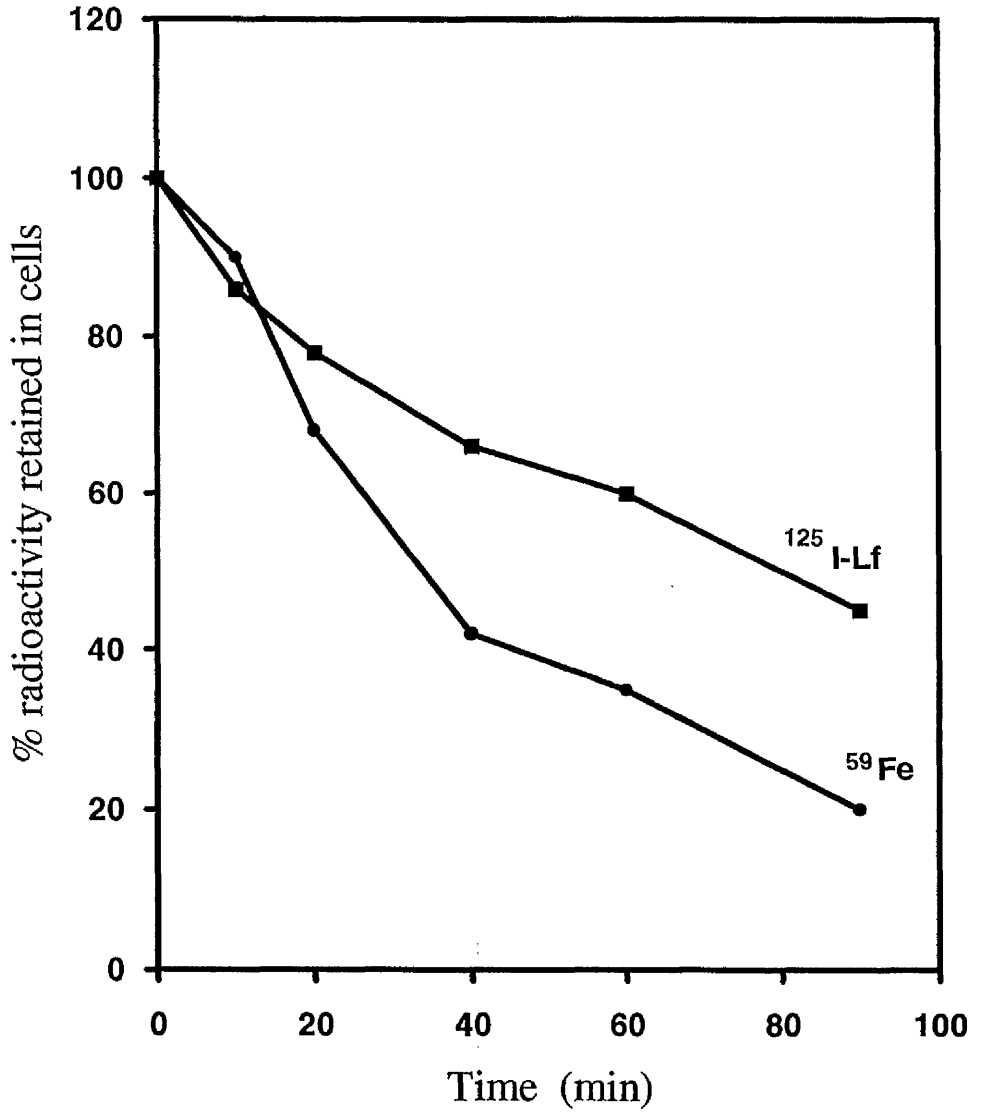


Fig. 33 : Dissociation of bound ^{125}I,^{59}Fe-labelled lactoferrin from U937 cells with time. Each point is a mean from three separate experiments.

desferrioxamine may be due a slow return of some of the released iron to the cells from transferrin.

In contrast there was no release of iron previously bound to transferrin (**Fig. 34**) even though about 35 % of the bound transferrin was released, suggesting that most of the iron had already been internalised. More than 92 % of the supernatant ^{125}I activity was TCA precipitable at all time intervals for both proteins, suggesting that little protein catabolism occured during the incubation.

2.4.7 Studies on Cell Proliferation

2.4.7.1 Effect of Lactoferrin and Transferrin on the Proliferation of U937 Cells

Cells grown in serum free medium in the presence of either lactoferrin or transferrin incorporated more ^{3}H-thymidine ($p < 0.01$) (**Fig. 35**). The effect of Fe-containing lactoferrin and transferrin on thymidine uptake was similar, with an increase of 61 % and 72 % over the control. Addition of apolactoferrin or apotransferrin also increased thymidine uptake but to a lesser extent, the figures being 41 % (apolactoferrin) and 39 % (apotransferrin) greater than the control.

2.4.7.2 Effect of Heat Treated Lactoferrin and Milk Proteins on the Proliferation of U937 Cells

Bovine lactoferrin also caused a marked increase in ^{3}H-thymidine uptake by the cells (**Fig. 36**). Apolactoferrin and Fe-lactoferrin were equally effective. Heat treatment of Fe-lactoferrin at 72 °C for 20 sec or at 137 °C for 8 sec

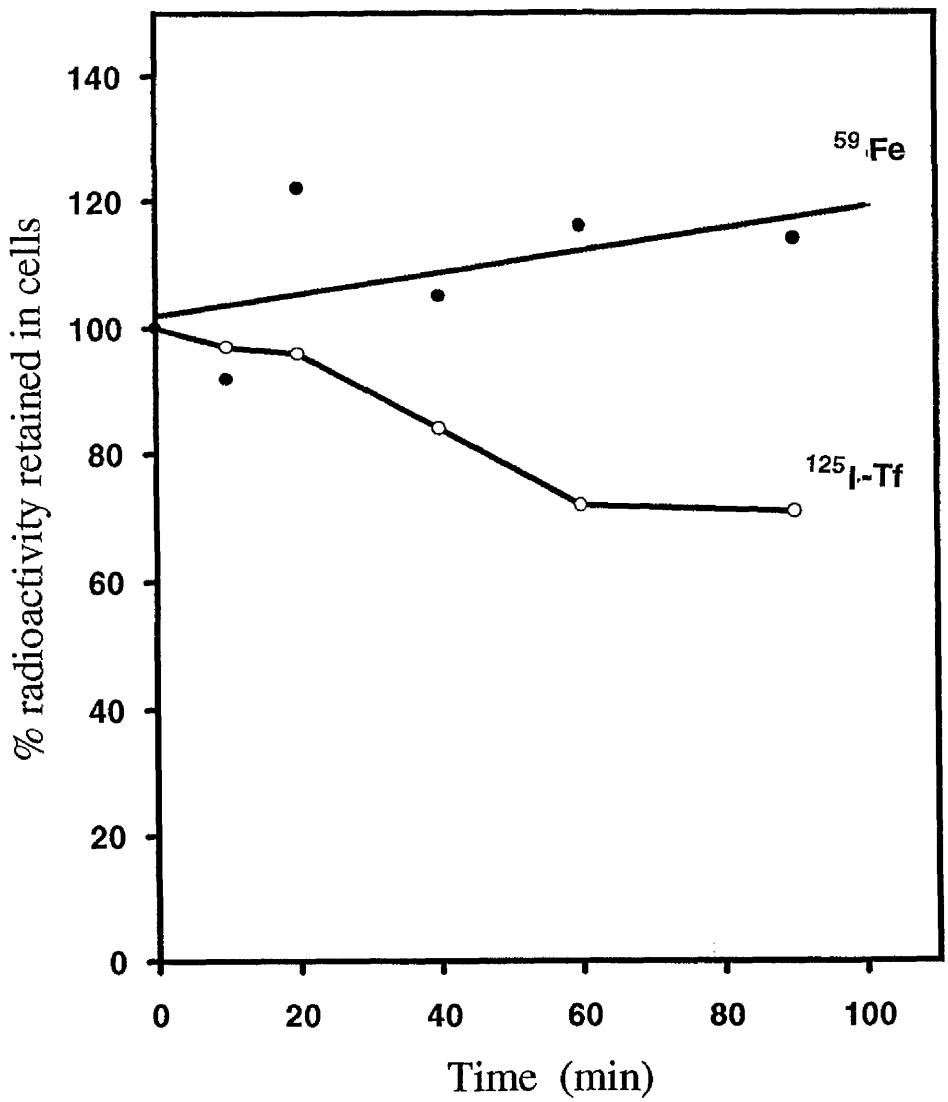


Fig. 34 : Dissociation of bound $^{125}I,^{59}Fe$-transferrin from U937 cells with time. Each point is a mean from three separate experiments.

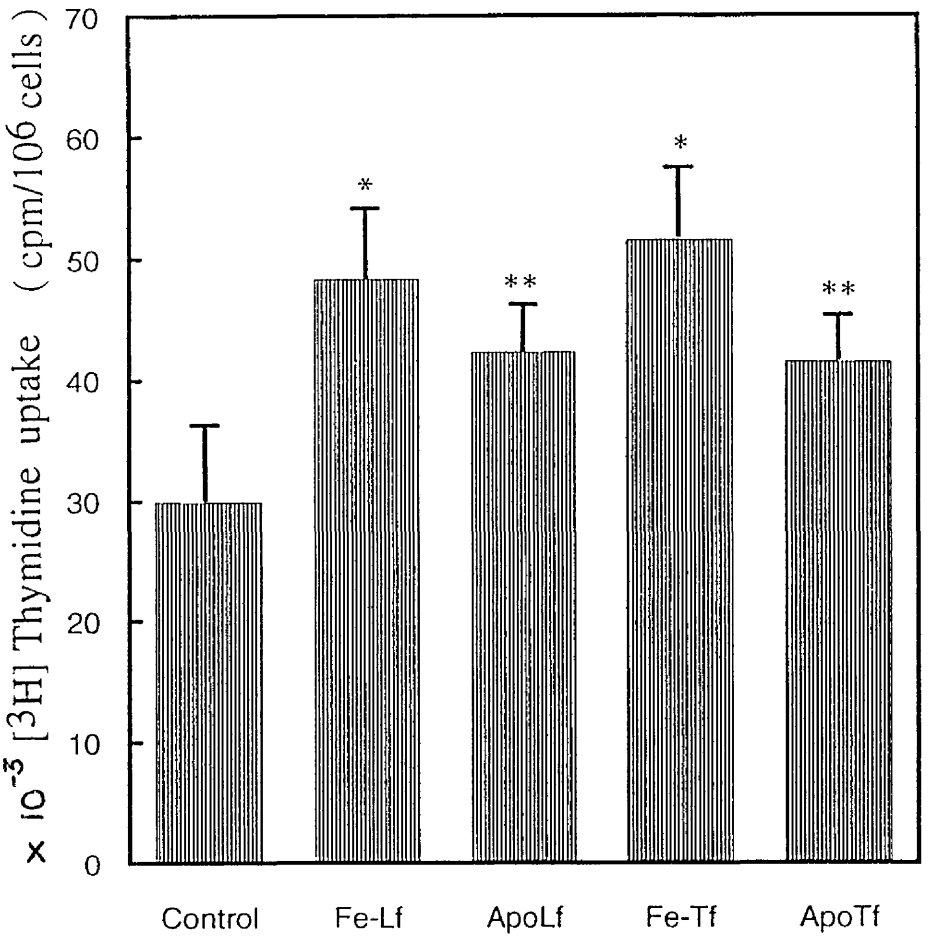


Fig. 35: The effect of lactoferrin and transferrin on the incorporation of ^{3}H-thymidine by U937 cells. Columns and vertical bars represent means ± SD of five replicate wells from two separate experiments.
(* $p < 0.01$; ** $p < 0.05$)

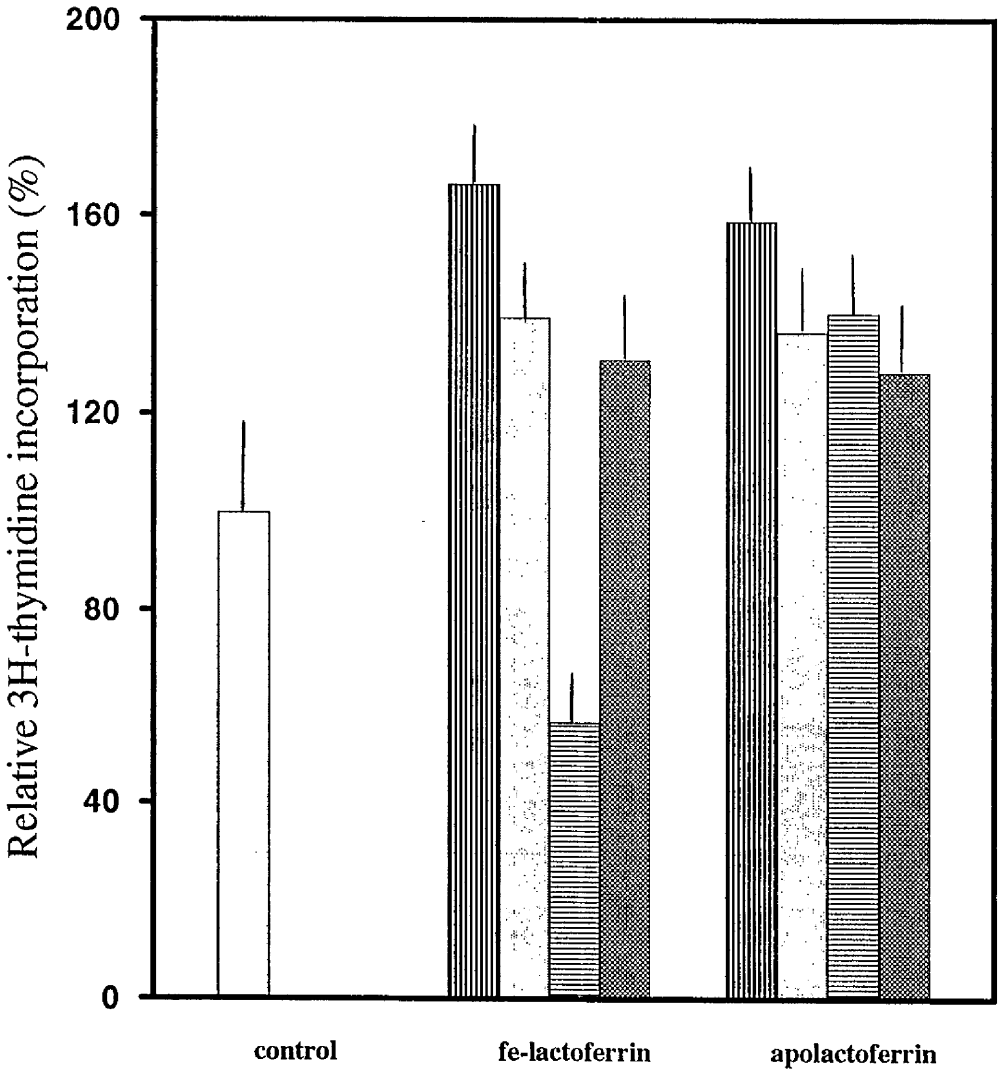


Fig. 36 : Effect of heat-treated Fe-lactoferrin and apolactoferrin on ^{3}H-thymidine incorporation by U937 cells. The cells were incubated with 50 µg/ml of either untreated lactoferrin ▥, or lactoferrin heat-treated at 72 °C for 20 sec□, 85 °C for 20 min ▤, or 137 °C for 8 sec ▩. Results are means ± SD (n = 5).

did not abolish the stimulatory effect, but when Fe-lactoferrin was subjected to the most severe treatment (85 °C for 20 min) it became slightly but significantly inhibitory rather than stimulatory. In contrast none of the heat treatments altered the stimulatory effect of lactoferrin.

None of the milk proteins by themselves had any significant effect on ^{3}H-thymidine incorporation, nor did they modify the stimulatory effect of lactoferrin (**Fig. 37**).

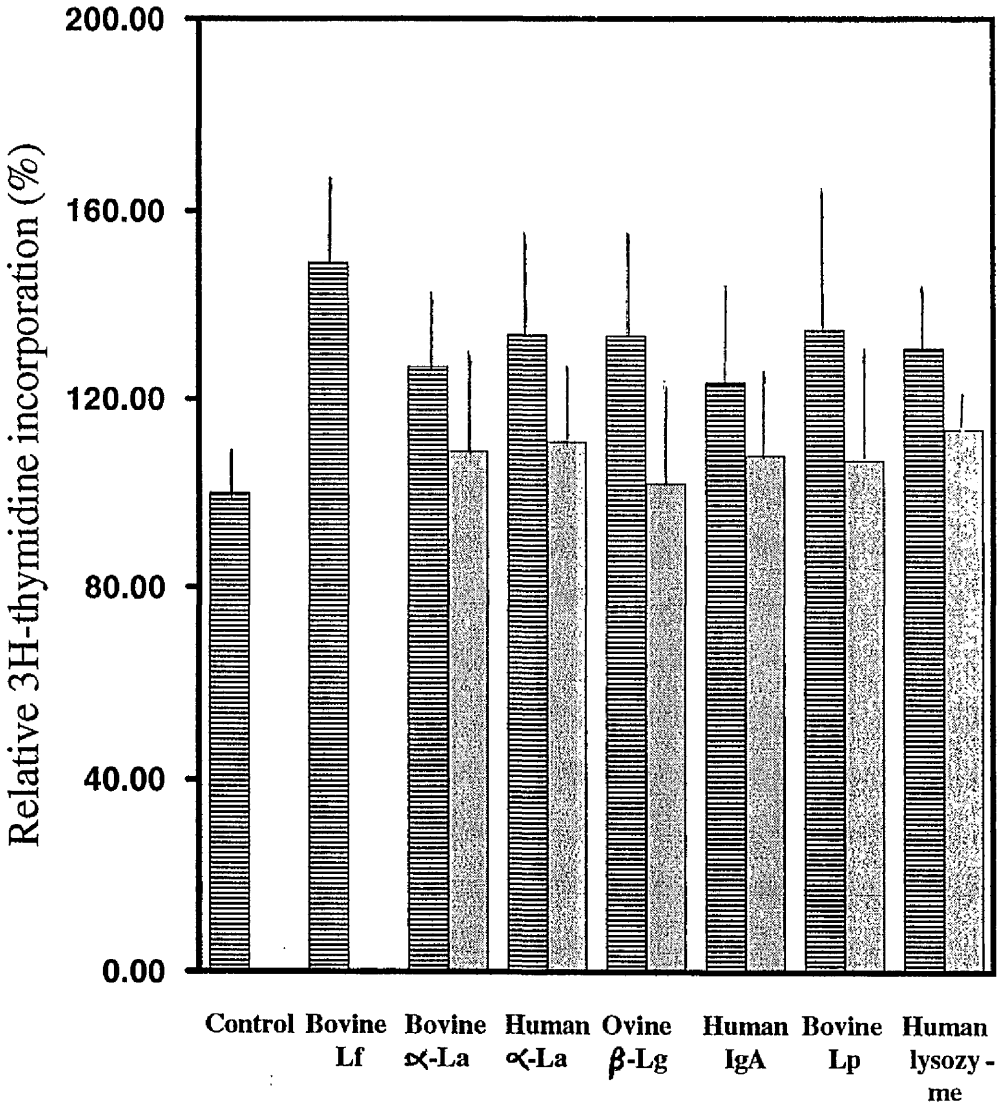


Fig. 37 : Effect of lactoferrin and milk proteins on the uptake of ^{3}H-thymidine by U937 cells. The cells were incubated with 50 µg/ml native lactoferrin plus 50 µg/ml milk proteins, ▤ or 100 µg/ml native lactoferrin ▤ milk protein alone, ▒ . Controls contained no added lactoferrin. Values are means ± SD (n = 5).

2.5 DISCUSSION

This chapter has examined the molecular basis of interaction of lactoferrin with a promonocytic cell line, U937 and its effect on these cells.

2.5.1 Binding of lactoferrin and transferrin

Lactoferrin has been reported to bind to a number of cell types including cells of the reticuloendotheliel system, lymphocytes, intestinal brush border membranes, a variety of cultured leukemic cell lines and a human colon adenocarcinoma cell line (reviewed by Birgens, 1991). Hepatocytes, Kupffer and liver endothelial cells have also been observed to bind lactoferrin. Binding of lactoferrin to some of these cells was reported to occur through specific receptors which have been demonstrated in human fetal intestinal brush border membranes (Kawakami and Lonnerdal, 1990) and in phytohaemagglutinin-stimulated peripheral blood lymphocytes (Mazurier *et al.,* 1989).

The binding of lactoferrin to the cells of the RES was first reported by Van Snick (1974). They found that binding of human milk lactoferrin to mouse peritoneal macrophages had occurred through a specific receptor. Since then many studies have confirmed the binding of lactoferrin to monocytes and macrophages (Bennet and Davis, 1981; Steinmann *et al.*, 1982; Bartal *et al.*, 1987; Oria *et al.*, 1988). Apart from Van Snick's group others have also reported receptor binding of lactoferrin to these cells. However, since then there has not been any membrane lactoferrin-binding molecules that have been identified. There are also others who have suggested that the binding is relatively non-specific (Goavec *et al.*, 1985).

In this study, lactoferrin and transferrin have been found to bind to U937 cells; binding was dependent on temperature as was also observed in the case of lactoferrin binding to human adenocarcinoma cells, HT29-D4 (Roiron *et al.*, 1989). The maximum binding at 37 °C was about 4 x as high as that at 4 °C (**Fig. 18**). Binding of transferrin at 37 °C was observed to be more rapid than that of lactoferrin, reaching a plateau in less than 2 h (**Fig. 19**). However at both temperatures, maximal binding of lactoferrin was about 10-fold greater than that of transferrin. When the binding curves for lactoferrin and transferrin at both temperatures are extrapolated to 0 h, the number of protein molecules bound per cell are estimated to be about 14.4 x 10^5 molecules of lactoferrin per cell and 1.5 x 10^5 molecules of transferrin per cell.

The binding pattern of lactoferrin differs from that of transferrin when the incubation was carried out at 37 °C. Transferrin binding showed saturation after 2 h incubation. The uptake of transferrin at this temperature is likely to represent, initially binding of transferrin to the receptors present on the cell surface at the time, and later binding to the receptors which are recycled to the surface from the interior during the incubation. The low saturating level of transferrin binding at 4 °C can be explained by the fact that receptors located inside the cell at this temperature do not recycle to the cell surface and therefore cannot rebind more transferrin. Once all the receptors have been occupied, no further increase in uptake is possible and a steady state is reached between binding, endocytosis and exocytosis of transferrin. In the case of lactoferrin, binding occurred more rapidly within the first 2 h of incubation, similar to transferrin, but more lactoferrin seemed to bind to the cells with time. The increase in lactoferrin binding is probably due to non-specific binding of lactoferrin to the cells as was observed from competition experiments.

The presence of cell membrane receptors for both proteins was suggested by the saturation studies performed in the presence of unlabelled proteins (**Figs. 20** and **21**). As predicted binding of labelled transferrin to the cells was found to be saturable and was inhibited by the presence of excess of unlabelled transferrin. Total binding of labelled lactoferrin on the other hand increased in an approximately linear manner within the range of concentration used in this study, indicating a non-specific, non-saturable process, but there was also a specific component to the lactoferrin binding demonstrated by adding a large excess of non-radioactive lactoferrin to the incubation medium. Although this represented only a small fraction of the total binding, the number of specific sites for lactoferrin (3.0×10^6 sites/cell) was actually slightly greater than for transferrin (1.9×10^6 sites/cell), as demonstrated from Scatchard analyses of the binding data (**Figs. 22** and **23**). The number of specific lactoferrin binding sites per cell found in this study is similar to that obtained by Roiron *et al.* (1989) ($N = 4.1 \times 10^6$) and falls between the values obtained by Van Snick *et al.*(1976) ($N = 20 \times 10^6$) using mouse peritoneal macrophages and that of Birgens *et al.*(1983) ($N = 1.6 \times 10^6$) using human blood monocytes.

The affinity of the specific binding of lactoferrin to U937 cells (K_d = 83 nM) is also within the range of values reported for binding to other cells, being comparable to that of activated lymphocytes (46 nM) (Rochard *et al.*, 1989), lower than that of the high affinity binding sites on hepatocytes (K_d 20 nM) (McAbee and Esebensen, 1991) and higher than the "high-affinity" binding sites on adenocarcinoma cells (K_d = 408 nM) (Roiron *et al.*, 1989). When compared to other studies using monocytes or macrophages, the binding affinity of lactoferrin to U937 was higher than that for macrophages (K_d = 600 nM) (Campbell, 1982; Van Snick *et al.* 1976) but lower than binding affinity for human monocytes (K_d = 0.22 nM) (Birgens

et al., 1983). Therefore it can be concluded that the quantitative binding data (affinity constants and number of binding sites/cell), obtained in this study falls between the values obtained by others. The slight differences in values could be explained on the basis of different cell types as well as due to the neoplastic characteristics or origin of U937 cells. In addition, the variation in values obtained could be attributed to the difference in the concentration of labelled lactoferrin employed in the binding assay. In this study the concentration range used was between 1 -150 nM, while in other studies with higher binding sites, higher concentration range was used, i.e., 0.2 - 5.0 mM (Van Snick *et al.,* 1976) and 0.1 - 10 mM (Campbell, 1982). According to Bennett and Davis (1981), concentration of lactoferrin > 3.1 μM may cause cell aggregation which could result in increased binding of labelled lactoferrin and likely to cause an over estimate of the number of binding sites.

The specificity of lactoferrin binding to U937 cells was demonstrated in competitive binding studies with various proteins. Cold apolactoferrin was able to inhibit the binding of Fe-lactoferrin to the cells, but the inhibition was lower than expected. This could be explained by the steric changes that lactoferrin undergoes when it combines with iron. The molecule of Fe-lactoferrin is more compact and more asymmetric than that of apolactoferrin. Such observation confirmed what was noted earlier by Van Snick *et al.*, (1976).

Although bovine lactoferrin could bind to U937 cells, interestingly its binding was not inhibited by human lactoferrin, indicating that different mechanisms of cellular recognition are involved between bovine and human lactoferrin. Such a difference has also been reported for the interaction of lactoferrin with monkey intestinal brush-border membranes which bound

human but not bovine lactoferrin (Davidson and Lonnerdal, 1988). Furthermore the pattern of inhibition of binding by other milk proteins also point out to a difference in the nature of the interaction between the milk proteins and the two lactoferrins; none of the the proteins with the exception of high concentrations of human IgA, inhibited binding of bovine lactoferrin, whereas binding of human lactoferrin was markedly inhibited by bovine lactoperoxidase.

Transferrin, despite its structural homology to lactoferrin, did not inhibit the binding of lactoferrin; a conclusion also reached in studies using other cell types (Yamada *et al.*, 1987; Bennett and Davis, 1981; Birgens *et al.*, 1983). Likewise, the binding of transferrin to the cells was not inhibited by either apolactoferrin or Fe-lactoferrin, suggesting that there are different binding sites are located for transferrin and lactoferrin on the cell surface.

The nature of the cell surface molecule(s) responsible for binding of lactoferrin has not been determined. Since lactoferrin is a cationic protein, it was considered that binding to the negatively charged cell surface might be of electrostatic in nature. Inhibition of binding by another cationic protein, lactoperoxidase (pI=9.6) seemed to point this way. However, the presence of lysozyme, a more basic protein (pI=11.4) did not result in a decrease lactoferrin binding to the cells. Similar findings were reported by McAbee and Esebensen (1991), who found that lactoperoxidase inhibited lactoferrin binding to intact hepatocytes, but not lysozyme or cytochrome c (pI=10.6). Removal of sialic acid, one of the major sources of negative charge on the cell surface, by neuramidase decreased binding of lactoferrin (Yamada *et al.*, 1987). On the other hand, Moguilevsky *et al.*(1985) found that sialic acid as well as glycosaminoglycans and phospholipids did not contribute to the binding. In contrast removal of sialic acid from lactoferrin

increased its binding affinity to mouse peritoneal macrophages (Van Snick and Masson, 1976). Others have reported that lactoferrin bound to alveolar macrophages in competition with two other cationic neutrophil granule glycoproteins, elastase and cathepsin G (Campbell, 1982).

Several mammalian receptors mediate glycoprotein binding through recognition of different terminal carbohydrate residues. Recognition of lactoferrin could be mediated by fucosyl residues as proposed by Prieels *et al.*, 1978; Goavec *et al.*, 1985). However in this study fucosyl residues were found not to play any significant role in the binding of lactoferrin to U937 cells. This conclusion is based on the following findings: firstly, competition studies with 30mM fucose did not inhibit binding of lactoferrin. Similar results were obtained with 30 mM glucose. Secondly, treatment of the cells with heparinase, which removes acidic sugar residues did not affect lactoferrin binding and thirdly, the fact that bovine lactoferrin which lacks fucose residues in the glycan chains could also bind to the cells. These results support the findings of Imber and Pizzo (1983) and Moguilevsky (1984), who concluded that fucosyl residues were not involved in the interaction of lactoferrin with peritoneal macrophages and cultured hepatocytes because the removal of fucose from the protein molecule did not affect its clearance from plasma.

Competition studies with monocyte reactive monoclonal antibodies suggest that the binding site does not appear to involve any of the surface reactive molecules defined by these monoclonal antibodies.

In conclusion, results from binding studies show that there are two types cell-lactoferrin of interaction; a large and non-saturable component which is likely to consist of low affinity binding sites that accounts for the non-

specific binding of lactoferrin and a smaller specific component which is made up of high affinity binding sites. The reason for the high level of non-specific binding of lactoferrin to U937 and other cells is not clear. It is possible that lactoferrin initially binds to a relatively high affinity binding molecule, such as that identified on activated lymphocytes (Mazurier *et al.*, 1989), thus giving rise to the specific element of the binding. Further lactoferrin may be bound as a result of polymerisation, a known feature of lactoferrin (Bennett and Davis, 1981; Britigan *et al.*, 1991) as mentioned earlier.

2.5.2 Uptake of iron from lactoferrin and transferrin

Even though the cells bound about 10 times more lactoferrin than transferrin, uptake of iron was much more rapid from transferrin than from lactoferrin (**Fig. 30**). The amount of iron taken up at the end of 55 h incubation is about 160 pmole/10^6 cells, which is only three times more than the amount taken up from lactoferrin (55 pmole/10^6 cells). However, when the molar ratio of iron:protein associated with the cells is plotted against time (**Fig. 31**), it clearly shows that while the molar ratio of iron:protein for lactoferrin remained almost constant, the molar ratio of iron:protein for transferrin increased linearly with time. A closer look at the molar ratio of iron: protein after 2 h incubation shows that for lactoferrin it was only 2.4 versus 12.7 for transferrin, while after 18 h, the figures rose to 5.9 and 66 respectively. These findings suggest that at 37 °C, there is definitely a significant uptake of iron from transferrin with time but only a slow uptake of iron from lactoferrin. A previous study by Brock and Esparza (1979) also reported that there was no uptake of iron from lactoferrin by reticulocytes in contrast to transferrin.

Since each protein is theoretically capable of delivering 2 iron atoms to the cell in each endocytic cycle, this represents only about 3 cycles in 18 h for lactoferrin, as against 33 for transferrin. This marked difference in iron uptake was reflected in a difference in the fate of the two proteins follwing binding to the cell membrane. Pulse chase studies showed that after binding of transferrin to the cells at 4 °C, > 65 % of the bound protein could be removed by a 5 sec acid wash with 0.25 M acetic acid/ 0.5 M NaCl, whereas only 44 % was releasable after incubation at 37 °C. In contrast the amount of lactoferrin released after incubation at 4 °C was similar to that at 37 °C (about 85 %). These findings suggest that while transferrin was internalised by the cell, in agreement with the previous work on other cell types (Iacopetta and Morgan, 1983; Klausner *et al.*, 1983), no net internalisation of lactoferrin could be detected. Transferrin has been known to release its iron intracellularly at acidic pH and then recycles to the plasma membrane still bound to its receptor (Dautry-Varsat, 1986). Its recycling has been shown to be inhibited by primaquine, a weak base which neutralises the acidic compartment thereby blocking the recycling and increasing its intracellular pool size (Schwartz *et al.*, 1984; Stoorvogel *et al*, 1987; Reid and Watts, 1990). Using 3 mM primaquine, it was shown that twice the amount of transferrin was retained in the cells in the presence of primaquine while there was only a negligible difference in the amount of lactoferrin retained by the cells in the presence or absence of primaquine. The molar ratio of cell-associated iron:transferrin was reduced by about 50 % in the presence of primaquine, whereas the ratio of cell-associated iron:lactoferrin remained at about 2 either in the presence or absence of primaquine, indicating that primaquine slowed down the process of iron uptake from transferrin but did not affect any possible iron uptake from lactoferrin. Roiron-Lagroux and Figarella (1990) also found no evidence for the internalisation of lactoferrin in adenocarcinoma cells. In contrast, it

is quite clear that hepatocytes do internalise lactoferrin; 80 % of cell-associated lactoferrin being intracellular after 60 min at 37 °C (McAbee and Esebensen, 1991). In addition it was found that hepatocytes bound and internalised lactoferrin regardless of its iron content suggesting that lactoferrin released into the circulation by neutrophils could be cleared from the circulation by these cells and hence the need for such a mechanism. The observed difference in the fate of lactoferrin bound to U937 as compared to hepatocytes must be related to its function.

Despite the lack of endocytosis of lactoferrin, slow uptake of iron by U937 cells nevertheless occurred (**Fig. 30**). This slow apparent uptake could have been due to the prior transfer of iron from lactoferrin bound to the cells to transferrin present in the medium which then transfers this iron to the cell in the same manner mentioned earlier. Although this might appear to be no more than an experimental artifact resulting from "scrambling" of iron during culture, the fact that more iron was transferred from lactoferrin to transferrin than vice versa suggests that this was not the case; lactoferrin binds iron with a greater affinity than transferrin (Aisen and Leibman, 1972), so scrambling in solution ought to result in transfer of iron from transferrin to lactoferrin. Furthermore results indicate that lactoferrin bound to the cells releases some of its iron to the medium, which would then be available for binding by either protein. The dissociation studies reported here showed that cells preincubated with Fe-lactoferrin released iron more rapidly than they release lactoferrin itself. The molar ratio of Fe:lactoferrin determined from the graph in fact shows a ratio of < 1, indicating that some of the iron has been removed from the protein molecule. It also seems unlikely that the radioactivity associated with the cells is due to release of iron which had been taken up by the cells as no evidence of internalisation of lactoferrin was obtained. In contrast there

was no release of iron occurred when transferrin rather than lactoferrin was used, despite the fact that transferrin-bound iron is accumulated by the cells. The release of iron from cell-bound lactoferrin implies some destabilisation of the lactoferrin iron-binding site, a phenomenon known to occur when transferrin binds to the transferrin receptor (Bali and Aisen, 1991: Sipe and Murphy, 1991). Alternatively, iron may be removed enzymatically through the action of membrane NADH reductase (Sun *et al.*, 1991; Thorstensen and Romslo, 1988), although this seems less likely given the experiment was carried out at 4 °C.

These results appear to contradict earlier reports (Van Snick *et al.*, 1977; Moguilevsky *et al.*, 1987; Birgens *et al.*, 1988) of iron uptake from lactoferrin by macrophages and monocytes and its incorporation into ferritin. However, the rates of uptake reported in these studies were very slow, and comparable to the slow apparent uptake from lactoferrin by U937 cells; Moguilevsky *et al.* (1987) reported a "cycling time" of about 11 h, in comparison to as little as 2-3 min for transferrin recycling in reticulocytes (Intragumtornchai *et al.*, 1988). Although transferrin was not added to the medium in the previous studies, fetal calf serum was present and this contains bovine transferrin, which can donate iron to cells in culture (Brock and Rankin, 1981). It is therefore possible that iron uptake in those studies occurred via the indirect mechanism proposed here. Indeed an earlier study by Oria *et al.*, (1988) carried out in this laboratory clearly indicated that in a short term cultures of a murine macrophage cell line, there was no net accumulation of iron from transferrin (Oria *et al.*, 1988). Birgens *et al.* (1988) reported that lactoferrin was released from monocytes more rapidly than iron, in contrast to the present findings, but as their studies were performed at 37 °C in serum-containing medium it seems possible that transferrin could have promoted cellular uptake of iron released from

lactoferrin during incubation. However, a difference in behaviour between the monocytic cell line U937 used here and monocytes/macrophages used by others cannot be completely ruled out.

2.5.3 Effect of lactoferrin on uptake of iron from other sources

In addition to iron-transferrin, iron chelates such as FeNTA can also supply iron to U937 cells (**Fig. 32**). When uptake of labelled iron from FeNTA and Fe-transferrin were compared directly, it was found that the amount of iron taken up from FeNTA was much greater than from transferrin. These results are in accordance with the findings of Brock and Rankin (1981) and Djeha and Brock (1992b) that mouse lymphocytes took up irom more rapidly from FeNTA, and iron citrate complexes than from transferrin. Similar findings were reported by White and Jacobs (1978) using Chang liver cells which took up 30-fold more iron from FeNTA than from transferrin and distribute the iron differently in the cell. In contrast Taylor *et al.*,(1988) found that iron uptake from FeNTA added directly to mitogen-stimulated human peripheral blood lymphocytes was quantitatively very similar to that from transferrin. However, their cultures contained 5 % serum, so it is likely that transferrin in the serum mediated the uptake of ^{59}Fe (Djeha and Brock, 1992b).

The mechanism by which non-physiological iron chelates enter the cell in the absence of physiological iron binding proteins is not clear. One mechanism might simply be fluid phase endocytosis. Another mechanism is suggested by studies of iron transport into hepatocytes indicating that non-transferrin bound iron is transported into these cells by a membrane carrier for iron and this process is driven by the membrane potential difference between the interior and exterior of the cell (Wright *et al.*, 1988).

One thing that is clear from the binding studies is that lactoferrin does not donate iron to the cells, instead, when it was added to the cell cultures containing ^{59}Fe-labelled transferrin or FeNTA at iron concentration reported to be within the range that characterise iron overload condition (Hershko *et al.*, 1987), it was found that lactoferrin inhibited iron uptake from FeNTA. This effect was found with both apolactoferrin and Fe-lactoferrin, but uptake from transferrin was not affected which confirms the earlier findings. Furthermore the total amount of iron as FeNTA was 6 times greater than that required to saturate all the lactoferrin present, so the effect was not simply due to sequestration of all the iron in the medium by lactoferrin. These observations coupled with the return of iron from membrane-bound lactoferrin to the medium (**Fig. 33**), suggest that lactoferrin may serve to regulate uptake of potentially toxic non-transferrin bound iron.

There is now increasing evidence that the non-transferrin bound iron may be important in producing conditions of iron loading and cellular damage (Wright *et al.*, 1986). The non-transferrin bound iron circulates in plasma and may be complexed to a variety of low molecular weight compounds which are effective in the formation of harmful oxygen derivatives (Halliwell and Gutteridge, 1986). Superoxide dismutase, catalase and glutathione peroxidase, which are useful in limiting such toxicity within cells, are virtually absent from the extracellular compartment. Thus the major protective mechanism of human plasma in preventing iron from participating in the catalysis of hydroxyl radical formation is its binding to the proteins or the therapeutic use of effective iron chelator (Hershko, 1987).

2.5.4 Effect of lactoferrin on cell proliferation

Iron plays an important role in cell growth and transferrin has been shown to promote the cell growth. The ability of transferrin to promote cellular proliferation is closely related to its ability to donate iron to the growing cells (Aisen and Listowsky, 1980; Brock *et al.*, 1986). More recent studies have suggested that lactoferrin may also promote cell growth.

Data obtained from this study by measurement of incorporation of radiolabelled precursors i.e. ^{3}H-thymidine into cellular DNA indicate that transferrin and lactoferrin, both apolactoferrin and Fe-lactoferrin have stimulatory effect on the growth of U937 cells. Both bovine and human lactoferrins can enhance the cell proliferation. The stimulatory effect of transferrin is due to its iron donating property as iron uptake studies have indicated this ability. Earlier work in this laboratory has also shown that lactoferrin increased the proliferation of a murine macrophage-like cell line, P388D1 (Oria *et al.*, 1988). Interaction of lactoferrin with lymphoma cell lines has also demonstrated some enhancement of the rate of cellular proliferation (Hashizume *et al.*, 1983). Subsequent work by Amouric *et al.* (1984) on adenocarcinoma cell line HT29 also reported stimulatory effect of lactoferrin in the presence of low concentration of iron. The mechanism by which lactoferrin is able to stimulate the cell growth is unclear (Hashizume *et al.*, 1987; Roiron-Lagroux and Figarella, 1990). Unlike transferrin which donates iron to the cell, lactoferrin does not seem to promote growth in a similar manner. Little or no iron is taken up from lactoferrin for this purpose. The fact that apolactoferrin was as effective as Fe-lactoferrin proves this point.

Heat treatment of bovine lactoferrin at 72 °C for 20 sec or at 137 °C for 8 sec had little effect on its ability to enhance proliferation, although some reduction in its ability to competitively inhibit binding of ^{125}I-labelled native lactoferrin was observed. These temperatures correspond to those used for mild pasteurisation or to spray-drying, respectively, suggesting that neither of these treatments would materially affect the biological activity of lactoferrin. However, a more severe heat treatment (85 °C for 20 min) abolished the proliferation enhancing effect of Fe-lactoferrin, but had no effect on that of apolactoferrin. Moreover the effect on cell binding was similar to that of the more mild treatments. These findings suggest that lactoferrin still in a biologically active form could be supplemented to cow's milk based infant formula.

CHAPTER THREE

ROLE OF LACTOFERRIN IN IRON ABSORPTION

3.1 INTRODUCTION

Total body iron balance is regulated by iron absorption which occurs in the proximal end of the small intestine. The uptake of iron from the diet by the intestinal cells is sensitive both to physiological needs as well as to iron stores. The mechanisms involved in the processing of iron taken up from the diet at the brush border membranes and its release at the basolateral membrane as well as its intracellular storage are not fully understood.

The greater bioavailability of iron and higher concentration of lactoferrin in human milk compared to cow's milk have led to the proposal that lactoferrin might play a role in iron absorption. This proposal is further supported by the finding that lactoferrin receptors have been identified on the brush border membranes of some species, including human fetal intestinal membrane (Davidson and Lonnerdal, 1988; Hu *et al.*, 1990). However, there is so far no evidence that these receptors actually mediate uptake or transport of lactoferrin bound iron in the mucosal cell. In fact, there have been reports suggesting that lactoferrin may inhibit iron absorption and may actually serve as an additional control of iron absorption during neonatal period (reviewed by Brock, 1980).

If lactoferrin is transported across the intestinal cell and enters the circulation, this ought to result in higher plasma levels of lactoferrin. Since cow's milk contains very little lactoferrin, there would be a difference in the plasma lactoferrin levels of infants fed cow's milk based formulas as compared to breast-fed infants. Indeed recent studies in preterm infants have demonstrated the presence of lactoferrin derived from maternal milk in the urine of breast-fed infants, suggesting that lactoferrin can be absorbed

(Hutchens *et al.*, 1989). In contrast a study by Scott (1989) found no difference in plasma lactoferrin levels between these two groups. However this study was conducted in full-term infants and it is not known whether similar events occur in term infants as their gastrointestinal tracts are more developed.

Due to these controversies, a study was undertaken to investigate whether lactoferrin plays any role in iron absorption. The objectives of the study were:

(i) to investigate the interaction of lactoferrin with an *in vitro* model of the human intestinal mucosa, and to study iron and protein (lactoferrin and transferrin) transport across these cells.

(ii) to determine plasma lactoferrin levels in breast-fed versus formula-fed newborn infants.

Iron and protein transport studies were carried out using Caco-2 cells, a human colon adenocarcinoma cell line, which was first established in 1974 by Jorgen Fogh (Memorial Sloan-Kettering Cancer Centre, New York) (reviewed by Rousset, 1986). The cells have been shown by many workers to exhibit enterocyte like differentiation patterns, such as brush border micorvilli and associated enzymes and the formation of domes which are typical of transporting epithelial monolayers. The Caco-2 cell line has been used to examine a variety of intestinal function (Audus *et al.*, 1990) and is useful to study iron transport as the cells can be grown in bicameral chambers, and form a highly polarised monolayer which can be used to demonstrate vectorial transport (Alvarez-Hernandez, *et al.*, 1991).

The plasma lactoferrin levels were determined using a solid phase enzyme-linked immunoabsorbent assay (ELISA). The method is well suited to measure low levels of lactoferrin and the reagents are stable, since alkaline phosphatase-conjugated antiserum may be kept for at least 6 months without losing activity (Kolsto Otnaess *et al.*, 1983). Since lactoferrin is also present in the secondary granules of neutrophils, extreme care must be taken in obtaining plasma to ensure that lactoferrin levels are not artificially raised due to neutrophil degranulation.

3.2 MATERIALS

3.2.1 Cell lines

Two cell lines were used in this experiment. The human colon carcinoma cell line Caco-2, was kindly provided by Dr. R.I. Freshney, Department of Medical Oncology, University of Glasgow. INT 407, a human embryonic intestinal epithelial cell line was obtained from Flow laboratories, Irvine, Scotland.

3.2.2 Blood Samples

Venous blood was obtained from 15 infants, delivered at the Department of Paediatrics, Queen Mother's Hospital, Glasgow. Approval from the Ethical Committee of the Hospital and parental consent were granted before taking samples. Samples were taken only from healthy infants without any jaundice, 48 to 72 hours after delivery. The infants were selected according

to their method of feeding since birth. One group consist of infants who were exclusively breastfed and the other was a group of formula-fed infants.

3.2.3 Materials for ELISA

3.2.3.1 Lactoferrin antibody

Rabbit anti-human lactoferrin antibody was raised in two rabbits at the Department's animal house, Western Infirmary, Glasgow.

3.2.3.2 Lactoferrin antibody-enzyme conjugate

Lactoferrin antibody alkaline phosphatase conjugate was prepared from antibody (3.2.3.1) and alkaline phosphatase (Serotec Ltd., Kidlington, Oxford, U.K.), as described below in section 3.3.6.

3.2.3.3 Myoglobin (1-2 %), Human serum albumin (5 %) and Bovine serum albumin (1-2 %) solutions

These solutions were prepared from appropriate amount of myoglobin from horse skeletal muscle (Sigma), human serum albumin (Behringwerke) and bovine serum albumin (Sigma) dissolved in distilled and dionised water, which were then filter sterilised.

3.2.3.4 Polystyrene beads

Polystyrene beads, white etched, 6.4 mm diameter, surface enhanced, were purchased from Northumbria Biologicals Ltd., Northumberland, U.K.

3.2.3.5 Enzyme substrate

One tablet (5 mg) of *p*-nitrophenyl phosphate (Sigma) was added to 5 ml of diethanolamine buffer (see section 3.2.4.4). The substrate solution was prepared fresh for each assay.

3.2.3.6 Standard lactoferrin solution for ELISA

Lactoferrin used for calibration was isolated and purified by the procedure described in section 2.3.1. The standard solutions were prepared from a stock lactoferrin solution (1 mg/ml) diluted in 5 % human serum albumin to give a range of concentrations from 6.12 ng/ml to 300 ng/ml. The diluted standards were kept at -20 °C in small aliquots.

3.2.3.7 Other materials

Polystyrene test tubes, 55 x 12 mm, were purchased from Sarstedt Ltd., Leicester, U.K. Ninty six well microtitre plates were purchased from Millipore, U.K. Plastic microtubes (Scotlab, U.K.) containing 0.6 mg (1.5 mg/ml) potassium EDTA (BDH) were used for blood collection.

3.2.4 Buffers

All solutions for ELISA were prepared using distilled and deionised water which were then filter sterilised.

3.2.4.1 Phosphate buffers (200 mM , 100 mM, pH 7.2)

27.0 g Na_2HPO_4 .12 H_2 O (BDH) and 4.0 g NaH_2 PO_4. $2H_2$ O (BDH) was dissolved in 400 ml water and pH was adjusted with 1 M HCl, and the solution was then made up to 0.5 litre to make 200 mM solution. This buffer was diluted with equal volume of distilled and deionised water to make 100 mM buffer.

3.2.4.2 Tris/HCl (0.5 M, pH 8.0)

Tris (60.5 g) was dissolved in 800 ml water and pH was adjusted to 8.0 using 1 M HCl and the solution was made up to 1 litre.

3.2.4.4 Diethanolamine buffer

97 ml diethanolamine (BDH)

0.2 g NaN_3

100 mg $MgCl_2.6H_2O$ (BDH)

800 ml H_2O

pH was adjusted to 9.8 and the solution was made up to 1 litre.

3.3 METHODS

3.3.1 Cell Cultivation

3.3.1.1 Routine culture

Caco-2 cells were cultivated for routine use in Dulbecco's modified Eagles medium (DMEM, Gibco, Paisley, U.K.) supplemented with 10 % fetal calf serum (Northumbria), 1 % non-essential amino acids (Flow), 1 μg/ml bovine insulin (Sigma), 100 μg/ml streptomycin (Flow) and 100 U/ml (Flow) in 25 cm^2 flasks in a humidified incubator at 37 °C with 5 % CO_2. They were subcultured every 2-3 days using 5 ml EDTA (1 mM, BDH) and 5 ml trypsin (0.25 %, Sigma).

INT 407 cells were grown in RPMI medium (without HEPES, Gibco), supplemented with 10 % fetal calf serum (Northumbria), 100 μg/ml streptomycin (Flow), 100 U/ml penicillin (Flow) and 250 ng/ml fungizone (Flow). The cells were subcultured every 3 days using EDTA and trypsin as above.

3.3.1.2 Cell Culture in Transwell Bicameral Chambers

For transport experiments, cells were cultured in Transwell inserts **(Fig.38)** until they formed complete monolayers. Each insert has polycarbonate membrane and the inside wall has been treated for uniform cell attachment. The surface area of the membrane is 0.33 cm^2 with 3 μm pore size. Each insert was precoated with a thin layer of collagen by applying evenly 50 μl

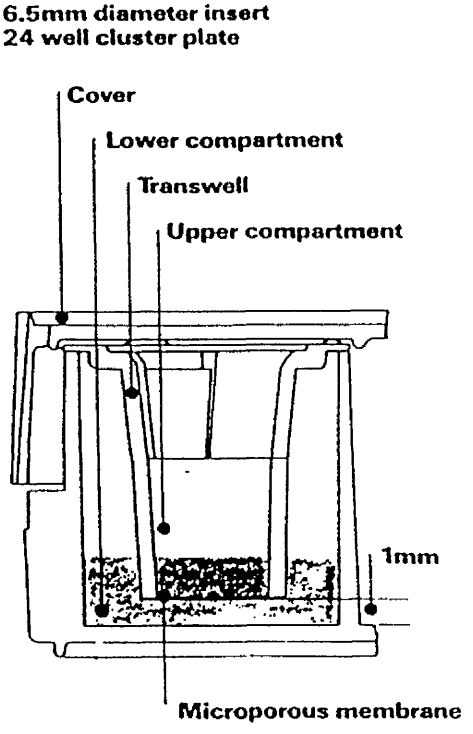


Fig. 38: A diagram of a bicameral chamber containing a Transwell insert with polycarbonate membrane on to which Caco-2 cells are cultured

of Type 1 collagen from rat tail (2 mg/ml; Boehringer Mannheim).

Excess collagen was carefully removed and the insert was allowed to stand inverted to let it dry in a sterile laminar air flow for 30-45 min. Collagen mediates cell attachment and enhances the growth as well as differentiation of many cell types including epithelial cells. Cells were seeded at a density of 10^5 cells/cm^2 i.e. 33,000 cells per insert. The culture medium was added: 200 µl in the upper and 800 µl in the lower compartments, and changed daily. Formation of cell monolayers was monitored every day by measuring the transepithelial electrical resistance (TEER) and diffusion of phenol red across the monolayers, as described below.

3.3.2 Monolayer Formation and Cell Morphology

3.3.2.1 Transepithelial Electrical Resistance Measurements (TEER)

Transepithelial electrical resistance measurements of Caco-2 cell monolayers were recorded daily before changing the culture medium and also before and after performing iron and protein transport studies. The device utilised in obtaining TEER values was an Epithelial Voltohmeter (EVOM), (World Precision Instruments, U.K.). The device consists of a pair of chopstick-like electrodes, 4 mm wide and 1 mm thick. The small size of the electrode sticks facilitates placement of the electrodes in the culture wells and one electrode tip, being 2.5 mm longer than the other fits into the deeper outer chamber of the well. Each electrode contains a silver/silver chloride pellet for voltage changes and a silver electrode for passing current. Before taking electrical resistance measurements, the electrodes which had been kept in sterile PBS, pH 7.2,

were placed in 70 % ethanol for a few minutes and then transferred to a culture medium without fetal calf serum and the resistance set at zero. Electrical resistance values of the cell monolayers in each Transwell insert were measured directly and were then multiplied by the surface area of the polycarbonate membrane (0.33 cm^2). Electrical resistance values obtained in the absence of cells (caused by the electrical system and the collagen coated polycarbonate membrane) were considered as background. For each experiment, total electrical resistance values were corrected for background which ranged from 35 to 50 Ω cm^2, to obtain the TEER values.

3.3.2.2 Diffusion of Phenol Red

Another way to assess the confluency of the cell monolayer is to determine the degree of diffusion of phenol red across the cell monolayer to the lower compartment. The medium in the lower compartment was removed and replaced with phenol-red free HBSS (Flow) after carefully washing the lower compartment several times with the same medium. The medium in the upper compartment was the culture medium which contained phenol red. After incubation at 37 °C for 2 h, aliquots of 100 µl were removed from the upper and lower compartments and A_{546} readings were taken using an ELISA reader.

3.3.2.3 Transmission Electron Microscopy

Caco-2 cells on the polycarbonate membrane were fixed for 24 h at room temperature with 2 % glutaryldehyde (Agar Scientific, Stansted, Essex, U.K.) in Sorensens phosphate buffer, pH 7.38 and postfixed in 1 % osmium tetroxide. After dehydration through a graded series of alcohols, the

complete membrane from the Transwell insert was cut from the insert walls and embedded in Araldite (Agar Scientific) which was allowed to polymerise overnight at 60 °C. Sections 2 μm thick were cut on a Reichert Jung Ultracut E, stained with toluidine blue and examined by light microscopy. Ultrathin sections 80-90 nm thick were then cut from suitable areas, mounted on 200 mesh copper grids and then stained with uranyl acetate and lead citrate. Sections were then examined in a Philips CM10 electron microscope.

3.3.3 Iron and Protein Transport Studies

Experiments on iron and protein transport across Caco-2 and INT 407 cell monolayers were carried out only after cultures gave percent diffusion of phenol red, < 5 %, and in the case of Caco-2 cells, the values of TEER > 200 Ω cm^2, indicating that intact monolayers had been formed. This was normally achieved after 15-20 days of seeding the cells in the Transwell inserts.

In this experiment, the media in the upper and lower compartments were removed and replaced with a similar medium but without fetal calf serum. The total volume of medium plus addition in the upper compartment was 200 μl and in the lower compartment was 600 μl. To the upper compartment was added ^{59}Fe-labelled lactoferrin or transferrin (50 μg/ml) or a similar amount of iron as ^{59}Fe-citrate. Unlabelled apotransferrin (1 mg/ml) was added to the lower compartment to serve as an iron acceptor. The cells were incubated at 37 °C with 5 % CO_2 for various time intervals. At each interval, the medium from the lower compartment was removed for counting and was replaced with an equal amount of fresh medium. For

protein transport studies, the procedure was similar except that ^{125}I-labelled lactoferrin and transferrin were used.

At the end of the experiment, the medium in the upper compartment was collected and the cells were dissolved in 2 % sodium dodecyl sulphate. The medium, cells and the polycarbonate membrane were immediately assayed for radioactivity. The integrity of labelled lactoferrin and transferrin in the upper and lower compartments was determined by precipitation with 10 % (w/v final concentration) trichloroacetic acid and also by using SDS-PAGE (see section 2.3.2) and autoradiography which was carried out using a Kodak XAR-5 film at -70 °C for 3-7 days.

3.3.4 Preparation of Plasma Samples from Newborn Infants

The presence of lactoferrin in the secondary granules of neutrophils calls for specific precautions in the collection and treatment of blood. The determination of blood lactoferrin must be performed on plasma and not on serum. Venous sampling was considered to be more suitable than capillary sampling (Maacks *et al.* 1990). Venous blood (0.3-0.5 ml) was collected in prelabelled microtubes and shaken to mix with EDTA contained in the tubes and immediately placed in ice water. The blood sample was centrifuged for 15 sec at 3000 g. The plasma was carefully extracted, making sure not to include any cellular particles and transferred to a fresh microtube and centrifuged for another 8 min. The upper two-thirds of the plasma were collected and placed immediately at -20 °C. For long storage the samples were transferred to -70 °C.

3.3.5 Preparation of Rabbit Anti-Human Lactoferrin IgG solution

The IgG fraction of the anti-serum was used for coating the polystyrene beads as well as labelling the enzyme. It was prepared from the anti-serum by ammonium sulphate precipitation as described in section 2.3.5.2.

3.3.6 Preparation of Alkaline Phosphatase-Anti Lactoferrin Conjugate

The alkaline phosphatase-IgG conjugate was prepared according to the company's instruction based on one step glutaryldehyde method (Avrameas, 1969). Alkaline phosphatase was supplied as a precipitate in ammonium sulphate. An appropriate volume (0.5 ml) containing 5 mg enzyme (activity > 1000 U/mg protein) was centrifuged at 2000 g for 10 min at 4 °C, and the supernatant was discarded. A volume, < 1.0 ml containing 2 mg IgG solution in PBS (pH 7.2) was added to the enzyme pellet and these were dialysed together against PBS overnight at 4 °C with several changes in order to remove all the ammonium sulphate present in the enzyme extract. The volume of conjugate was made up to 1.25 ml with PBS to which was added 10 µl glutaryldehyde solution (25 %), and it was allowed to react for 2 h at room temperature with constant mixing on a rotator. The mixture was dialysed against two changes of PBS at 4 °C overnight and lastly against two changes of Tris buffer (pH 8.0). The conjugate was collected and diluted to 4 ml with Tris buffer containing 1 % BSA and 0.02 % sodium azide. The conjugate was filter sterilised and dispensed into small volumes and stored at -20 °C.

3.3.7 Procedure for ELISA

3.3.7.1 Principle of Assay

The method used to determine plasma lactoferrin is based on a non-competitive sandwich antigen assay as reported by Figarella (1986) and Scott (1989). The principle of the assay is shown in **Fig. 39**. Human lactoferrin is extracted from the samples by a specific antibody bound to polystyrene beads. After washing, solid phase-bound lactoferrin reacts with alkaline-phosphatase labelled anti-lactoferrin IgG. Excess of non-reacted enzyme conjugate is removed by washing and the bound enzymic activity is revealed with subsequent addition of substrate, *p*-nitrophenyl phosphate (*p*-NPP), which develops a yellowish colouration that can be measured at λ_{405} nm. The concentration of lactoferrin in the sample is thus directly proportional to the enzymic activity present on the solid phase.

3.3.7.2 Coating of the solid phase

In initial attempts to set up the ELISA assay, different types of polystyrene microtitre plates kindly provided as trial samples by Dynatech., Sussex, U.K. were used as a solid phase. This was not susseccful due to high background readings. Polystyrene beads were therefore used as the solid phase (Figarella, 1986 and Scott, 1989).

Fifty polystyrene beads were placed in a clean glass universal which had been rinsed several times with sterile water and dried by alcohol to remove any traces of detergent. The beads were washed with absolute ethanol and rinsed three times with filtered, distilled and deionised water and lastly with

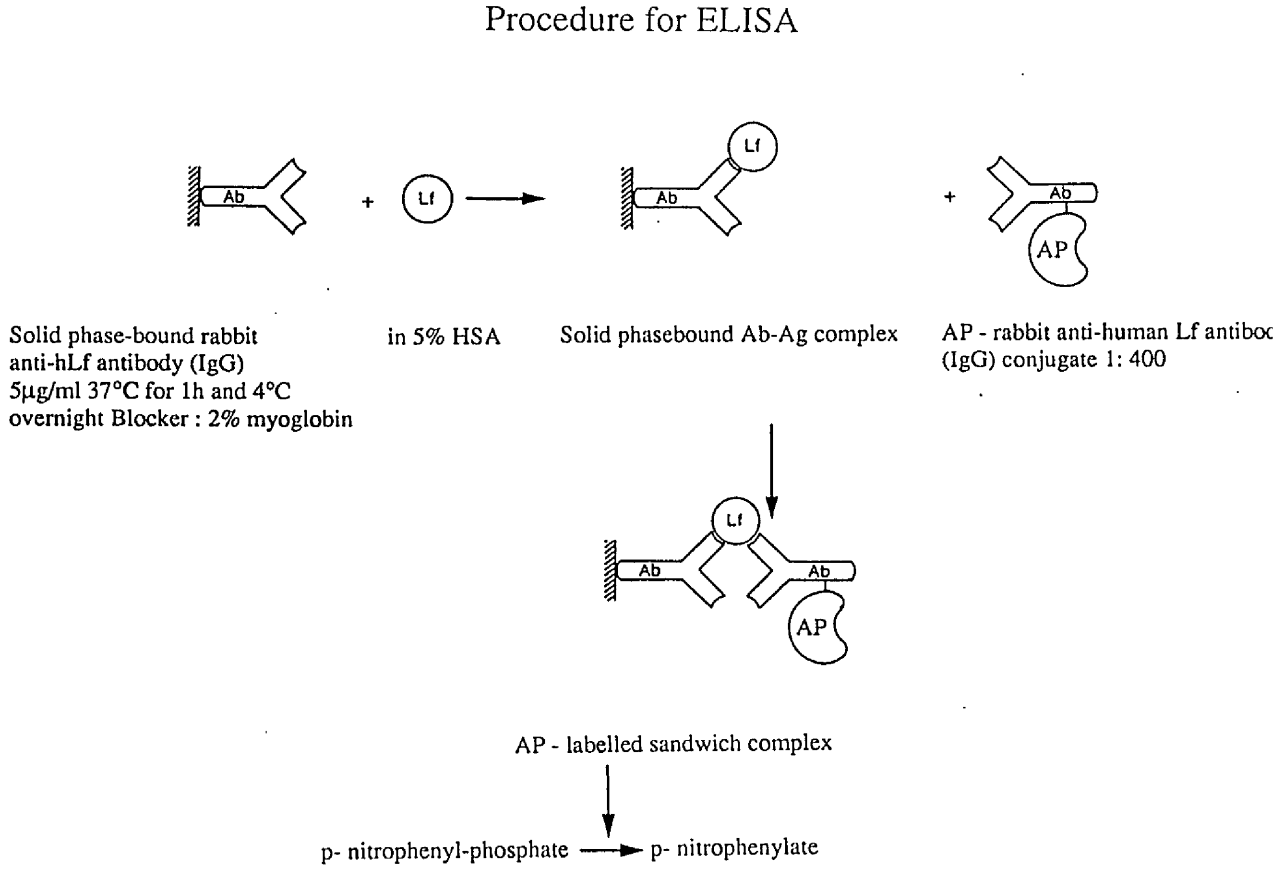


Fig. 39: Principle of ELISA according to the design of sandwich antigen assay for the determination of plasma lactoferrin

phosphate buffer (200 mM). About 7.5 ml (150 µl per bead) of anti-lactoferrin IgG (5 µg/ml) was steadily poured to cover the beads and mixed gently on a roller for an hour at 37 °C, then at 4 °C overnight without further mixing. The beads were washed four times with phosphate buffer (100 mM) and were either used immediately or stored at 4 °C in the same buffer.

3.3.7.3 Assay procedure

Into the required number of polystyrene test tubes was pipetted 200 µl of HSA solution (5 %) and 20 µl standards, controls and samples that diluted with HSA (5 %). To each tube was added one coated bead and these were incubated at 37 °C for 2 h. Each bead was washed three times with 4 ml filtered, distilled and deionised water. Three hundred microlitre of diluted enzyme conjugate (1:400) was added to each bead and incubated at 37 °C for 1.5 h. Excess enzyme conjugate was removed and the bead was washed as before and transferred to a fresh polystyrene test tube. To each tube was added 200 µl *p*-nitrophenyl phosphate solution followed by incubation in the dark at room temperature for 30 min, at the end of which the reaction was quenched by adding 50 µl sodium hydroxide (3 M). A volume of 200 µl of the coloured product was pipetted out from each tube and placed in a 96-well microtitre plate to be read at 405 nm, using an ELISA reader.

3.3.8 Assay Optimisation

3.3.8.1 Optimal antibody coating concentration

As the capacity of polystyrene beads to bind the antibody is limited,

it was necessary to determine an optimal concentration that would coat the bead fully, but at the same time not be present in too much excess. Therefore, various concentrations of anti-human lactoferrin IgG (1, 5, 10 and 30 μg/ml) were used to coat the polystyrene beads, and the corresponding beads were tested for their ability to bind lactoferrin following the procedure described in 3.3.7.3.

3.3.8.2 Choice of blockers

A blocker is used to fill any vacant sites on the bead in order to reduce non-specific binding of lactoferrin. Blockers that were tested included 1 % and 2 % BSA, 1 % and 2 % myoglobin and incubation buffer (200 mM phosphate buffer containing 10 % rabbit serum; Figarella, 1986). These blockers were added to the coated beads and the assay was continued as described in 3.3.7.3.

3.3.8.3 Optimal dilution for alkaline phosphatase-anti lactoferrin conjugate

Several dilutions of enzyme conjugate were tested in the assay to obtain a range of optical densities giving a maximum A_{405} value of 1.5 for the highest standard solution. The stock enzyme conjugate was diluted in appropriate volumes of PBS for the following dilutions; 1:200, 1:400, 1:600 and 1:1000.

3.3.8.4 Selection of assay conditions

For the first incubation period, two procedures were tested. These involved incubation for either 2 h at 45 °C (Figarella, 1986), or at 37 °C for 1 h followed by overnight incubation at 4 °C (Scott, 1989). The incubation period with the enzyme conjugate was at 37 °C for either 1, 1.5 or 2 h. The enzyme subtrate reaction was allowed to continue for 30, 45 and 60 min.

3.3.8.5 Accuracy of the assay

The accuracy of lactoferrin detection was checked by adding known quantities of lactoferrin (50-200 ng/ml) to rabbit plasma and comparing the measured values to the expected ones.

3.3.8.6 Specificity of the assay

Cross reactivity of the lactoferrin antibody was checked against bovine lactoferrin and human transferrin by measuring diferrent amounts (50-200 ng/ml) of human lactoferrin in rabbit plasma to which had been added equal amount of bovine lactoferrin and human transferrin respectively.

3.4 RESULTS

3.4.1 Iron and Protein Transport Studies

3.4.1.1 Transepithelial electrical resistance measurements (TEER)

Caco-2 cells grown on polycarbonate membranes of the Transwell inserts demonstrated a gradual increase in TEER values (**Fig.40**). Electrical resistance across the collagen-coated, polycarbonate membranes without cells ranged from 35-49 $\Omega.cm^2$. Tests on uncoated membranes indicated that the electrical resistance due to the collagen layer was only 6-8 $\Omega.cm^2$. For the first four days after seeding the cells to the polycarbonate membranes, the TEER values of the cell monolayers were only slightly higher than the background, about 80 $\Omega.cm^2$. The values started to increase on the fifth day with a gradual increase and reaching maximum at day 16 (251.2 $\Omega.cm^2$) and remained almost constant until day 20.

With INT 407 cells, observations under the light microscope showed a good monolayer was formed by day 7. However, the TEER values did not show any increase and in fact, they were only slightly higher than the background values (78.1 $\Omega.cm^2$) **(Fig. 40)**.

The TEER values of the monolayers were also measured before and after every transport experiment. These values were found to be unchanged after the experiment, suggesting that the monolayers remained intact during and after the experiment.

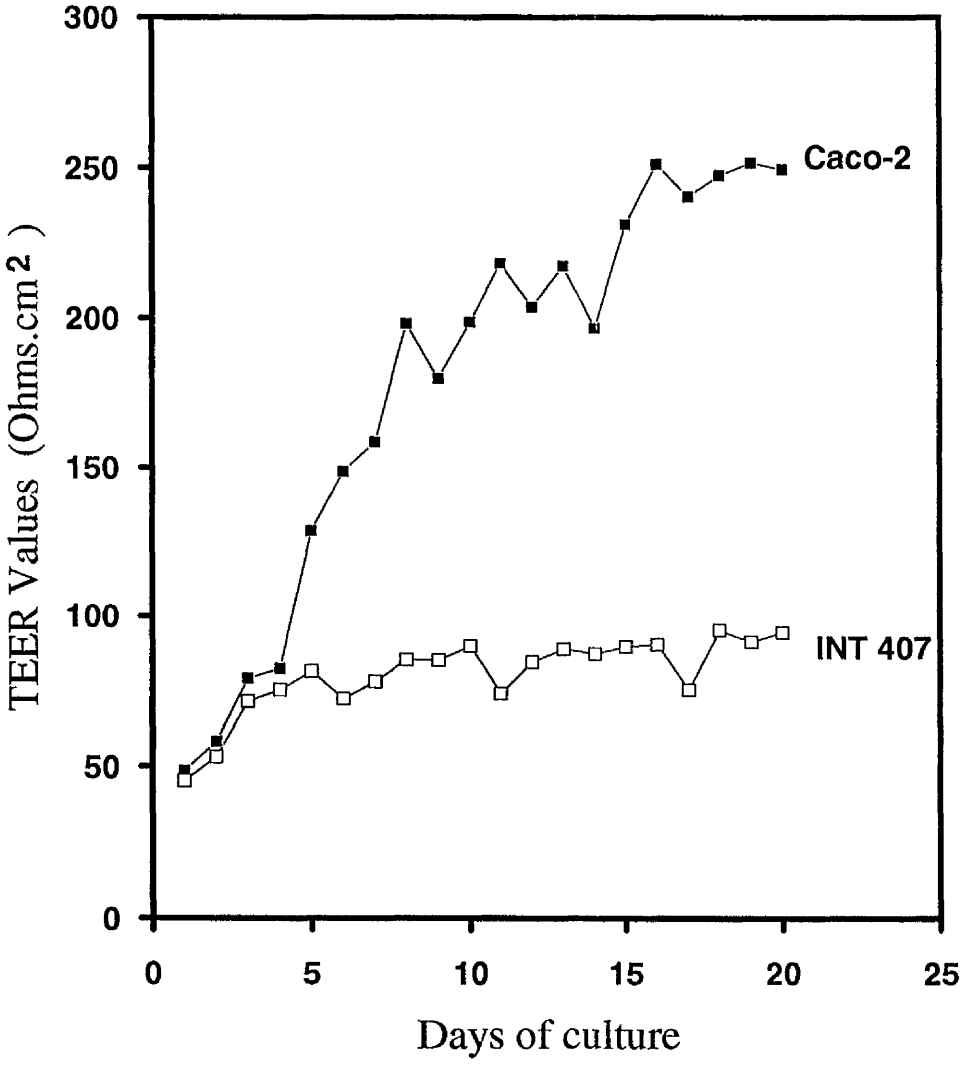


Fig. 40 : Transepithelial electrical resistance values of cell monolayers cultured in Transwell bicameral chambers. Each point represents a mean of at least 4 inserts.

3.4.1.2 Diffusion of phenol red across the cell monolayers

Passage of phenol red already present in the culture medium from the upper compartment to the lower one, filled during the assay with phenol red-free medium was used as another simple and non-destructive test to determine the confluency of the cell monolayers. Diffusion of phenol red through Caco-2 cells is shown in **Fig. 41**. After two days of culturing in the Transwell inserts, transport of phenol red was about 15.4 %. This value was close to that of background i.e. in the absence of cells (17.1 ±1 %; n=5). The diffusion of the phenol red gradually decreased, reaching a minimum from day 15 onwards.

Percent diffusion of phenol red across INT 407 cell monolayer (**Fig. 41**) showed a rapid decrease from the initial 14.8 ± 0.5 % at day 2 to 6.9 ± 0.2% at day 7.The decrease in percent diffusion was faster for INT 407 than for Caco-2 cells, indicating that INT 407 monolayers were formed earlier. From day 7, the amount of phenol red excluded changed only slightly and remained low until day 17 and onwards.

3.4.1.3 Electron microscopy of Caco-2 cells

The degree of morphologic differentiation of Caco-2 cells with the expression of microvilli and tight junctions and the ability of the cells to form complete monolayers on collagen coated, polycarbonate membrane were assessed by examining the cell morphology after day 17. **Figures 42** and **43** show representative pictures of Caco-2 cells after 17 days of culture in the Transwell inserts and on the same day the transport studies were carried out. The cells demonstrated highly polarised structure with

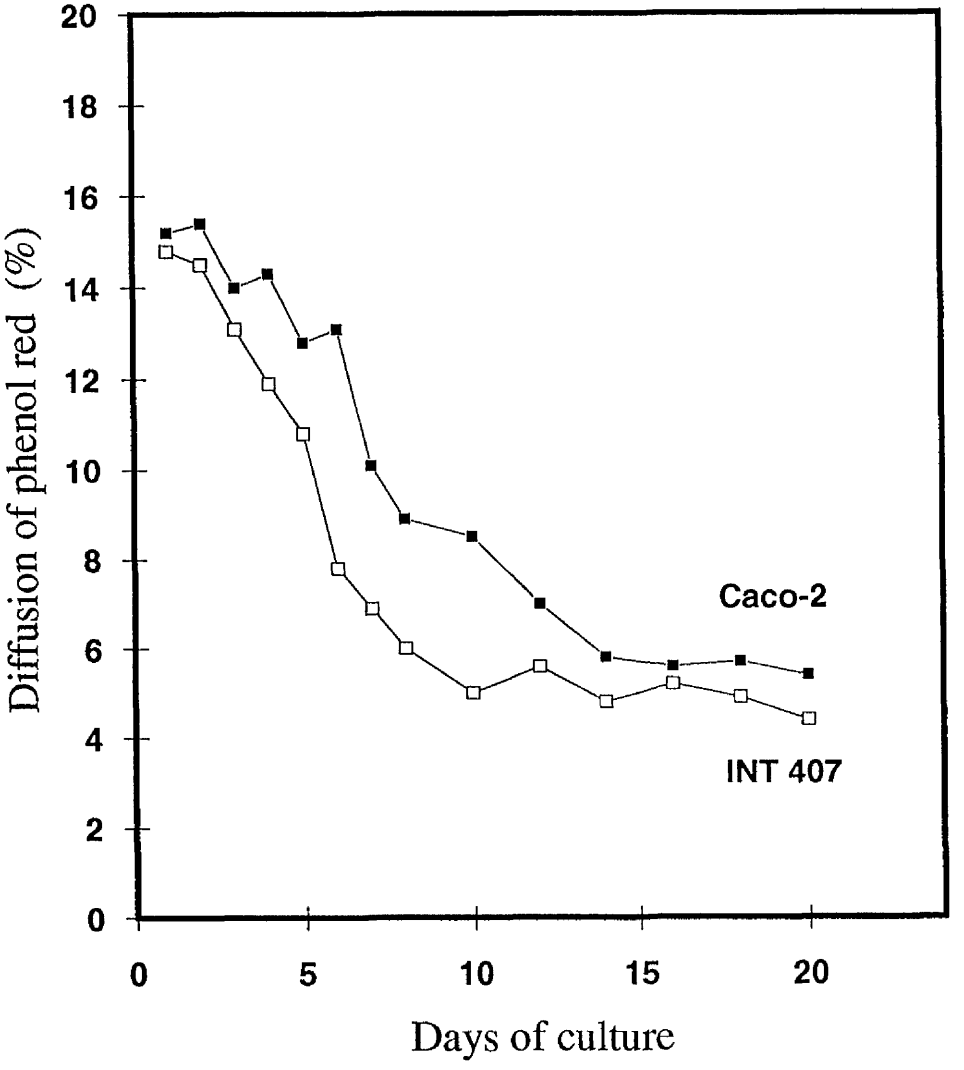


Fig. 41 : Passage of phenol red across the cell monolayers cultured in the Transwell bicameral chambers. Each point represents a mean of at least three determinations.

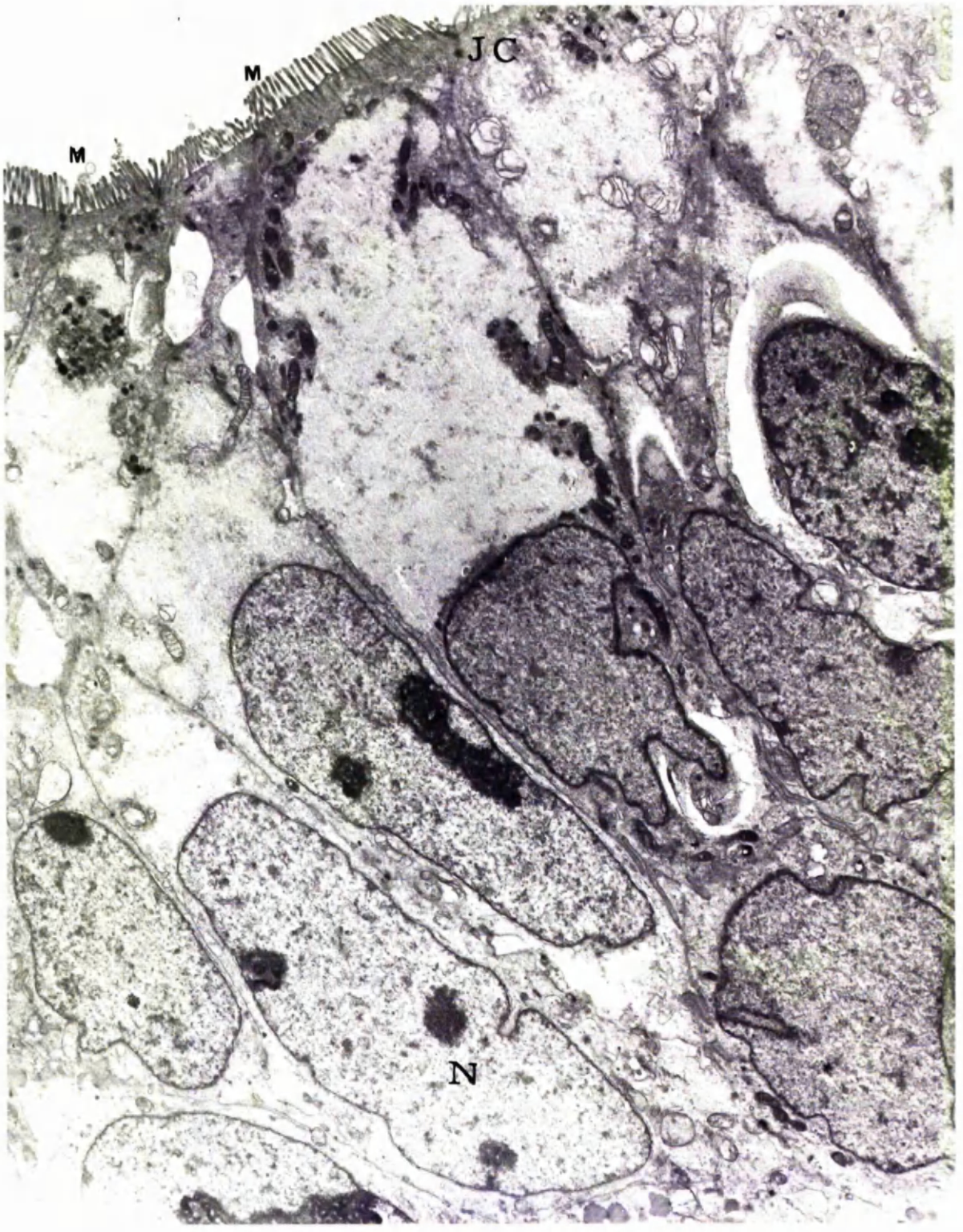


Fig. 42: A transmission electron micograph of Caco-2 cells which had been cultured in a Transwell insert for 17 days: magnification x 89000. (M, microvilli; JC, junctional complex; N, nucleus).

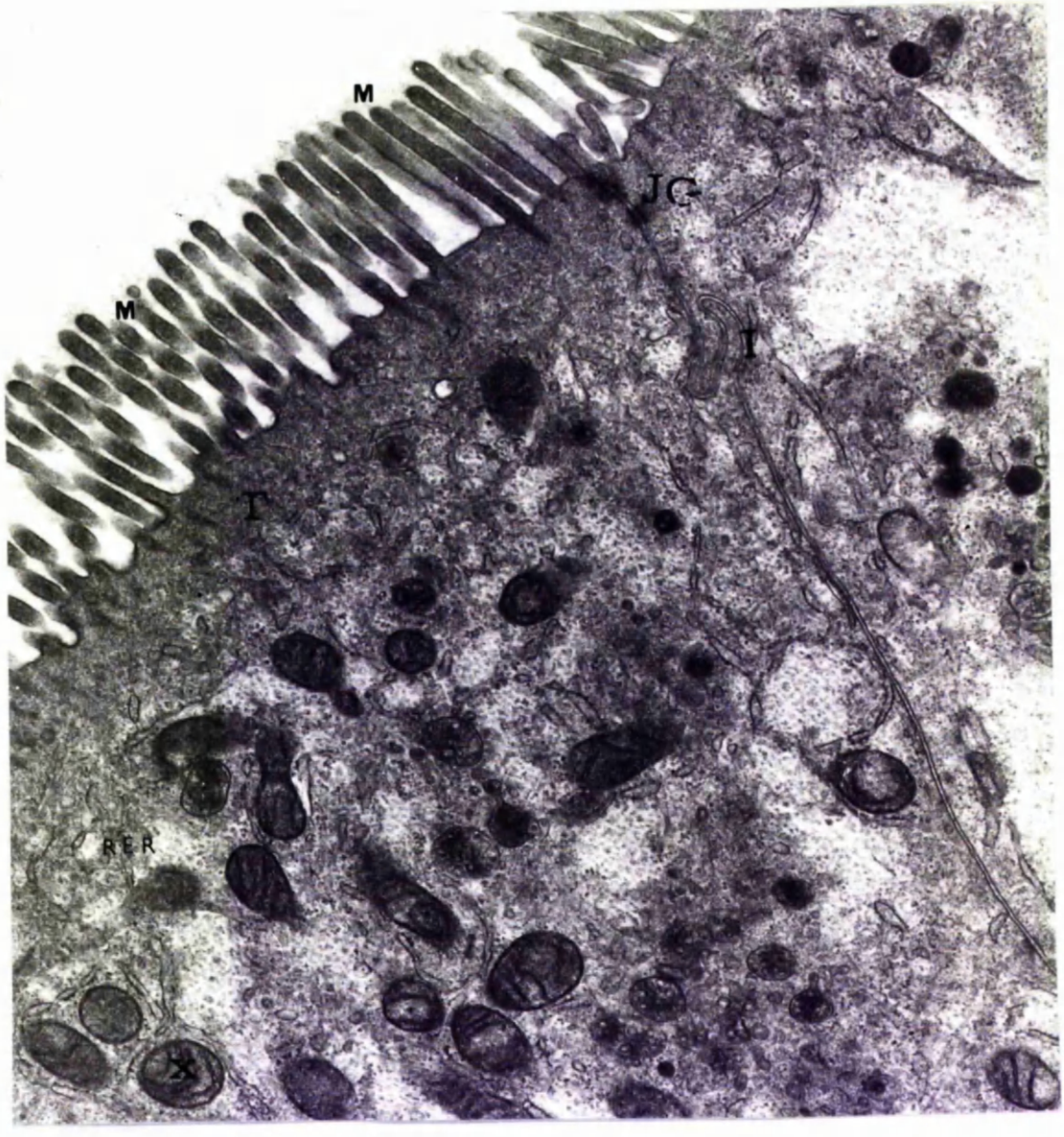


Fig. 43: A higher magnification of the same cell showing clearly enterocytic-like differentiation features which include microvilli (M), terminal web (T), junctional complex (JC) and interdigitation (I). × and RER refer to mitochondria and rough endoplasmic reticulum.

abundant microvilli in the apical surface, well developed tight junctions and the presence of desmosomes.

3.4.1.4 Transport of Iron Across Caco-2 Monolayers

Figure 44 demonstrated that the transport of ferric iron across Caco-2 monolayers to the lower compartment increased with time. There was a large increment in the amount of iron transported from lactoferrin and citrate but an insignificant increase from transferrin from 1 h to 23 h of incubation. The amount of iron transported was highest from lactoferrin whereas transport of transferrin-bound iron was almost undetectable. In contrast to these results, there tended to be greater amount of iron retained by the cells when the metal was initially bound to transferrin rather than to lactoferrin, although the greatest retention occurred with ferric citrate **(Table VII)**.

3.4.1.5 Transport of Lactoferrin and Transferrin Across Caco-2 monolayers

The passage of lactoferrin and transferrin across Caco-2 monolayers to the lower compartment demonstrated that the transport of both proteins, like ferric iron, increased with time, but the amount of transferrin transported was slightly more than lactoferrin at the end of 23 h incubation (**Table VIII**). However, about 80 % of the proteins that had been transported to the lower compartment was in a low molecular weight form, suggesting that there was very little transport of intact lactoferrin or transferrin across the monolayer as determined by trichloroacetic acid precipitation and SDS-PAGE (**Fig.45**).

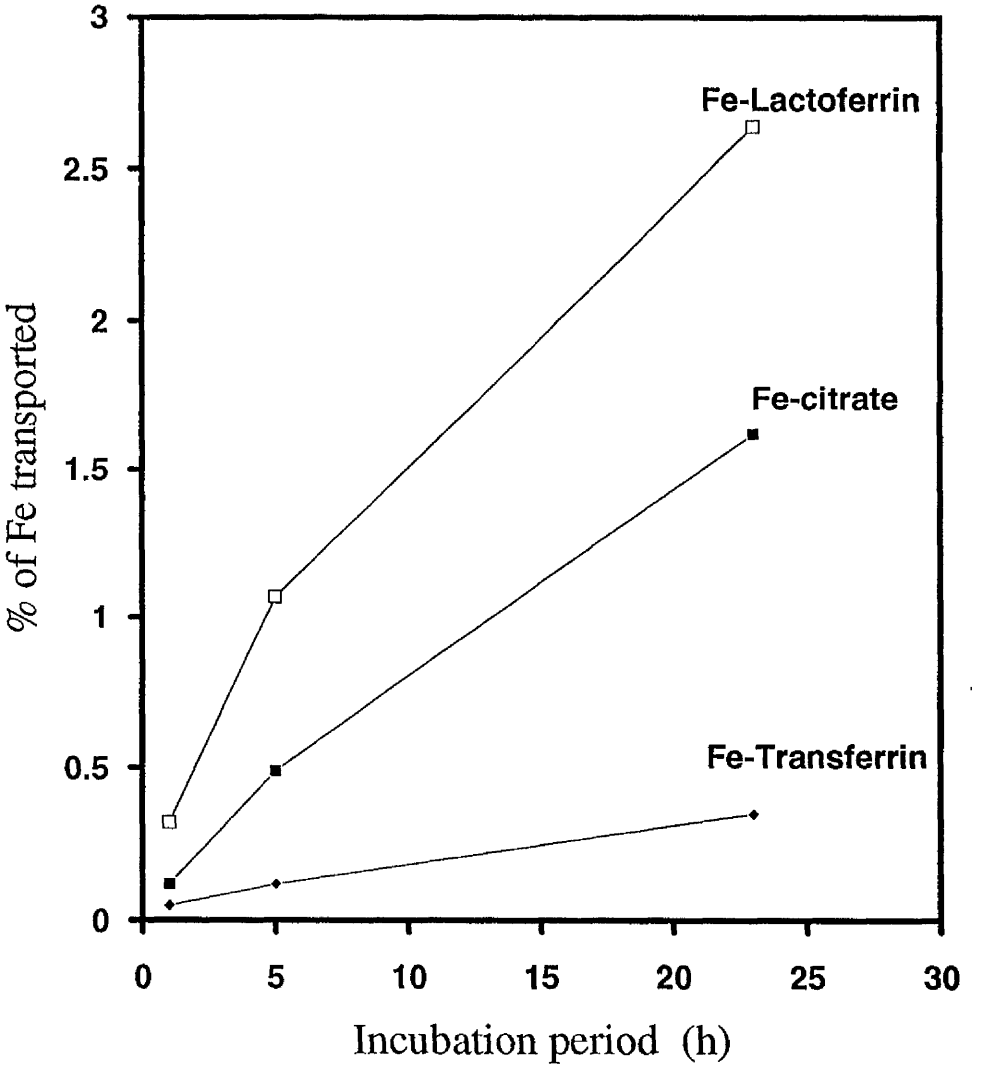


Fig. 44 : Transport of iron across the Caco-2 cell monolayers grown in the Transwell bicameral chambers (n = 2).

TABLE VII

Iron uptake by Caco-2 and INT 407 cell cultured in bicameral chambers

Iron uptake from 50μg/ml of 80% saturated Fe-transferrin, Fe-lactoferrin, or an equivalent amount of Fe-citrate determined after 23h culture in bicameral chambers

	% of added Fe incorporated by	
Source of iron:	Caco-2	INT 407
Fe-citrate	8.7±1.4	9.8±1.5
Fe-lactoferrin	2.6±1.0	1.4±0.8
Fe-transferrin	3.6±0.8	2.9±1.0

TABLE VIII

Transport of lactoferrin and transferrin across Caco-2 and INT 407 cellmonolayers cultured in bicameral chambers

Incubation time(h)	% of radioactivity detected in the lower chamber			
	125I-Lf		125I-Tf	
	Caco-2	INT 407	Caco-2	INT 407
1	0.3±0.03	6.3±0.12	0.4±0.03	4.8±0.12
5	1.1±0.08	12.2±0.83	1.4±0.09	10.3±0.98
23	3.5±0.10	42.1±0.5	4.5±0.11	35.4±3.4

Fig. 45: Autoradiograph of culture supernatant of Caco-2 cells from both the upper and lower chambers containing ^{125}I-labelled proteins. Lanes are referred to as follows: (1) Lf in the upper chamber after 23 h incubation (2) Lf in the lower chamber at 3 h (3) Lf in the lower chamber at 5 h (4) Lf in the lower chamber after 23 h (5) Lf solution used in the study. Lanes 6-10 refer to labelled transferrin corresponding to lactoferrin in lanes 1-5 respectively.

The amount of lactoferrin and transferrin found in the upper compartment was similar, about 95 % of the total proteins initially added. In contrast to the proteins found in the lower compartment, more than 97 % of these proteins were still intact (**Fig.45**).

More lactoferrin (1.7 %) remained bound to the cells at the end of incubation as compared to transferrin (0.4%).

3.4.1.6 Transport of Lactoferrin and Transferrin Across INT 407 Cell Monolayers

The TEER values of INT 407 cells grown in the Transwell inserts had been found to be low and did not increase like Caco-2 cells, even though the percentage of phenol red diffusion to the lower compartment was < 5 %, suggesting confluency. Thus a parallel study was conducted to see the pattern of transport of lactoferrin and transferrin across these cells. As shown in **Table VIII**, the transport of both proteins increased with time and by 23 h, about 42 % and 35 % of lactoferrin and transferrin were found in the lower compartment. In contrast to Caco-2, these proteins were found mainly intact (62.6 % and 76.3 %, respectively).

3.4.1.7 Transport of Iron Across INT 407 Cell Monolayers

Just as more protein was transported across INT 407 cells than Caco-2 cell monolayers, a higher percentage of iron was also found to be transported to the lower compartment. More iron was transported as Fe-citrate and Fe-lactoferrin than as Fe-transferrin (**Fig.46**). As in the case of Caco-2 cells, the amount of iron retained by INT 407 cells was higher when iron was

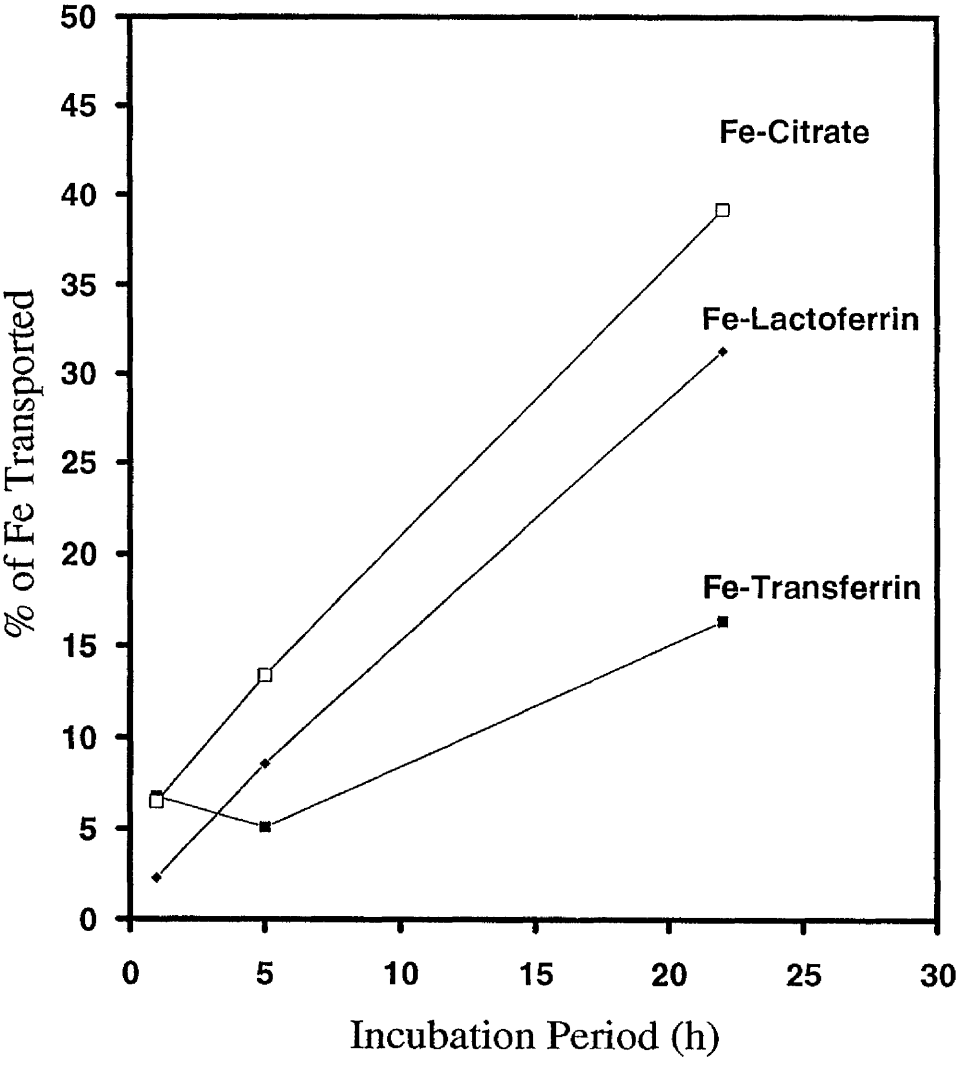


Fig. 46 : Transport of iron across INT 407 cell monolayers cultured in Transwell bicameral chambers (n = 2).

initially bound to transferrin as compared to lactoferrin, the difference was double (**Table VII**).

3.4.2 Plasma Lactoferrin Levels in the Newborn

3.4.2.1 Detection Range

The assay used was found to be linear up to 200 ng/ml lactoferrin. Above this concentration, the graph started to form a plateau. The lower detection limit i.e. the lowest cencentration of lactoferrin used in the calibration assay was 6.1 ng/ml, which was found to be 1.5 x the value of the background. The standard curve for lactoferrin in this assay is shown in **Fig.47**.

3.4.2.2 Accuracy of the Assay

The percentage recovery of added lactoferrin to rabbit plasma was found to be more than 94 % at lactoferrin concentrations between 50-200 ng/ml.

3.4.2.3 Specificity of the Assay

Cross reactivity of the rabbit-anti human lactoferrin antibody against bovine lactoferrin and human lactoferrin was found to be less than 8 % and 7 % respectively.

3.4.2.4 Concentration of Anti-lactoferrin Antibody

The concentration of anti-lactoferrin antibody which gave the steepest dilution curve (**Fig. 48**) was chosen to coat the polystyrene beads.

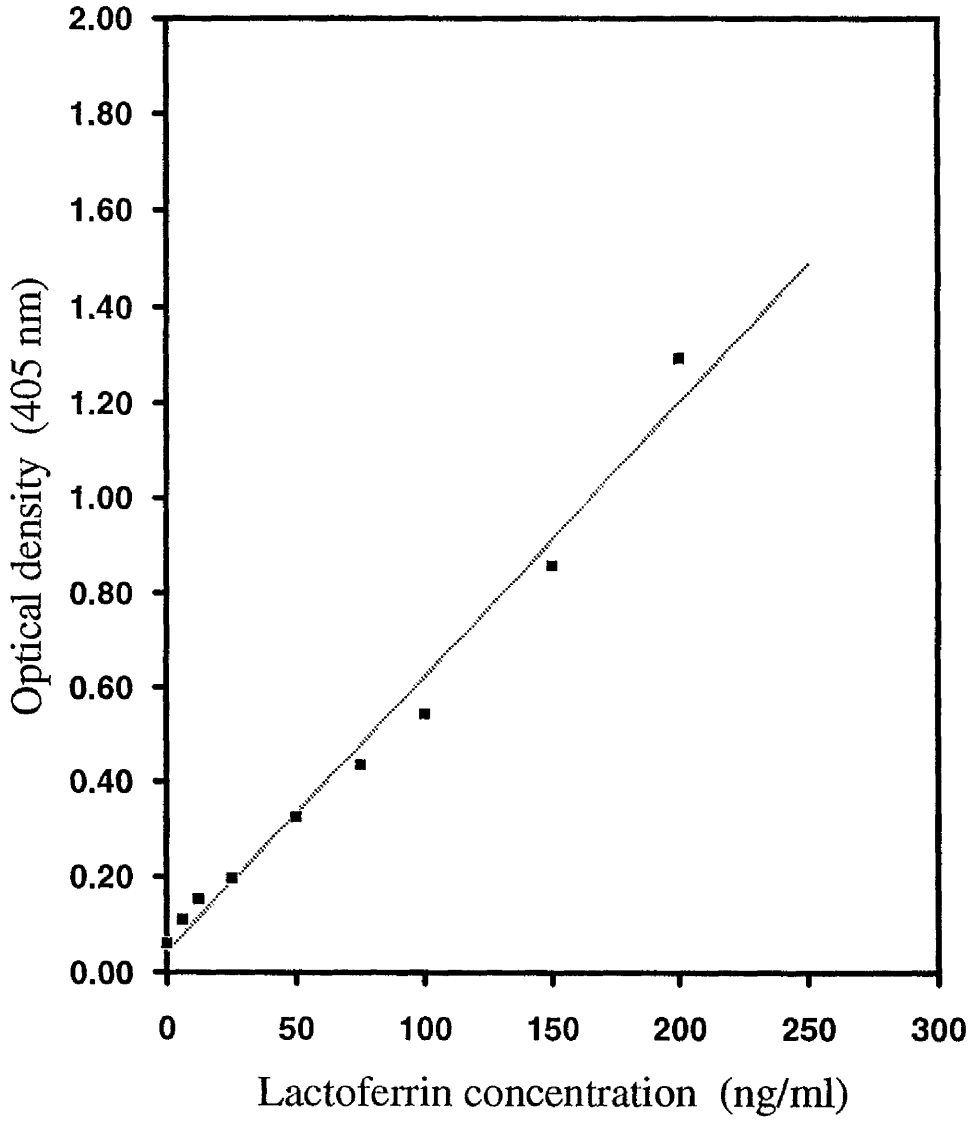


Fig. 47 : A standard curve for lactoferrin using ELISA

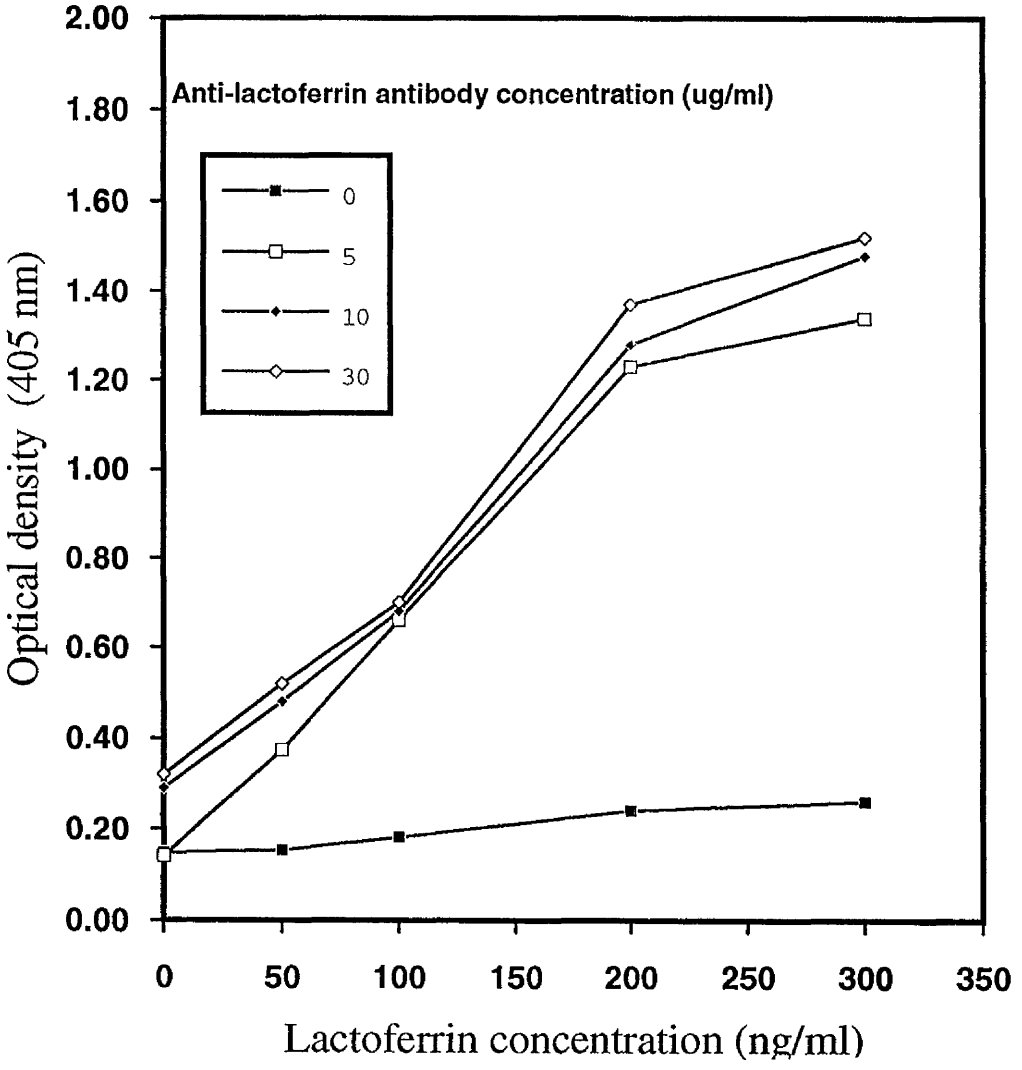


Fig. 48 : Determination of optimal concentration of anti-human lactoferrin antibody for coating the polystyrene beads (n = 3).

The concentration of the antibody was 5 μg/ml which showed linearity up to 200 ng/ml lactoferrin. Lower concentrations were not sufficient to coat the polystyrene beads whereas at higher concentrations of antibody, the graph reached a plateau at the same lactoferrin concentration (5 μg/ml).

3.4.2.5 Choice of Blocking Agent

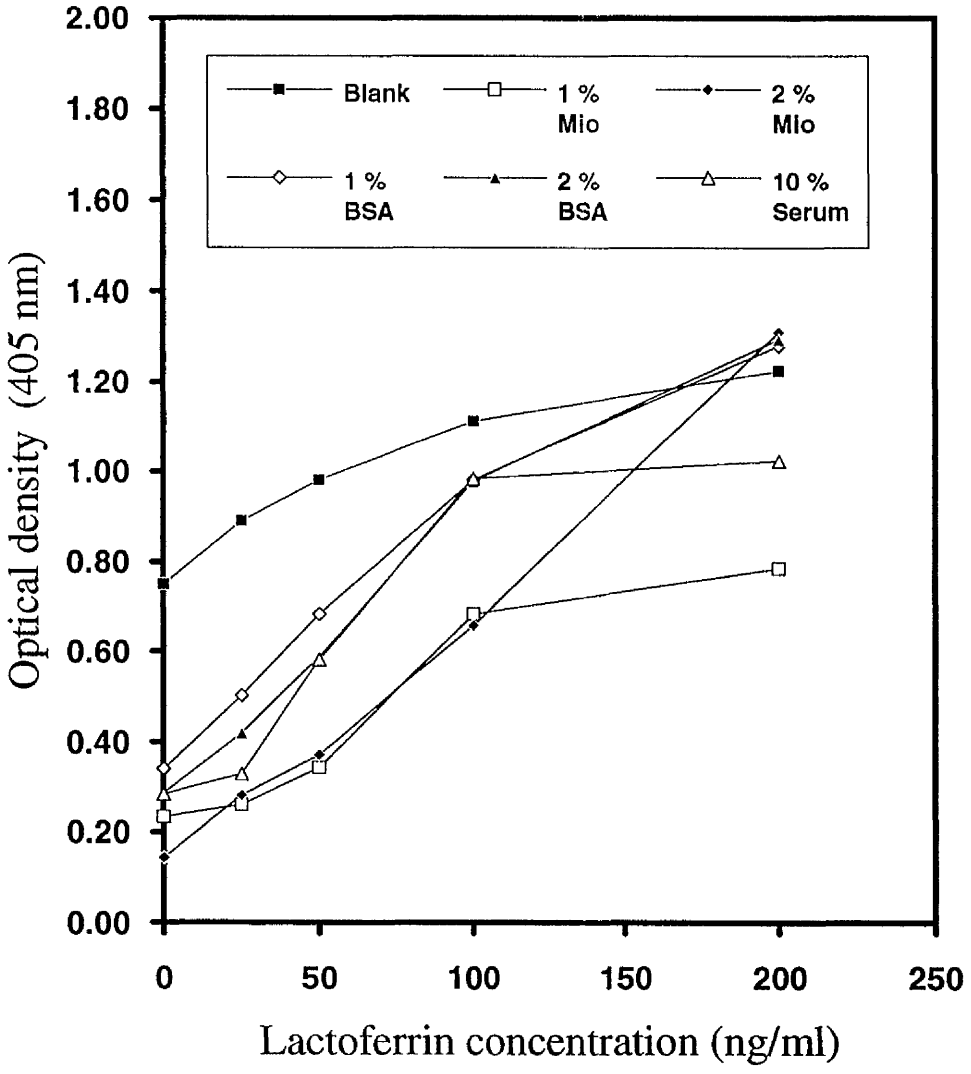
Two percent myoglobin was chosen as a blocking agent as this resulted in a considerable reduction of blank values compared to the other blockers **(Fig.49).**

3.4.2.6 Concentration of Enzyme Conjugate

The dilution of enzyme conjugate chosen for the assay was 1:400. Further dilution had resulted in insufficient of *p*-nitrophenylate being produced, whereas a lower dilution was not found to increase sensitivity but to raise the value of blank absorbance **(Fig.50)**.

3.4.2.7 Assay conditions

Coating the polystyrene beads with the antibody at 37 °C for 1 h and then overnight at 4 °C was found to be satisfactory and the results obtained were identical to those with beads coated at 45 °C for 2 h. Incubation with anti-lactoferrin enzyme conjugate for 1.5 h at 37 °C was found to be optimal. The enzymic reaction to produce a coloured product giving a maximum absorbance of 1.5 took about 30 min. Longer reaction resulted in maximum absorbance approching 2.0 and less sensitivity to variation in lactoferrin concentration.



Key: Mio, myoglobin

Fig. 49 : Determination of different types and concentration of blocking agents used for ELISA (n = 3).

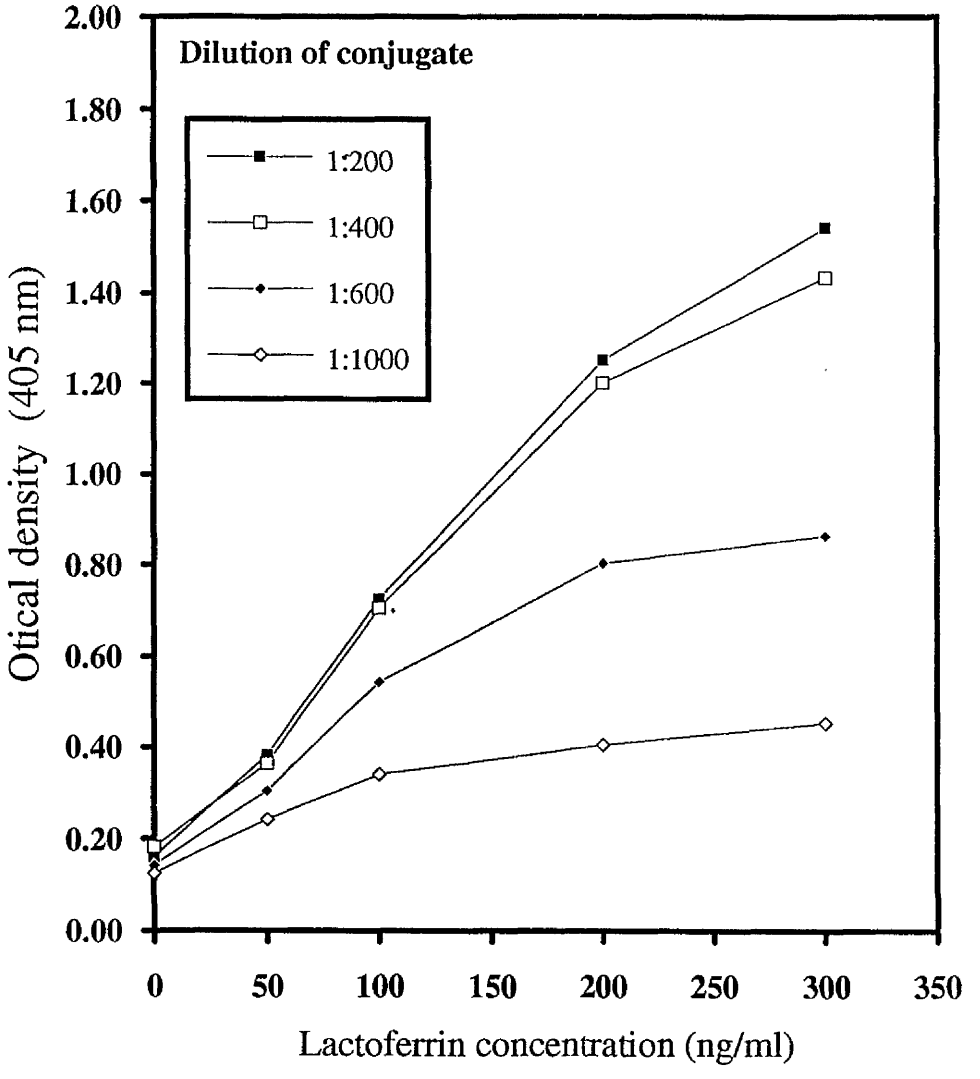


Fig. 50 : Titration of anti-human lactoferrin antibody alkaline phosphatase conjugate used for ELISA (n = 3)

3.4.2.8 Background information on the infants

For the study of plasma lactoferrin in the newborn, 15 venous blood specimens were obtained from Caucasian babies born at 37 weeks gestation or over with normal delivery. Their birthweights were greater than 2.2 kg. The babies were healthy with no clinical signs of infection. The blood specimens were taken between 48-72 h after delivery. The infants were divided into two groups, the first group (n=7) consisting of babies who were exclusively breast-fed, and the second group (n=8) was made up of babies who received standard stage 1 infant formulas: SMA Gold Cap (Wyeth Ltd., U.K.), Cowgate Milk (Cow Milk Ltd., U.K.), and Oster Milk (Farleys Ltd., U.K.) which were provided by the Hospital.

3.4.2.8 Plasma lactoferrin levels in full-term breast-fed versus formula-fed infants

The range of plasma lactoferrin levels measured in the combined group of full-term infants was found to between 100 and 336 ng/ml with a mean value of 178 ± 46.6 ng/ml, corresponding to 2.1 nM.

The effect of feeding regime on the plasma lactoferrin levels of these infants is shown in **Fig. 51**. Plasma lactoferrin levels of breast fed infants were found to be between 136-336 ng/ml, with a mean of 191.9 ± 46.4 ng/ml. Infants fed with commercial formulas had plasma lactoferrin level in the range of 100-216 ng/ml, and a mean of 155.4 ±30.0 ng/ml. Even though the mean plasma lactoferrin level of breast fed infants was higher than that of formula fed infants, a t-test showed no significant difference between the means of the two groups ($p \leq 0.05$)

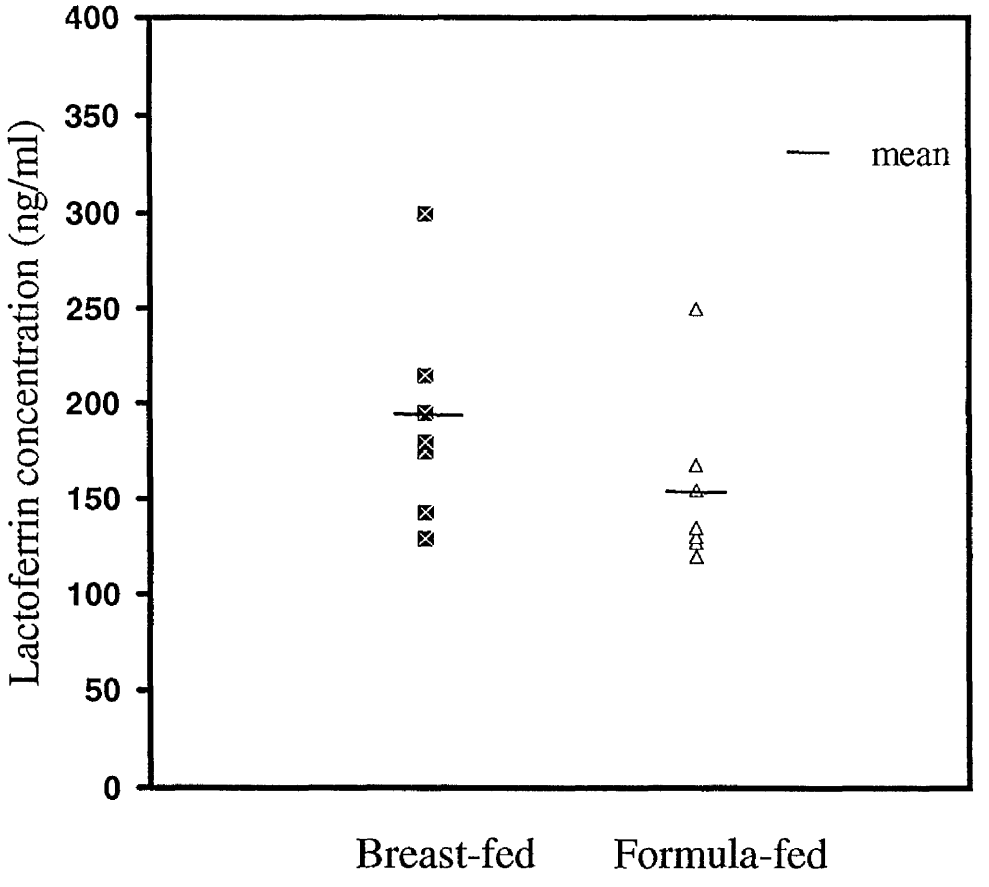


Fig. 51 : Plasma concentration of fifteen full term newly born infants, exclusively breast-fed or formula-fed. Each point is a mean of duplicate determinations.

3.5 DISCUSSION

3.5.1 Iron and Protein Transport studies

The human colon carcinoma cell line, Caco-2 grown on collagen coated polycarbonate membranes mounted in Transwell bicameral chambers was found to exhibit structural and functional differentiation features normally associated with the characteristics of mature enterocytes as have been reported by earlier workers (Pinto *et al.*, 1983, Grasset *et al.*, 1984, Hidalgo *et al.*, 1989; Alvarez-Hernandez *et al.*, 1991). The Caco-2 cell monolayers were seen from transmission electron micrograph to be covered by brush border microvilli. The structural and functional differentiation of the brush border microvilli is associated with polarisation of the epithelial monolayers as displayed by the presence of tight junctions and the appearance of desmosomes which are known to be specific features of polarised epithelia (Cereijido, 1978).

The electron micrographs of these cells were taken on day 17 after seeding on to the polycarbonate membranes, when they have already become columnar in shape. Therefore changes in cell dimensions and formation of these features during the earlier stages of culture were not examined. However, Hidalgo *et al.* (1989) had reported that the height of the cell had increased as much as 489 % from day 3 to day 16. The microvilli started to appear on day 3 but were poorly organised.

The development of polarity of the Caco-2 monolayers was determined from measurements of TEER across the monolayers. The TEER values of

day 1 to post confluence obtained in this study were similar to those observed by Alvarez-Hernandez (1991) but slightly higher than the values obtained by Hidalgo (1989) at day 8 which then remained almost constant up to day 18. The TEER values increased above 250 $\Omega.cm^2$ on day 16 suggesting that Caco-2 monolayers had then formed a tight barrier. The tightness of the barrier was further confirmed by the impermeability of the cells to phenol red. The minimum percentage of phenol red diffusion obtained was 5.8 ± 0.4 % from day 14. Halleux and Schneider (1991) obtained the minimum percentage of phenol red diffusion as 6 % after day 7 of cultivation, suggesting that confluency was reached a few days earlier. The use of different passages of cells plus a different type of membrane could contribute to the slight difference.

Unlike Caco-2 cells, INT 407 cells did not differentiate in culture after cultivation of more than 20 days. Results from phenol red exclusion indicate that these cells formed a complete monolayer, in fact the minimum percentage of phenol red diffusion (6.9 ± 0.2 %) was reached after only 7 days in culture. However TEER values across these cells did not increase and never reached more than 90.5 ± 0.3 $\Omega.cm^2$. Therefore, although on the one hand INT 407 cells could be cultured on the polycarbonate membrane and could form a monolayer, the cells did not differentiate as Caco-2. The finding is not totally unexpected because attempts to either culture intestinal epithelial cells or establish cell lines derived from enterocytes have not been successful (Qudroni *et al.* 1979; Raul *et al.*, 1978). Similar results were obtained by Chantret *et al.*(1988) when using various colon carcinoma cell lines and most of these cells do not develop enterocytic differentiation in culture, with the exception of a few cell lines, including Caco-2 cells.

Having established that Caco-2 cultured in the Transwell inserts had undergone enterocytic differentiation, they were then used as a model of enterocytes to study the transport of iron and proteins (lactoferrin and transferrin) across the brush border into the cell as well as across the cell. Undifferentiated INT 407 cells were used as a control. Since INT 407 cells also formed good monolayers, the difference in the pattern of transport could be attributed to special features of differentiated Caco-2 cells.

Transport of lactoferrin and transferrin across Caco-2 monolayers is similar, with only 4 % of the proteins being transported within 24 h. The remaining proteins were found in the upper compartment with only a small percentage of transferrin (0.5 %) and lactoferrin (1.7 %) being retained by the cells and the membrane. Even though only one-third of transferrin was bound by the cells as compared to lactoferrin, more iron was incorporated into the cells when it was initially bound to transferrin.

Most of the ^{125}I activity that have been detected in the lower compartment was TCA soluble, indicating that these proteins had been degraded and only < 20 % of the protein was still intact. With the present set up, it is not known exactly where protein degradation has taken place, either in the cytosol or as the proteins cross over the basal membrane. In contrast almost half of the proteins were found to have been transported across INT 407 cells. At the end of 23 h, about 42 % of lactoferrin and 49 % of transferrin was detected in the lower compartments and unlike the earlier finding with Caco-2 cells, only 43 % and 37 % of these proteins were degraded. These findings suggest that neither lactoferrin nor transferrin was transported across Caco-2 cell monolayers and the passage

of these proteins across INT 407 cells could occur through intercellular spaces rather than transcellularly.

Transport of lactoferrin bound iron by Caco-2 cells was comparable to transport of iron presented as ferric citrate, and greater than that of iron transported from transferrin. If iron transport across the cells, rather than release from lactoferrin is the rate-limiting step, then Fe-lactoferrin and ferric citrate would be expected to behave similarly. In contrast, since Caco-2 cells express transferrin receptors (Schreider and Halleux, 1991), iron bound to transferrin may be taken up by endocytosis of the transferrin-iron complex and used for cell metabolism. The fact that rather more iron from transferrin than from lactoferrin was found within the cells, despite the minimal transport of transferrin-iron across the monolayer, tends to support this hypothesis, though proof would require examination of the intracellular distribution of iron. As there was little evidence that either lactoferrin or transferrin themselves are transported across the cell monolayers, this suggests that iron from lactoferrin detected across the monolayer was due to the release of this iron from membrane bound lactoferrin and not due to it being transported by the protein itself. If this is true, then iron from lactoferrin or citrate would be expected to behave similarly and being taken up by the cell following the same route.

These findings are in line with the *in vivo* study of iron absorption from ^{58}Fe-labelled bovine lactoferrin in newly born infants which did not demonstrate any difference from the absorption of ferrous chloride (Fairweather-Tait *et al.*, 1987). These workers concluded that lactoferrin-bound iron was handled by the body in exactly the same way as other non-haem dietary iron. In addition other *in vivo* studies show that lactoferrin

neither inhibits nor enhances absorption of inorganic iron (Fransson *et al.*, 1983; Davidson *et al.*, 1990).

3.5.2 Plasma Lactoferrin levels in newborn infants

Further work to see the relevance of lactoferrin in iron absorption was to determine its presence in the plasma of newborn infants. In general it is thought that lactoferrin is not absorbed, but recent studies in preterm infants have demonstrated the presence of lactoferrin derived from maternal milk in the urine, suggesting in this case that lactoferrin can be absorbed. It is not known whether similar events occur in term infants, but it has been shown that premature infants absorb large amounts of intact macromolecules from milk into their plasma (Jakobsson *et al.*, 1986).

For this purpose, the non-competitive sandwich ELISA was used to determine the plasma lactoferrin levels in newborn infants, based on the combination of the methods devised by Figarella (1986) and later modified by Scott (1989). Polystyrene beads were used as the solid phase instead of the microtitre plates in order to reduce the high background readings due to non-specific binding of lactoferrin to the sides of the plates. Other steps were also taken to counter this problem such as transferring the polystyrene beads to fresh polystyrene test tubes before the enzymic reaction. Use of 2 % myoglobin as a blocking agent further reduced the blank values when compared to rabbit serum (10 %), which was used in Figarella's method.

The concentration of plasma lactoferrin detected by ELISA in this study ranged from 6.1 to 200 ng/ml. Considering the small amount of blood sample that can be obtained from the infants, this range of concentration is

suitable for the detection of plasma lactoferrin with some dilution of the plasma. Within this range of concentration, the percentage recovery of lactoferrin from the plasma is > 94 %

Plasma lactoferrin levels of all the full term infants tested in this study were between 100-336 ng/ml with a mean value of 170.8 ± 46.6 ng/ml, corresponding to 2.1 nM. The range of plasma lactoferrin level found in this study was comparable to that found by Kolsto Otnaess *et al.* (1983) who reported that lactoferrin levels in cord plasma ranged between 20-300 ng/ml. They also found that the values in cord plasma were identical to those in the plasma taken from day 5 post partum. Scott (1989) on the other hand reported higher mean value of plasma lactoferrin in term infants up to the age of 21 days (385 ± 113 ng/ml). However in this case, capillary blood was taken instead of venous sampling which had been shown to give higher lactoferrin concentration (Maacks *et al.*, 1990) as the capillary sampling technique could have resulted in neutrophil activation and release of lactoferrin. In this study extreme care had been taken to ensure that degranulation of neutrophils had been kept to a minimum.

Plasma lactoferrin levels of infants found in this study were higher than the levels in adults which had been reported by many workers. Figarella (1986), reported mean adult value of 113 ± 20 ng/ml, with a range of 30-240 ng/ml. Scott (1989) using similar procedure as Figarella, but using venous sampling instead of capillary sampling as in the infants reported a similar mean adult value of 122 ± 40 ng/ml, with a range of 42-202 ng/ml. Kolsto Otnaess *et al.* (1983) also reported a slightly higher values in infants plasma but they were not significantly different from the adult values (20-200 ng/ml).

As the breast-fed infants consume much higher levels of lactoferrin than formula-fed infants, a comparison was made between plasma lactoferrin levels of these two groups. The plasma lactoferrin levels of exclusively breast-fed infants were found to be higher (191 ± 46.8 ng/ml) than the formula fed infants (155 ± 30 0 ng/ml). However t-test showed no significant difference between the mean values of the two groups. Despite the higher concentration of lactoferrin in human milk than in cow's milk and therefore cow's milk based feeding formulas, this study and that of Scott (1989) were unable to demonstrate that this difference has any effect on plasma lactoferrin levels in the newborn. This suggests that in term newborn infants, lactoferrin despite its high concentration in breast milk and its resistance to proteolytic degradation is not absorbed by the intestinal cells. On the other hand, studies in preterm infants have shown that molecular forms of lactoferrin have been detected in the stool and urine of those infants who were breast-fed (Goldman *et al.*, 1990; Hutchens *et al.*, 1991). However the development of gastrointestinal tract and renal tubular function in preterm infants is not well characterised, and therefore it is not known whether there is an increased permeability of lactoferrin in this case.

In conclusion the present work seems to indicate that lactoferrin does not enhance the absorption of iron nor it itself being absorbed by the intestinal cells of healthy newly born term infants. This observation could be explained on the present understanding that in the early period of infancy, at least up to 6 months, they have sufficient endogenous iron to meet their requirements (Aggett *et al.*, 1989). This is reflected in the low incidence of anaemia among healthy full term infants irrespective of whether they are breast-fed or fed with iron supplemented or unsupplemented formulas

(Wharton, 1989). Iron absorption is known to be lower in the early postnatal period than at any later time in childhood, and the amount and bioavailability of dietary iron are therefore less important before 4 months of age (Cook and Bothwell, 1984). The findings of Kolsto Otnaess *et al.* (1983) that lactoferrin levels in the cord plasma of healthy full term infants were the same as the levels after 5 days post partum supports this view.

In contrast preterm infants have less total body iron than term infants since premature birth interrupts iron acquisition from the mother during the period of maximal iron absorption by the fetus. Despite the lower total body iron pool, transferrin saturation of 100 % often occur due to low level of transferrin (Sullivan, 1992). This puts the premature infants at risk of oxygen radical injury and the absorption of lactoferrin from maternal milk (Hutchens *et al.*, 1991) may be a strategy for combating this problem. Thus absorbed apolactoferrin may be a partial explanation for the reported lower incidence of retinopathy of prematurity in prematurity in breast-fed infants (Cunningham, 1987).

CHAPTER FOUR

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The work reported in this thesis has attempted to examine the possible roles of lactoferrin in cellular iron uptake and iron absorption. For this purpose, the molecular basis of the interaction of lactoferrin with a monocytic and an enterocyte-like differentiated colon carcinoma cell line has been studied.

Cellular uptake of iron may occur by various routes but the most studied and best characterised mechanism is that of uptake from transferrin which occurs via receptor mediated endocytosis (Dautry-Varsat, 1986; Crichton and Charloteaux-Wauters, 1987; Theil and Aisen, 1987). The present work has shown that transferrin can indeed deliver its iron, most probably via a similar pathway to U937 cells. Lactoferrin on the other hand does not seem to donate its iron despite its ability to bind to these cells. This finding appears to contradict that of Van Snick *et al.* (1974; 1976) who reported some uptake of iron from lactoferrin by peritoneal macrophages. The group used this observation as a basis for explaining the hypoferraemia associated with inflammation. They proposed that the rapid drop of serum iron accompanying acute inflammation results from the release of unsaturated lactoferrin from stimulated neutrophils into the extracellular environment. Lactoferrin that is released removes iron from transferrin or it competes with transferrin for the iron released by the macrophages. It then returns this iron to the macrophages of the reticuloendothelial system, where it becomes incorporated into iron stores. Although a transfer of iron from transferrin to lactoferrin is favoured thermodynamically the kinetics of iron exchange from transferrin at physiological pH are very slow (Morgan, 1977) and this coupled with the rapid elimination of lactoferrin by the liver (Moguilevsky *et al.*, 1987); McAbee and Esebensen, 1991) suggests that little iron transfer actually occurs before lactoferrin is cleared from the circulation.

A closer look at the rates of iron uptake reported in in those studies revealed that they are indeed extremely slow compared to transferrin and in fact the iron uptake measured was actually due to the lactoferrin being ingested by the cells (Oria *et al.* 1988, Ismail and Brock, 1993). Therefore it seems unlikely that the mechanism proposed by Van Snick *et al.* (1974, 1976) would operate at the systemic level as at this physiological pH the affinity for iron by transferrin is high, resulting in no movement of iron to lactoferrin in plasma (Fletcher, 1989). Furthermore the amount of lactoferrin-iron taken up by the reticuloendothelial system from the general circulation is probably too small to account for the fall in serum iron circulation (Kluger and Bullen, 1987). However there is a possibility that lactoferrin might be involved in the hypoferraemia at the local inflammatory lesions, where the acidic condition favours the binding of "free" iron to lactoferrin instead of transferrin, as lactoferrin has a higher affinity for iron at this pH, but the question of slow rate of iron uptake from lactoferrin still remains.

A more likely conclusion from the present work, and that of others seems to indicate that lactoferrin released from neutrophils may have the following alternative functions and/or fate. Firstly, lactoferrin may bind to monocytes and macrophages and in so doing may offer a protective mechanism against uptake of "free" iron released from transferrin or from the phagocytosed microorganisms. This protective mechanism offers a mean of reducing the possible involvement of "free" iron in the generation of potentially harmful hydroxyl radical which may cause tissue damage. This work has shown that lactoferrin inhibited the uptake of excess non-transferrin bound iron in the form of FeNTA. The fact that this effect was found with both apolactoferrin and Fe-lactoferrin suggests that the inhibitory effect was not simply due to binding of all the iron by lactoferrin in the medium. Rather it

appears that lactoferrin in some way regulates uptake of this potentially toxic non-transferrin bound iron at the cell membrane. Further evidence for such a role is provided by the ability of lactoferrin to abolish the inhibitory effect of FeNTA on lymphocyte proliferation (Djeha and Brock, 1992b), and prevent membrane damage to monocytes (Britigan *et al.*, 1991). Substantial binding of lactoferrin by a relatively non-specific mechanism, as found in this study would allow lactoferrin to perform this function not only with monocytes and macrophages but with a wider range of cell types.

The likely fate of the released lactoferrin is that it may be cleared rapidly from the circulation by the liver (Imber and Pizzo, 1983) with hepatocytes accumulating > 90% (McAbee and Esebensen, 1991; Ziere *et al.* 1992) and the remaining lactoferrin being cleared by Kupffer and liver endothelial cells. Studies by McAbee and Esebensen (1991) have shown that hepatic clearance of plasma lactoferrin may occur by a high-capacity, clathrin dependent endocytic pathway whose biochemical features resemble in every way a receptor-mediated process, in contrast to the present finding and that of Birgens *et al.* (1988) which indicate that lactoferrin binding to monocytes was not followed by endocytosis of the protein. The presence of such an efficient pathway in hepatocytes thus provides a continuous clearance of plasma lactoferrin and its iron from the circulation especially from the inflammatory sites where the concentrations of both lactoferrin and iron have increased many folds.

Others have evidence that hypoferraemia is due not to an accelerated removal of iron from the serum transferrin pool but rather to an altered processing of iron within the reticuloendothelial system which limits the supply of iron to the extracellular iron pool (Letendre and Holbein, 1983). Konijin and Hershko (1977) put forth the idea that hypoferraemia results

from surplus apoferritin synthesis which binds a larger-than-normal proportion of iron entering the cell. Enhanced ferritin synthesis in response to inflammation precedes the block of iron release and is not simply the result of increased tissue iron concentrations (Cartwright and Lee, 1971).

There are many aspects of this work that still need to be examined and expanded. This study has shown that human lactoferrin binds to two populationsof sites on U937 cells: a relatively small number of high affinity receptor binding sites and a large number of low affinity binding sites which constitute the non-specific binding. Although the binding seems complex, investigating the nature of the specific binding is crucial in understanding its involvement in regulating cell functions. For this purpose, the specific receptor has to be identified, isolated and characterised. Receptor isolation might provide the basis for generation of monoclonal anti-receptor antibodies, which would be an extremely valuable tool for these functional studies. The role of lactoferrin in hypoferraemia is still unsettled especially at the systemic level for the reasons mentioned earlier and also because the lactoferrin hypothesis does not provide a good explanation for the defective iron metabolism in intestinal cells and hepatocytes. It is not known how neutrophilic lactoferrin is involved in the reduction of iron absorption and iron reutilisation from hepatocytes, both of which are associated with hypoferraemia. *In vivo* study involving animals lacking in specific granules in their neutrophils may help enlighten the problem. If lactoferrin is an essential element in hypoferraemia, such animals should not develop the usual disturbances in iron metabolism.

The present work has also attempted to determine the possible involvement of lactoferrin in iron absorption. Although lactoferrin receptors have been identified in the mucosal membranes (Davidson and Lonnerdal, 1988; Hu *et*

al., 1990) there is still no direct evidence that these membrane proteins actually mediate uptake or transport of the lactoferrin-bound iron across the mucosa. This work using Caco-2 cells which can undergo enterocyte-like differentiation in culture has shown that lactoferrin does not enhance the absorption of iron nor it itself being transported transcellularly. In fact the small amount of iron taken up might follow the same route as other non-haem iron such as ferric citrate. This observation is further supported by an *in vivo* study which shows that plasma lactoferrin levels of breast-fed infants are not significantly different from those who were formula fed, despite the higher amount of lactoferrin in breast milk. These findings are consistent with the idea that lactoferrin may serve as a regulator rather than as an enhancer of iron absorption as proposed by Brock (1980). This may be true especially during the early period of infancy when there is an adequate level of iron stores. Later in infancy, when the iron stores have been depleted and the proteolytic activity of the infant's gut is increased, lactoferrin can be degraded and the bound iron can be released and hence be absorbed. The presence of lactoferrin binding molecules in the brush border membrane might aid in this regulating activity. The advantage of such a mechanism might be a protection against the toxic effects of excess iron as in the case of monocytes mentioned earlier.

If lactoferrin does aid in regulating the uptake of iron by the enterocyte, more work is needed to understand the regulatory mechanism and factors that are involved in either promoting or inhibiting the uptake of the lactoferrin-bound iron. Since studies have repeatedly shown that iron absorption is inversely proportional to the body's iron stores (reviewed by Conrad, 1987), information regarding the state of iron repletion of the body must be communicated to the enterocyte which must then be able to process dietary iron absorption. The precise means by which these two aspects of

intestinal iron metabolism are interrelated are also not known. The present work could be expanded to study some of these uncertainties. Caco-2 cells which have been considered by many workers as a good *in vitro* model for the iron transport system can be employed to follow the uptake of lactoferrin-bound iron at various levels of iron stores of the cells. The *in vivo* study on plasma lactoferrin between breast-fed and formula-fed infants could be extended to cover a larger sample in order to obtain a more confident statistical analysis. A longitudinal study to measure both plasma lactoferrin and iron status of these infants would be desirable. In addition the effect of lactoferrin-supplemented formulae on these aspects could also be studied.

Lactoferrin in milk plays a role in stimulating tissue growth and development, but the growth promoting effect of lactoferrin has been found to be somewhat different from the iron donating effect of transferrin. The mechanism of this growth enhancement is not known and more research along this line is needed.

Last but not least, in view of the functional properties of lactoferrin, especially its protective effects, it would be beneficial to supplement cow's milk based infant formula with lactoferrin. To do this more feasibility studies of adding lactoferrin in a biologically active form are needed.

In conclusion this work has shown that lactoferrin-cell interactions either with monocytic or enterocyte-like cell lines, may serve to regulate rather than facilitate iron uptake by these cells. Two properties of lactoferrin, namely its ability to bind iron extremely tightly even at low pH, and its readiness to bind to a variety of cells and other molecules, in most cases in a relatively non-specific manner may be critical to its functions. The first

property would allow lactoferrin to sequester any "free" iron in situations or locations where low pH predominates and cause transferrin to lose its iron such as the upper gastrointestinal tract or in the inflammatory lesions. The ability of lactoferrin to bind to a wide range of cells and tissues could focus this protective effecty on to cell surfaces, and hence protect them against membrane lipid peroxidation and excessive uptake of non-transferrin bound iron which can be taken up very rapidly and could be cytotoxic.

CHAPTER FIVE

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